UNIVERSITY OF SÃO PAULO
SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO

**Produtos Naturais Antifúngicos e Antileishmania a partir de
Actinobacterias Associadas a Formigas Cultivadoras de Fungos do Brasil**

**Antifungal and Antileishmanial Natural Products from Actinobacteria
Associated to Brazilian Fungus-Growing Ants**

Doctoral thesis presented to the Graduate Program of School of Pharmaceutical Sciences of Ribeirão Preto/USP for the degree of Doctor in Sciences.

Concentration Area: Natural and synthetic products

Student: Humberto Enrique Ortega-Domínguez

Supervisor: Mônica Tallarico Pupo, PhD

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2018

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In dedication

To my father and mother, Jaime and Rosa, to be an example, an inspiration and to help me in my education;

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*“Discouragement is an enemy of your perseverance.
If you don't fight against discouragement you will
become pessimistic first, and lukewarm afterwards.
Be an optimist.”*

- St. Josemaría Escrivá.

ABSTRACT

ORTEGA-DOMÍNGUEZ, H. E. **Antifungal and Antileishmanial Natural Products from Actinobacteria Associated to Brazilian Fungus-Growing Ants**. 2018. 160 p. Doctoral Dissertation. School of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo, Ribeirão Preto, 2018.

There is a quadripartite symbiosis in the fungus-growing ant ecosystem between three mutualist (Attine ant, fungal garden and symbiotic actinomycetes) and one parasite (specialized pathogenic fungus *Escovopsis* sp). The actinobacteria associated to the ant host produce secondary metabolites to inhibit this pathogen but not the crop fungus. Interesting natural products have been reported from these bacteria with a wide spectrum of biological activities. In this thesis, several actinobacteria were isolated from the exoskeleton and garden of fungus-growing ants to isolate active compounds against different targets such as *Leishmania donovani* and *Escovopsis*. The known antibiotic and cytotoxic compounds griseorhodin A (**1**), griseorhodin C (**2**), griseorhodin G (**3**) and dinactin (**4**) were produced in solid ISP-2 culture by *Streptomyces puniceus* AB10, which was isolated from the leaf-cutter ant *Acromyrmex rugosus rugosus*. The absolute configurations of **1** and **2** were unambiguously established as 6*S*,6*aS*,7*S*,8*S* and 6*R*,6*aS*,7*S*,8*R*, respectively, using vibrational circular dichroism (VCD) and density functional theory (DFT) calculations. The bacterium *Streptomyces puniceus* AB10 produced in broth A-medium only one family of antibiotics as dinactin (**4**). Compound **4** showed inhibition against *Escovopsis* and a higher activity against *L. donovani* promastigotes and intracellular amastigotes than miltefosine. Two stereoisomers strepchazolin A (**5**) and strepchazolin B (**6**), the antibiotic streptazolin (**7**), its *E*-isomer (**8**), and the inorganic compound cyclooctasulfur (**9**) were produced in solid ISP-2 culture by *Streptomyces chartreusis* AC70, which was isolated from the fungal garden of the leaf-cutter ant *Acromyrmex subterraneus brunneus*. Compound **9** showed antagonist activity against the specialized pathogenic fungus *Escovopsis* sp. This is the first report of **8** as natural product. The absolute configurations of **5** and **6** were unambiguously established as 5*S*,6*S*,9*R* and 5*S*,6*S*,9*S*, respectively, using vibrational circular dichroism (VCD) and density functional theory (DFT) calculations. The bacterium *Candidatus Streptomyces philanthi* ICBG292, isolated from the exoskeleton of a worker of a *Cyphomyrmex* colony, produced the antibiotics Mer-A2026B (**10**), piericidin-A₁ (**11**) and nigericin (**12**). Compounds **10-12** showed activity against *Escovopsis* sp and against *L. donovani*. Compound **12** showed higher activity against *L. donovani* promastigotes and intracellular amastigotes than miltefosine. Compound **10** was also active against the fungus *Trichoderma* sp. *Streptomyces sioyaensis* ICBG311, isolated from winged male ants of *Cyphomyrmex* colonies, produced a new naphtoquinone named cyphoquinone (**13**), two new antifungal compounds named cyphomycin (**14**) and epoxycyphomycin (**15**), and the known antifungal GT-35 (**16**). Compounds **14-16** displayed activity against several strains of *Escovopsis* sp and *Candida albicans* K1 with a MIC of 1.0, 0.5 and 0.25 µg/mL, and a higher activity against *L. donovani* promastigotes and intracellular amastigotes than miltefosine, while **13** a weak activity against *L. donovani*. Cyphomycin (**14**) also showed potent *in vitro* activity against the resistant human pathogens *Aspergillus fumigatus* 11628 (echinocandin resistance), *C. glabrata* 4720 (triazole resistance), and *C. auris* B11211 (echinocandin, triazole, and amphotericin B resistance), with MIC of 0.5, 0.5 and 4 µg/mL, respectively. A single-dose study of cyphomycin (**14**) in a neutropenic mouse disseminated candidiasis model exhibited a dose-like response with 0.56 and 0.66 log reduction of infectious burden when treated with 20 and 40 mg/kg cyphomycin (**14**), respectively, and epoxycyphomycin (**15**) exhibited 0.53 log

reduction with 40 mg/kg, demonstrating clinical relevance and effectiveness of **14** and **15** in this industry-standard model of *Candida* infection. On the other hand, GT-35 (**16**) killed the mice 1 hr post dose at 40 mg/kg.

Keywords: Fungus-growing ant, *Escovopsis*, Actinobacteria, *Cyphomyrmex*, *Acromyrmex*, *Leishmania donovani*, antifungal, polyketides.

RESUMO

ORTEGA-DOMÍNGUEZ, H. E. **Produtos Naturais Antifúngicos e Antileishmania a partir de Actinobacterias Associadas a Formigas Cultivadoras de Fungos do Brasil**. 2018. 160 p. Tese de doutorado. Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2018.

Há uma simbiose quadripartida no ecossistema das formigas cultivadoras de fungos entre três mutualistas (Formiga da tribo Attini, jardim fúngico e actinomicetos simbiotes) e um parasita (fungo patogênico especializado *Escovopsis* sp). As actinobactérias associadas à formiga hospedeira produzem metabólitos secundários para inibir este patógeno, mas não o fungo mutualista. Produtos naturais interessantes foram relatados a partir destas bactérias com um amplo espectro de atividades biológicas. Portanto, várias actinobactérias foram isoladas do exoesqueleto e do jardim das formigas agricultoras para isolar compostos ativos contra diferentes alvos como *Leishmania donovani* e *Escovopsis*. Os antibióticos e compostos citotóxicos conhecidos griseorhodina A (**1**), griseorhodina C (**2**), griseorhodina G (**3**) e a dinactina (**4**) foram produzidos em cultivo sólido de ISP-2 por *Streptomyces puniceus* AB10, que foi isolada da formiga cortadeira *Acromyrmex rugosus rugosus*. As configurações absolutas de **1** e **2** foram inequivocamente estabelecidas como 6*S*,6*aS*,7*S*,8*S* e 6*R*,6*aS*,7*S*,8*R*, respectivamente, usando dicroísmo circular vibracional (VCD) e cálculos da Teoria do Funcional de Densidade (DFT). A bactéria *Streptomyces puniceus* AB10 produziu em meio-A líquido apenas uma família de antibióticos como a dinactina (**4**). O composto **4** mostrou inibição contra *Escovopsis* e uma atividade maior contra *L. donovani* em promastigota e amastigota intracelular que a miltefosina. Dois estereoisômeros, strepchazolina A (**5**) e strepchazolina B (**6**), os antibióticos streptazolina (**7**), seu isômero-*E* (**8**), e o composto inorgânico octa-enxofre (**9**) foram produzidos em cultivo sólido de ISP-2 por *Streptomyces chartreusis* AC70, que foi isolada do jardim fúngico da formiga cortadeira *Acromyrmex subterraneus brunneus*. O composto **9** mostrou atividade antagonista contra o fungo patogênico especializado *Escovopsis* sp. Este é o primeiro relato de **8** como produto natural. As configurações absolutas de **5** e **6** foram inequivocamente estabelecida como 5*S*,6*S*,9*R* e 5*S*,6*S*,9*S*, respectivamente, usando dicroísmo circular vibracional (VCD) e cálculos da Teoria do Funcional de Densidade (DFT). A bactéria *Candidatus Streptomyces philanthi* ICBG292, isolada do exoesqueleto de operária de colônia de formiga *Cyphomyrmex*, produziu os antibióticos Mer-A2026B (**10**), piericidina-A₁ (**11**) e nigericina (**12**). Os compostos **10-12** mostraram atividade contra *Escovopsis* sp e contra *L. donovani*. O composto **12** mostrou uma atividade maior contra *L. donovani* em promastigota e amastigota intracelular que a miltefosina. O composto **10** também foi ativo contra o fungo *Trichoderma* sp. *Streptomyces sioyaensis* ICBG311, isolada de machos alados de colônia de formiga *Cyphomyrmex*, produziu uma nova naftoquinona chamada cyphoquinona (**13**), dois novos compostos antifúngicos denominados cyphomycina (**14**) e epoxicyphomycina (**15**), e o antifúngico conhecido GT-35 (**16**). Os compostos **14-16** mostraram atividade contra diferentes linhagens de *Escovopsis* sp e *Candida albicans* K1 com MIC de 1.0, 0.5 e 0.25 µg/mL, e uma atividade maior contra *L. donovani* em promastigota e amastigota intracelular que a miltefosina, enquanto **13** apresentou atividade baixa contra *L. donovani*. A cyphomycina (**14**) também mostrou uma potente atividade *in vitro* contra os patógenos humanos resistentes *Aspergillus fumigatus* 11628 (resistente à equinocandina), *C. glabrata* 4720 (resistente ao triazol), e *C. auris* B11211 (resistente à echinocandina, ao triazol, e à anfotericina B), com MIC de 0.5, 0.5 e 4 µg/mL, respectivamente. Um estudo de dose única de cyphomycina (**14**) no modelo de camundongos neutropênicos de candidíase disseminada exibiu uma dose-resposta

com um log de redução de 0.56 e 0.66 do carga infecciosa quando é tratado com 20 e 40 mg/kg da cyphomycina (**14**), respectivamente, e epoxicyphomycina (**15**) exibiu um log de redução de 0.53 com 40 mg/kg, demonstrando relevância clínica e eficácia de **14** e **15** neste modelo padrão da indústria de infecção por *Candida*. Por outro lado, GT-35 (**16**) matou os ratos 1 hora após a dose de 40 mg/kg.

Palavras-chave: Formigas cultivadoras de fungos, *Escovopsis*, Actinobacterias, *Cyphomyrmex*, *Acromyrmex*, *Leishmania donovani*, antifúngico, policetídeos.

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LIST OF SYMBOLS AND ABBREVIATIONS

Acetone- d_6	Deuterated Acetone
br d	Broad doublet
br s	Broad singlet
°C	Celsius degree
CAMHA	Cation-adjusted Mueller-Hinton Agar
CC ₅₀	50% cytotoxic concentration
CDCl ₃	Deuterated chloroform
¹³ C NMR	Carbon-13 nuclear magnetic resonance
δ	Chemical shift
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
DFT	Density functional theory
dq	Doublet of quartets
DMSO	Dimethyl sulfoxide
DMSO- d_6	Deuterated dimethyl sulfoxide
E.COSY	Exclusive correlation spectroscopy
ELSD	Evaporative Light Scattering Detector
ESI-MS	Electrospray ionization mass spectrometry
EtOAc	Ethyl acetate
<i>g</i> COSY	Gradient correlation spectroscopy
<i>g</i> HSQC	Gradient heteronuclear single-quantum correlation
<i>g</i> HMBC	Gradient heteronuclear multiple-bond correlation
GI%	Growth inhibition in percent
Hax	Hydrogen in axial position
Heq	Hydrogen in equatorial position
¹ H NMR	Proton nuclear magnetic resonance
HPLC	High performance liquid chromatography
HRESIMS	High resolution electrospray Ionization mass spectrometry
HSQC-TOCSY	Heteronuclear Single Quantum Coherence-Total Correlation Spectroscopy
IC ₅₀	The half maximal inhibitory concentration
IR	Infrared
ISP-2	International Streptomyces Project medium 2
K	Kelvin
LB medium	Luria-Bertani medium
m	Multiplet
MeOH	Methanol
MeOH- d_4	Deuterated methanol
mg	Miligram
MHz	Megahertz
MIC	Minimal inhibitory concentration
mm	Milimeter

μL	Microliter
μM	Micromolar
nM	Nanomolar
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser effect
NOE DIFF	Nuclear Overhauser effect differential
NOESY	Nuclear Overhauser effect spectroscopy
NOESY 1D	One-dimensional Nuclear Overhauser effect spectroscopy
q	Quartet
quint	Quintuplet
®	Register trade-mark
ROE	Rotating frame nuclear Overhauser effect
ROESY	Rotating frame nuclear Overhauser effect spectroscopy
RPMI medium	Roswell Park Memorial Institute medium
s	Singlet
SDS	Sodium dodecyl sulfate
sep	septet
SPE	Solid Phase Extraction
t	Triplet
THP-1 cell line	Human leukemia cells
TSE	Tris/Saline/EDTA
t_R	Retention time
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
VCD	Vibrational Circular Dichroism
YPM medium	Yeast-Peptone-Mannitol medium

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1. Introduction

1.1 The fungus-growing ants

Fungus-growing ants (tribe Attini) are native to the Neotropics and comprise more than 230 species, all of which depend on the cultivation of a fungus (phylum Basidiomycota, genus *Leucoagaricus*) for food. They can be divided into five distinct agricultural systems: lower agriculture; coral fungus agriculture; yeast agriculture; generalized higher agriculture; and leaf-cutter agriculture that has evolved more recently to become the dominant herbivores of the New World tropics. The leaf-cutter agriculture involves different species of the two major genera, *Atta* and *Acromyrmex*, with the ability to cut and process fresh vegetation as a nutritional substrate for their fungal garden (Branstetter et al., 2017; Schultz & Brady, 2008). Attine ants have a symbiotic bacterium (phylum Actinobacteria, genus *Pseudonocardia*) that produces secondary metabolites, as dentigerumycin (Oh, Poulsen, Currie, & Clardy, 2009), to suppress the growth of the specialized pathogenic fungus of the genus *Escovopsis* to protect their fungal garden and avoid the extermination of ant colony (**Figure 1**). The actinobacteria are carried in different regions of the ants' cuticle and is transmitted from parent to offspring colonies (Currie, Mueller, & Malloch, 1999). Therefore, fungus-growing ants ecosystem is an interesting model to study symbiosis, defined by "de Bary" as close, long-term associations between different organisms (Wilkinson, 2001).

Figure 1 - Quadripartite symbiotic relationship between three mutualists (Attine ants, actinobacteria and fungal garden *Leucoagaricus*) and one parasite (*Escovopsis*) in fungus-growing ant ecosystem (Pupo, Currie, & Clardy, 2017).

A second conflict theory has been emerged to explain the mutualism between fungus-growing ants and actinobacteria. This model suggests that attine ants could select other antifungal-producing actinobacteria from the environment, including the genera *Streptomyces* and *Amycolatopsis*, to avoid resistance in the fungal pathogens (Barke et al., 2010; Haeder, Wirth, Herz, & Spiteller, 2009; Kost et al., 2007; Sen et al., 2009). One work described *Acromyrmex octospinosus* co-evolved with a *Pseudonocardia* strain and then recruited useful *Streptomyces* strain from the soil (Barke et al., 2010). Other actinomycete bacteria such as *Mycobacterium* and *Microbacterium* have been also found in attine gardens, as well as in queen-pellets of *Atta texana* (Mueller, Dash, Rabeling, & Rodrigues, 2008). Additionally, the bacteria of the genus *Burkholderia* has been described to be involved in this multitrophic interaction (Santos, Dillon, Dillon, Reynolds, & Samuels, 2004), providing evidence of highly diverse of microbial community to protect this ecosystem against pathogens.

Actinobacteria strains have been a source of 2/3 of all naturally derived antibiotics used in therapeutics, and for many anticancer, anthelmintic, and antifungal drugs. Therefore, these microorganisms are of major importance for biotechnology, medicine and agriculture (Barka et al., 2016).

Several actinobacteria strains, such as *Streptomyces* and *Amycolatopsis*, have been also described to produce different types of secondary metabolites that defend other insects as fungus-growing termites, beetles and wasps against pathogens (Beemelmans et al., 2017; Kim et al., 2014; Kroiss et al., 2010; Oh, Poulsen, Currie, & Clardy, 2011; Oh, Scott, Currie, & Clardy, 2009). This provides additional evidence that insect-associated actinobacteria are a promising sources of new bioactive natural products.

1.2. Natural products produced by microorganisms isolated from fungus-growing ant colonies

The first natural product described as a mediator in this ecosystem is the cyclic depsipeptide, dentigerumycin (**Figure 2**), produced by the symbiotic *Pseudonocardia* strain, isolated from the exoskeleton of *Apterostigma dentigerum*. This compound shows selective inhibition against the pathogenic fungus *Escovopsis* sp. and no inhibition against the fungal cultivar. Dentigerumycin also inhibits *Candida albicans* wild type, *C. albicans* ATCC10231 and amphotericin-resistant *C. albicans* ATCC200955 with minimum inhibitory concentration (MIC) values of 1.1 μ M (Oh, Poulsen, et al., 2009). New smaller analogs of dentigerumycin, named gerumycins A-C (**Figure 2**), were isolated from *Pseudonocardia* spp. strains associated

with *Apterostigma* spp and *Trachymyrmex cornetzi* ants. These compounds are at least three orders of magnitude less potent than dentigerumycin at suppressing *Escovopsis* growth (Sit et al., 2015).

Some studies have demonstrated that *Pseudonocardia* strains can inhibit growth of other *Pseudonocardia* isolated from different colonies, showing a possible competition among strains for establishing the symbiotic relationship with an ant colony. Further studies have led to the discovery of a new indolocarbazole, named 9-methoxyrebeccamycin (**Figure 2**), produced by a *Pseudonocardia* strain isolated from *A. dentigerum*. It showed potent activity against a small panel of *Pseudonocardia*. Several structurally related indolocarbazole have been used in clinical trials for different cancer types (Van Arnam, Ruzzini, Sit, Currie, & Clardy, 2015).

Three new angucyclines, pseudonocardones A-C (**Figure 2**), together with the known antibiotic 6-deoxy-8-O-methylrabelomycin and X-14881 E, have been described from a *Pseudonocardia* strain isolated from the same ant species *A. dentigerum*. The new angucyclines did not show significant biological activity, while 6-deoxy-8-O-methylrabelomycin and X-14881 E showed activity against *Bacillus subtilis* 3610 and liver-stage of *Plasmodium berghei* (Carr, Derbyshire, Caldera, Currie, & Clardy, 2012).

A new antifungal polyene macrolide, selvamycin (**Figure 2**), was reported from *Pseudonocardia* strains isolated from *A. dentigerum*. This compound showed MIC of 23 μ M against *C. albicans*, and similar activity against fungi *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, and *Trichoderma harzianum* (Van Arnam et al., 2016).

Figure 2 - Compounds produced by *Pseudonocardia* strains isolated from fungus-growing ants.

Some known antibiotic compounds, candicidin D, actinomycin D, actinomycin X₂, valinomycin, antimycin A₁-A₄ (**Figure 3**) have been identified from *Streptomyces* strains isolated from different colonies of leaf-cutter ants of the genus *Acromyrmex*. These compounds showed high inhibition activity against *Escovopsis*. The presence of valinomycin on the integument of *Acromyrmex* workers and in the waste of some colonies was shown, while the actinomycins were only observed in the waste; supporting the importance of these compounds to keep colonies healthy against pathogenic microorganisms. Actinomycins also have the capability to affect the growth of soil bacteria, as well as other *Streptomyces* and *Pseudonocardia* symbionts. It was also observed that antimycins inhibit the mutualistic fungal garden, *L. gongylophorus* (Haeder et al., 2009; Schoenian et al., 2011). Additionally, the rare

antimycins urauchimycins A and B were identified from *Streptomyces* sp. TD025 isolated from workers of *Trachymyrmex* ants. These compounds were evaluated for antifungal activity against a panel of *Candida* species. Urauchimycin B (**Figure 3**) showed a high activity with MIC values (1-2 $\mu\text{g/mL}$) equivalent to nystatin (Mendes et al., 2013).

Figure 3 - Compounds produced by *Streptomyces* strains isolated from fungus-growing ants.

Several known natural products, melinacindin III and IV, chetracin B and C, shearinine A, B, D and E, 22,23-dehydroshearinine A, cycloarthropsone, emodin, and two novel shearinine derivatives, shearinine L and M (**Figure 4**), were produced by the fungus *Escovopsis weberi* isolated from *Acromyrmex* leafcutter ant colonies. Melinacindin IV and shearinine D can inhibit the growth of mutualist bacterium *Pseudonocardia*. Additionally, shearinine D can reduce worker behavioral defense. Emodin and cycloarthropsone showed potent inhibition against the fungal cultivar *Leucoagaricus gongylophorous*. Emodin was also active against bacterium *Streptomyces* sp. (Dhodary, Schilg, Wirth, & Spiteller, 2018; Heine et al., 2018). This could explain how fungal gardens are sometimes overwhelmed by *Escovopsis*, and also the death of workers in a colony.

Figure 4 - Compounds produced by *Escovopsis weberi* strains isolated from *Acromyrmex* leafcutter ant colonies.

The ecological function and biological activities of natural products reported from microorganisms isolated from fungus-growing ant colonies have increased the interest to explore new antimicrobial compounds that could be aligned to pharmacological activities.

1.3. Leishmaniasis disease

Leishmaniasis is designated as a Neglected Tropical Diseases (NTDs) by the World Health Organization (WHO). There are four clinical forms: cutaneous, mucocutaneous, visceral, and post kala-azar dermal leishmaniasis (PKDL). The visceral leishmaniasis is the most serious clinical form, caused by two leishmanial species, *Leishmania infantum* and *L. donovani* (Chappuis et al., 2007). Around 15 species of *Leishmania* cause the cutaneous leishmaniasis, with species such as *L. major*, *L. tropica* and *L. aethiopica* in the old world and *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. panamensis* and *L. guyanensis* in the new world (Reithinger et al., 2007). The leishmaniasis is transmitted by a sandfly *Phlebotomus* spp. in the old world and *Lutzomya* spp. in the new world. This disease is presented on all continents except Oceania. There are around 250-300 thousands cases, and 20-30 thousands death per year (World Health Organization, 2015). The treatment of leishmaniasis is still incomplete. No drugs were approved against leishmaniasis between 1981 and 2014 (Newman & Cragg, 2016), except for miltefosine (**Figure 5**), originally approved as anticancer and then approved to treat leishmaniasis in 2006 (Dorlo, Balasegaram, Beijnen, & de Vries, 2012). The current drugs used for the treatment of this disease, such as meglumine antimoniate, sodium stibogluconate, amphotericin B, paramomycin, and pentamidine isethionate and miltefosine (**Figure 5**), suffer the limitations of toxicity, variable efficacy, requirements for parenteral administration and/or length of treatment regimens (Barrett & Croft, 2012). Therefore, the discovery of new chemical entities and development of new drugs is important to overcome the impact of this protozoan disease.

Figure 5 - Drugs used for the treatment of the leishmaniasis

1.4. Human fungal infections

The most common fungal diseases in humans happen in the skin and nails, affecting around 1.7 billion of people worldwide, caused by dermatophytes. This incidence increases with age of 70 year and older (Havlickova, Czaika, & Friedrich, 2008; Thomas et al., 2010). Mucosal infections of the oral and genital tracts are also common, especially vulvovaginal candidiasis (Sobel, 2007). Many patients with HIV/AIDS in regions with limited care suffer oral thrush (10 million cases) and esophageal fungal infections (2 million cases) annually. Oral infections have been found in babies, denture wearers, in individuals who use inhaled steroids for asthma, in leukemia, transplant and radiotherapy patients. These superficial infections are caused mainly by several species of *Candida* (Brown et al., 2012).

The incidence of invasive fungal infections is lower than superficial infections, but is responsible for a high mortality rate (≈ 1.5 million per year). It could be similar or higher than

tuberculosis or malaria, worldwide. The most common genera of fungal-related deaths are *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis* (Brown et al., 2012).

The early antifungals used in the 1950s, such as amphotericin B (**Figure 5**), were characterized with limited efficacy and toxicity. In the 1980s the triazoles were developed, available in intravenous and oral formulations, with more effectivity against fungal pathogens. The last decades the newest classes of antifungals were developed, named echinocandins. They are the first class of antifungal agents that act against a specific component of the fungi and not mammalian cells (Ostrosky-Zeichner, Casadevall, Galgiani, Odds, & Rex, 2010). The echinocandins (caspofungin, anidulafungin and micafungin) and the third-generation triazoles (voriconazole and posaconazole) (**Figure 6**), are some of the best current drugs used for treatment of many fungal diseases (Ostrosky-Zeichner et al., 2010). But, complications such as toxicity, undesirable drug interactions and antifungal resistant problems have emerged (Victoria Castelli, Gabriel Derita, & López, 2017), therefore there is an urgent need for new antifungal agents.

Figure 6 – Antifungal drugs in clinical use for humans

2. AIM OF THIS STUDY

Considering the high potential of bacterial symbionts of fungus-growing ants in producing selective antifungal compounds, the aims of this PhD thesis are:

- To study the natural products produced by actinobacteria associated to Brazilian fungus-growing ants.
- To identify active compounds against the pathogenic fungus *Escovopsis* sp, *Leishmania donovani* and human pathogenic fungi as *Aspergillus fumigatus*, *Candida albicans*, *C. glabrata* and *C. auris*.

3. MATERIAL AND METHODS

3.1. General Experimental Procedures

Reversed Phase HPLC was performed using a Shimadzu Prominence HPLC system. The HPLC purification of **1-3**, **5-8** and **10-12** were carried out with a C₆-Phenyl semipreparative column (Phenomenex Luna, C₆-Phenyl 5µm, 250 x 10 mm). The HPLC purification of **13-16** were carried out with a C₁₈ semipreparative column (Phenomenex Luna, C₁₈(2), 5µm, 250 x 10 mm). Mass spectrometry data of **1-3**, **5-8** and **15** were acquired with a UPLC (Shimadzu) coupled to a micrOTOF II mass spectrometer (Bruker Daltonics); MS data of **4**, **10** and **13** were acquired with an Accela UHPLC (Thermo Scientific, USA) apparatus with an 80 Hz photodiode array detector (PDA) coupled to a head electrospray ionization source and an Exactive Plus mass spectrometer with an Orbitrap analyzer (Thermo Scientific, USA); and the gas chromatography mass spectrum of **9** was recorded in a Gas Chromatograph-Mass Spectrometer (Shimadzu) in the School of Pharmaceutical Sciences of University of São Paulo-Ribeirão Preto, SP, Brazil (FCFRP-USP). HR-ESI-MS data and of **11**, **12**, **14** and **16** were acquired with a Bruker MaXis Ultra-High Resolution Quadrupole Time-of-Flight MS coupled to Waters Acquity UPLC system operated by Bruker Hystar software in the School of Pharmacy of University of Wisconsin (UW)-Madison, WI, USA. The 1 and 2D NMR spectra of **4** and **10-13** were obtained in a Varian Unity-Inova 500 MHz spectrometer (School of Pharmacy, UW-Madison); while data of **14-16** were obtained with a Bruker Avance 500 MHz (500I) with 5 mm a ¹³C/¹⁵N(¹H) cryogenic probe (National Magnetic Resonance Facility at Madison, UW-Madison). ROESY, NOESY and NOESY-1D data of compounds **1**, **2**, **4**, **5**, **6** and **13-16**; and 1

and 2D NMR spectra of **1-3** and **5-8** were recorded with 500 MHz (DRX-500) (Bruker UK, Coventry, UK) in the Department of Chemistry of University of São Paulo-Ribeirão Preto (FFCLRP-USP), SP, Brazil. The E.COSY of **14** was recorded with a Bruker Avance III 600 MHz spectrometer in the Department of Chemistry, Federal University of São Carlos – UFSCar, SP, Brazil. Specific optical rotations were recorded in a P-2000 digital polarimeter (Jasco, Tokyo, Japan) at FCFRP-USP. IR and VCD spectra of **1, 2, 5** and **6** were recorded with a Single-PEM ChiralIR-2X FT-VCD spectrometer (BioTools, Inc.), in the Department of Chemistry, UFSCar, using a resolution of 4 cm^{-1} and a collection time of 8 h. The optimum retardation of the ZnSe photoelastic modulator (PEM) was set at 1400 cm^{-1} . Minor instrumental baseline offsets were eliminated from the final VCD spectrum by subtracting the VCD spectrum of each compound from that of the solvent obtained under identical conditions. The IR and VCD spectra were obtained in a BaF₂ cell with 100 μm path length using DMSO-*d*₆ (for **1** and **2**) and methanol-*d*₄ (for **5** and **6**) as solvents. Samples were prepared using 5 mg of each compound dissolved in 140 μL of solvent.

3.2. Collection and isolation of bacteria from fungus-growing ants

All ants and fungal gardens were collected between August of 2014 and October of 2015 at the campus of the University of São Paulo-Ribeirão Preto, as part of the ICBG-Brazil project (FAPESP/FIC-NIH Thematic Project 2013/50954-0) (Pupo et al., 2017), according to the permits SISBIO 46555-5 and CNPq 010936/2014-9. Workers of *Acromyrmex rugosus rugosus* colony were collected in location S 21° 09' 46.9"; W 47° 51' 40.0". Fungal garden and workers of the *Acromyrmex subterraneus brunneus* colony were collected in location S 21° 10' 13.0" ; W 47° 50' 49.4". Workers and winged male ants of a *Cyphomyrmex* colony were collected in location S 21°10'04.4" W 47°50'49.5".

Fungal gardens or ants collected were washed with 500 μL of sterile deionized water, vortexed for 30 seconds and then plated on International *Streptomyces* ISP-2 (per liter: 4 g yeast extract, 10 g malt extract, 4 g glucose) or chitin medium (per liter: 4g chitin) supplemented with the antifungals nystatin and cycloheximide (per liter: 0.7 g K₂HPO₄, 0.3 g KH₂PO₄, 0.5 g MgSO₄·5H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g ZnSO₄·7H₂O, 0.01 g MnCl₂·4H₂O, 20 g of agar, 0.04 g/L nystatin, and 0.05 g/L cycloheximide). After two weeks of growth at 28 °C, bacterial colonies were subcultured onto ISP-2 agar with antibiotics (0.04 g/L nystatin, and 0.05 g/L cycloheximide) (Poulsen & Currie, 2010) (**Figure 7**). The bacteria were placed in the freezer

at -80 °C in 1.5 mL Eppendorf vials or 2 mL screw top tubers containing 500 µL of ISP-2 with 20% of glycerol.

Seventy-seven bacteria were isolated from *Acromyrmex rugosus rugosus* and *Acromyrmex subterraneus brunneus* colonies at the beginning of this project (**Table 1**). Then, two additional bacteria (ICBG292 and ICBG311) were isolated from the *Cyphomyrmex* colony by Dr. Weilan (Postdoc of Prof. Mônica T. Pupo).

Figure 7 - Isolation procedure of actinobacteria associated to fungus-growing ants.

3.3. Bacterial identification procedure

3.3.1. DNA extraction for bacterial identification

3.3.1.1. *Streptomyces puniceus* AB10, *Nocardioides albus* AB12 and *Streptomyces chartreusis* AC70

Genomic DNA was extracted using a modified protocol (Sharma & Singh, 2005). Actinobacteria were grown in 4 mL of liquid medium ISP-2 at 30 °C for 72h. After this period, the cultures were centrifuged at 5,000 g for 5 minutes. The supernatant was discarded and the culture pellet transferred to a 2.0 mL Eppendorf tube with 500 µL of TE buffer 0.01X. It was added 3 µL of RNase to digest the RNA and then added 400 µL of solution I (1% Sarkosyl, 0.5M NaCl, 1% SDS). The tubes were kept for 10 min at 37 °C with continuous stirring. Then,

400 μ L of PCI (phenol: chloroform: isoamyl alcohol; 25:24:1) were added and mixed by inversion and centrifuged at 10,000 g for 5 min at 37 °C, followed by carefully transferring the supernatant to a new 1.5 mL Eppendorf tube. Then, 10% of the total volume of sodium acetate (3 M, pH 5.2) and 60% isopropanol were added and gently mixed by inverting tube 5 to 10 times. The tubes were centrifuged for 5 min at 10,000 g at 37 °C. The DNA was precipitated in the pellet and the liquid removed. The pellet was washed with 1 mL of 70% ethanol and centrifuged at 10,000 g for 3 min at 37 °C. The supernatant was removed and the DNA after dried was resuspended in 20 μ L of TE 0.01X.

3.3.1.2. *Streptomyces luteogriseus* AB11, *Amycolatopsis orientalis* AC44, *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292 and *Streptomyces sioyaensis* ICBG311

The DNA extraction procedure was modified from Kumar et al. (Kumar, Bharti, Gusain, & Bisht, 2010), in which the pellet was washed in 500 μ L of 10.3% sucrose, centrifuged for 1 min at 10,000 g and the supernatant discarded. Then 450 μ L of TSE + lysozyme were added and incubated for 20-30 min at 37 °C. After, 13 μ L of proteinase K was added and incubated for another 15 min at 55 °C and then 250 μ L of 2% SDS, gently mixed until to form a clear solution. Then 300 μ L of phenol: chloroform pH 8.0 were added and mixed and centrifuged for 10 min at 4 °C. The supernatant was transferred to another tube and 60 μ L of 3M NaOAc, pH 6.0 + 700 μ L of isopropanol was added. The contents were mixed until "white strings" appeared and then centrifuged for 1 min to 10,000 g, and the supernatant discarded. The pellet was washed with 70% ethanol and centrifuged again at 10,000 g for 1 min. After being left overnight to completely dry the ethanol, the DNA was resuspended in 30 μ L of deionized H₂O.

Some steps were added for sequencing of actinobacteria genomes. The pellets were resuspended in 500 μ L TE+10 μ L of RNase and the tubes were rocked for 15 min at room temperature to dissolve the pellet and then 100 μ L 5 M NaCl were added and mixed. Following, 70 μ L of CTAB/NaCl mixture were added, mixed and incubated for 10 min at 55 °C and then cooled to 37 °C. After this, 500 μ L of chloroform were mixed and centrifuged for 10 min at room temperature. The top layers were transferred to a new tube and 300 μ L of phenol and 150 μ L of chloroform were mixed and centrifuged for 10 min at 4 °C. The top layers were transferred with a wide bore tip to a tube containing 300 μ L of CHCl₃, mixed and centrifuged briefly for 1 min and then the top layers were transferred to a tube containing 50 μ L of NaOAc pH 6.0 + 400 μ L of isopropanol and mixed until "white strings" appear and solution to be uniform. The DNA were centrifuged briefly, poured off the supernatant and the remainder

pipetted off. And then, the DNA were resuspended in 100-200 μL of ultra-pure H_2O . The DNA were quantified and checked for quality in NanoDrop® (Thermo Scientific).

3.3.2. PCR Amplification

3.3.2.1. *Streptomyces puniceus* AB10, *Nocardioides albus* AB12 and *Streptomyces chartreusis* AC70

The 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction (PCR) using a pair of universal primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (Ludwig, 2007). The PCR reactions contained 2 μL of DNA (10 ng/ μL), 2.0 μL of each primer (10 μM), 2.5 μL of 10X buffer, 2.0 μL of MgCl_2 (25 mM), 4.0 μL of dNTPs (1.25 mM each base), 0.2 μL Taq-polymerase (5 U/ μL) and 10.3 μL of sterile milli-Q water for a total volume of 25.0 μL . PCR was performed in a thermocycler (Applied Biosystems), in which amplification conditions were initiated by denaturation step with one cycle at 95 °C for 3 minutes, followed by an annealing step and extension in 35 cycles at 95 °C for 1 minute, 60 °C for 1 minute and 72 °C for 2 minutes and a final extension cycle at 72 °C for 15 minutes, and maintained at 10 °C. Amplification products were subjected to agarose gel electrophoresis 1% in 1X TBE buffer and stained with Gel redTM (Biotium). Purification was performed with EDTA-ethanol.

3.3.2.2. *Streptomyces luteogriseus* AB11, *Amycolatopsis orientalis* AC44, *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292 and *Streptomyces sioyaensis* ICBG311

PCR amplification of the 16S rRNA gene of actinobacteria was performed using the same two primers: 27F and 1492R. The EconoTac® DNA Polymerase Kit (Lucigen, USA) was used and the final reaction volume of 15 μL contained: 8 μL Econotaq, 0.5 μL of each primer 27F and 1492R, 0.5 μL DMSO, 4.5 μL Deionized H_2O and 1 μL DNA (10 ng/ μL). Amplification followed the following profile: an initial denaturation step at 94 °C for 3 min followed by 32 cycles of amplification of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 minutes and a final extension step of 72 °C for 5 min. The PCR product was detected by agarose gel electrophoresis and visualized by ultraviolet (UV) fluorescence after staining with ethidium bromide.

3.3.3. Sequencing and purification reaction

3.3.3.1. *Streptomyces puniceus* AB10, *Nocardioideis albus* AB12 and *Streptomyces chartreusis* AC70

The same primers 27F and 1492R and the inner primer U519F (5'-CAGCMGCCGCGGTAATWC-3') were used for the sequencing reaction of 16S rRNA gene. The reactions containing 2.0 µL 5X buffer, 0.32 µL of primer (10 µM), 0.3 µL of BigDye 3.1, about 20 ng of purified DNA and sterile milli-Q water to complete a total volume of 10 µL. The thermocycler program consisted of: 28 cycles 95 °C for 15 seconds, followed by 50 °C for 45 seconds and 60 °C for 4 minutes, being completed and maintained at 10 °C. The sequencing reaction was purified according to the instructions in the manual BigDye (Applied Biosystems).

3.3.3.2. *Streptomyces luteogriseus* AB11, *Amycolatopsis orientalis* AC44, *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292 and *Streptomyces sioyaensis* ICBG311

The primers 27F and 1492R were used again for the sequencing of the 16S rRNA gene. The sequencing reaction of the PCR products contained: 1.5 µL 5X buffer, 1 µL primer (10 µM), 1 µL BigDye 3.1 (Applied Biosystems), 0.5 µL DMSO, 1 µL PCR product DNA and deionized water to make up the total volume of 10 µL. The program used consisted of 95 °C for 3 min, followed by 35 cycles of 96 °C for 10s, 58 °C for 3 min and a final extent of 72 °C for 7 min. The sequencing reaction was purified with the Axyprep Mag Dyclean purification kit (Axygen) in which 5 µL of magnetic beads solution and 31 µL of 85% ethanol were added for each reaction. The tubes were placed on a magnetic plate for 3 min and then the liquid was removed. 100 µL of 85% ethanol was added for 30s and then the liquid was discarded. 100 µL of 85% ethanol was added again for 30s and after discarded. The liquid was removed as much as possible with a pipette and left overnight to completely dry the ethanol. The DNA was resuspended in 25 µL of deionized H₂O.

3.3.4. Sequencing of the 16S rRNA gene and editing sequences

The sequencing of actinobacteria *Streptomyces puniceus* AB10, *Nocardioideis albus* AB12, *Streptomyces filamentosus* AC14 and *Streptomyces chartreusis* AC70 was performed in equipment ABI 3500 (Applied Biosystems) in the School of Pharmaceutical Sciences of

University of São Paulo-Ribeirão Preto, SP, Brazil. The sequencing of actinobacteria *Streptomyces luteogriseus* AB11, *Amycolatopsis orientalis* AC44, *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292 and *Streptomyces sioyaensis* ICBG311 was performed at the Center for Genetics and Biotechnology at the University of Wisconsin - Madison (Biotech Center, UW - Madison, WI, USA). The sequences were edited and used for assembly of the contigs in the BioEdit v7.2.5 and SecMan Pro Software (DNASTAR). Contigs were used to search for homologous sequences in the NCBI - GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Eztaxon (<http://www.ezbiocloud.net/eztaxon/> identify).

3.4. Culture of actinobacteria and isolation of compounds methodologies

3.4.1. Culture media compositions used in this work

- a) **Solid ISP-2 medium:** 4 g yeast extract, 10 g malt extract, 4 g glucose, and 20 g agar per liter.
- b) **Liquid ISP-2 medium:** 4 g yeast extract, 10 g malt extract, and 4 g glucose per liter.
- c) **Liquid YPM medium:** 5 g yeast extract, 3 g peptone, and 25 g mannitol per liter.
- d) **Liquid A-medium:** 20 g soluble starch, 10 g glucose, 5 g peptone, 5 g yeast extract, 5 g CaCO₃ per liter.
- e) **DSC medium:** 5 g soluble starch, 10 g glucose, 5 g peptone and 5 g yeast extract per liter.
- f) **CAMHA medium:** 1.5 g soluble starch, 17.5 g casein, 3 g beef extract, 15 g agar, 12.5 mg Mg²⁺, 25 mg Ca²⁺ per liter.
- g) **LB medium:** 10 g NaCl, 10 g tryptone, 5 g yeast extract per liter.

3.4.2. Small scale solid cultures of bacteria isolated from fungus-growing ants for antiparasitic bioassays

Seed cultures were initially grown in 10 mL of ISP-2 (1 falcon tube of 50 mL) and were mounted into a shaker for 4-7 days at 30 °C and 200 rpm. It was used 0.6 mL of seed culture to inoculate each ISP-2 agar plate (100 x 20 mm, 25 mL of medium). Five plates were used to culture each bacterium (77 strains isolated from *Acromyrmex rugosus rugosus* and *Acromyrmex subterraneus brunneus* colonies) during seven days at 30 °C. The solid agar was cut into small

cubes and soaked three times in EtOAc overnight. The solvent was filtered and dried under vacuum to give the crude extract. These extracts were tested against *L. donovani* and *T. cruzi*.

3.4.3. Small scale liquid cultures of *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292 and *Streptomyces sioyaensis* ICBG311 for antiparasitic bioassays (Carried out at Bugni lab, UW-Madison, USA).

Micro-scale fermentation: Each actinobacterium was initially grown in 300 μ L seed culture (Corning® 96 well cluster tubes) in DSC medium during 1 week at 30 °C and 300 rpm. Detoxified polypropylene square 96-deepwell microplates (Enzyscreen, The Netherlands) were autoclaved separately. Then, 50 μ L of these seed cultures were transferred to three micro-tube (2 mL) containing 500 μ L of the medium (ISP-2, YPM and A-medium). Culture plates were covered using low-evaporation sandwich covers (Enzyscreen, The Netherlands). Then, they were mounted into a shaker during two weeks at 30 °C and 300 rpm (Adnani et al., 2015).

Sample Preparation: An aliquot (500 μ L) of MeOH was added in each well of microplates used in micro-scale fermentation and kept during 1 h in the bench to kill the microorganisms. The plates were placed in the SpeedVac concentrator (Thermo electron corporation) for 90 min to remove the MeOH. The supernatants ($\approx$ 500 μ L) were subjected to SPE-C18 (Strata® C18-E, 55 μ m, 70 Å, 100 mg / well, 96-Well Plates), washed using H₂O (600 μ L) to remove media components/primary metabolites, eluted with MeOH (900 μ L) directly into megatiter™ plates (Neptune, ref 2405.X) and dried in the SpeedVac to get crude extracts (Hou et al., 2012). The amount of extract was quantified by Evaporative Light Scattering Detector (ELSD) (Adnani, Michel, & Bugni, 2012). These extracts were tested against *L. donovani* and *T. cruzi*.

3.4.4. Cultivation of *Streptomyces puniceus* AB10 and purification of compounds 1-4

Seed cultures were initially grown in 100 mL of ISP-2 (4 falcon tubes of 50 mL) and were mounted into a shaker for 4-7 days at 30 °C and 200 rpm. It was used 0.8 mL of seed culture to inoculate each ISP-2 agar plate (140 x 20 mm, $\approx$ 70 mL of medium). This bacterium was grown on solid ISP-2 medium in 70 Petri plates (5.0 L total) for 10 days at 30 °C. The solid agar was cut into small cubes and soaked three times in EtOAc overnight. The solvent was filtered and dried under vacuum to give the crude extract (1.281 g). The extract was dissolved in 90% MeOH-H₂O (200 mL), decanted and dried to give two fractions: *AB10.A* (soluble, 1061.00 mg) and *AB10.B* (insoluble, 160.00 mg).

Fraction *AB10.B* was purified by SPE-Si (55 μ m, 10 g/60 mL, Phenomenex) using the following gradient: 100 mL (100 % Hexane, *AB10.B1*: 6.62 mg); 100 mL (80% Hexane-EtOAc, *AB10.B2*: 2.42 mg); 100 mL (60% Hexane-EtOAc, *AB10.B3*: 4.25 mg); 100 mL (40% Hexane-EtOAc, *AB10.B4*: 6.13 mg); 100 mL (20% Hexane-EtOAc, *AB10.B5*: 3.61 mg); 100 mL (100% EtOAc, *AB10.B6*: 7.25 mg); and 100 mL (100 % MeOH, *AB10.B7*: 8.14 mg, compound **1**).

The fraction *AB10.A* was purified by SPE-C₁₈ (55 μ m, 10 g/60 mL, Phenomenex) using the following gradient: 150 mL (10% MeOH-H₂O, *AB10.A1*: 168.09 mg); 150 mL (20% MeOH-H₂O, *AB10.A2*: 46.77 mg); 150 mL (40% MeOH-H₂O, *AB10.A3*: 52.79 mg); 150 mL (60% MeOH-H₂O, *AB10.A4*: 219.08 mg); 150 mL (80% MeOH-H₂O, *AB10.A5*: 105.34 mg); 200 mL (100% MeOH, *AB10.A6*: 338.97 mg); and 200 mL (100 % acetone, *AB10.A7*: 110.19 mg).

The fraction *AB10.A5* was purified by semi-preparative HPLC using the column C₆-Phenyl (5 μ m, 250 x 10 mm) and the following gradient at 3 mL/min: 0-17 min, linear gradient from 30% CH₃CN-H₂O to 88.9% CH₃CN-H₂O; 17-17.5 min, linear gradient from 88.9% CH₃CN-H₂O to 100% CH₃CN; 17.5-20 min, isocratic flow of 100% CH₃CN; and 20-21 min, linear gradient from 100% CH₃CN to 30% CH₃CN-H₂O to give five fractions [*AB10.A5.1* (0.57 mg), *AB10.A5.2* (9.04 mg), *AB10.A5.3* (4.87 mg, compound **2**, *t_R* = 12.1 min), *AB10.A5.4* (5.05 mg, compound **3** and some impurities, *t_R* = 14.4 min) and *AB10.A5.5* (10.82 mg)].

The fraction *AB10.A6* was purified by SPE-Si (55 μ m, 10 g/60 mL, Phenomenex) using the following gradient: 100 mL (100% Hexane, *AB10.A6.1*: 13.80 mg); 150 mL (8:2 Hexane-EtOAc, *AB10.A6.2*: 194.4 mg); 100 mL (6:4 Hexane-EtOAc, *AB10.A6.3*: 15.46 mg, compound **4**); 100 mL (4:6 Hexane-EtOAc, *AB10.A6.4*: 6.25 mg); 100 mL (2:8 Hexane-EtOAc, *AB10.A6.5*: 3.63 mg); 200 mL (100% EtOAc, *AB10.A6.6*: 17.65 mg); and 200 mL (100% MeOH, *AB10.A6.7*: 66.43 mg).

3.4.5. Cultivation of *Streptomyces chartreusis* AC70 and purification of compounds 5-9

Seed cultures were initially grown in 100 mL of ISP-2 (4 falcon tubes of 50 mL) and were mounted into a shaker for 4-7 days at 30 °C and 200 rpm. It was used 0.8 mL of seed culture to inoculate each ISP-2 agar plate (140 x 20 mm, $\approx$ 70 mL of medium). This bacterium was grown on solid ISP-2 medium in 151 Petri plates (10.7 L total) for 10 days at 30 °C. The solid agar was cut into small cubes and soaked three times in EtOAc overnight. The solvent was filtered and dried under vacuum to give the crude extract (3.3 g). The extract was dissolved in 90% MeOH-H₂O (200 mL), decanted and dried to give two fractions: *AC70.A* (soluble, 1802.00 mg) and *AC70.B* (insoluble, 452.13 mg).

Fraction AC70.A was purified by SPE-C₁₈ (55 µm, 10 g/60 mL, Phenomenex) using the following gradient: 200 mL (10% MeOH-H₂O, AC70.A1: 613.97 mg); 200 mL (20% MeOH-H₂O, AC70.A2: 97.68 mg); 200 mL (40% MeOH-H₂O, AC70.A3: 43.93 mg); 200 mL (60% MeOH-H₂O, AC70.A4: 82.61 mg); 200 mL (80% MeOH-H₂O, AC70.A5: 165.22 mg); 200 mL (100% MeOH, AC70.A6: 511.37 mg); and 200 mL (100% Acetone, AC70.A7: 209.00 mg).

Fractions AC70.A1 - AC70.A5 were combined and purified by semi-preparative HPLC using C₆-Phenyl column (5µm, 250 x 10 mm) and the following gradient at 3 mL/min: 0-5 min, isocratic flow of 5% CH₃CN-H₂O; 5-23 min, linear gradient from 5% CH₃CN-H₂O to 50% CH₃CN-H₂O; 23-24 min, linear gradient from 50% CH₃CN-H₂O to 100% CH₃CN; 24-25 min, isocratic flow of 100 % CH₃CN; and 25-26 min, from 100% CH₃CN to 5% CH₃CN-H₂O to give six fractions [AC70.C1 (9.4 mg); AC70.C2 (19.19 mg); AC70.C3 (25.66 mg); AC70.C4 (42.91 mg); AC70.C5 (7.29 mg); and AC70.C6 (58.74 mg)].

Fraction AC70.C2 was further purified by semi-preparative HPLC using C₆-Phenyl column (Phenomenex Luna, 5µm, 250 x 10 mm) and an isocratic flow of 10% CH₃CN at 6 mL/min to yield strepchazolin A (**5**) (3.60 mg, *t_R* = 18.4 min) and strepchazolin B (**6**) (3.49 mg, *t_R* = 16.7 min).

Fraction AC70.C4 was purified by semi-preparative HPLC using C₆-Phenyl column (Phenomenex Luna, 5µm, 250 x 10 mm) and an isocratic flow of 10% CH₃CN at 6 mL/min to yield **7** (1.77 mg, *t_R* = 22.6 min) and **8** (4.47 mg, *t_R* = 24.8 min).

The fractions AC70.B, AC70.A6 and AC70.A7 were combined and purified by SPE-Si (55 µm, 10 g/60 mL, Phenomenex) using the following gradient: 100 mL (100% Hexane, AC70.B1: 280.38 mg); 100 mL (9:1 Hexane-EtOAc, AC70.B2: 498.90 mg); 100 mL (8:2 Hexane-EtOAc, AC70.B3: 85.72 mg); 100 mL (7:3 Hexane-EtOAc, AC70.B4: 22.14 mg); 100 mL (6:4 Hexane-EtOAc, AC70.B5: 55.07 mg); 100 mL (1:1 Hexane-EtOAc, AC70.B6: 7.33 mg); 100 mL (4:6 Hexane-EtOAc, AC70.B7: 5.29 mg); 100 mL (2:8 Hexane-EtOAc, AC70.B8: 5.62 mg); 100 mL (100% EtOAc, AC70.B9: 6.21 mg); and 100 mL (100% MeOH, AC70.B10: 117.44 mg). The fraction AC70.B1 was purified by solubility: AC70.B1.1 (Soluble in Acetone, 268.03 mg) and AC70.B1.2 (Insoluble in Acetone, 12.35 mg, compound **9**).

3.4.6. Cultivation of *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292 and purification of compounds 10-12

Seed cultures were initially grown in 40 mL of ISP-2 (four 25 × 150 mm tubes) and were mounted into a shaker for 4-7 days at 28 °C and 200 rpm. It was used 40 mL of seed culture to

inoculate 1 L of medium. This bacterium was grown in liquid ISP-2 medium using a Fernbach flask (1 L of medium in a flask of 2.8 L + 70 g of HP20) and placed in shakers for 7 days at 28 °C and 200 rpm. The HP20 was filtered and washed with distilled water and soaked with Acetone. The organic solvent was filtered and dried under vacuum. Then, a partition of Ethyl Acetate/Water was performed, and the organic phase was separated and dried to give the crude extract (271.70 mg). Extract was purified by SPE-C₁₈ (55 µm, 1 g) using the following gradient: 10 mL (20% MeOH-H₂O, *ICBG292.1*: 55.10 mg); 10 mL (40% MeOH-H₂O, *ICBG292.2*: 23.50 mg); 10 mL (60% MeOH-H₂O, *ICBG292.3*: 14.50 mg); 10 mL (80% MeOH-H₂O, *ICBG292.4*: 47.20 mg); and 10 mL (100% MeOH, *ICBG292.5*: 85.50 mg).

The fraction *ICBG292.4* and *ICBG292.5* were mixed and purified by semi-preparative HPLC using the column C₆-Phenyl (5µm, 250 x 10 mm) and the following gradient at 4 mL/min: 1-20 min, linear gradient from 70% MeOH-H₂O to 100% MeOH; 20-25 min, isocratic flow of 100% MeOH; 25-25.5 min, linear gradient from 100% MeOH to 70% MeOH-H₂O; 25.5-30.5 min, isocratic flow of 70% MeOH-H₂O to give 13 fractions [*ICBG292.5.1* (2.50 mg); *ICBG292.5.2* (5.00 mg, *t_R* = 14.0 min, compound **10**); *ICBG292.5.3* (3.10 mg); *ICBG292.5.4* (2.60 mg); *ICBG292.5.5* (3.10 mg); *ICBG292.5.6* (15.50 mg, *t_R* = 18.1 min, compound **11**), *ICBG292.5.7* (10.90 mg), *ICBG292.5.8* (2.20 mg), *ICBG292.5.9* (14.30 mg, *t_R* = 22.5 min, compound **12**), *ICBG292.5.10* (1.60 mg), *ICBG292.5.11* (0.90 mg), *ICBG292.5.12* (1.20 mg), *ICBG292.5.13* (4.30 mg)]. Compounds **10-12** were isolated during the BEPE project (FAPESP 2016/20154-0) at the University of Wisconsin-Madison in 2017.

3.4.7. Culture of *Streptomyces sioyaensis* ICBG311 and purification of compounds 13-16

Seed cultures were initially grown in 200 mL of ISP-2 (twenty 25 × 150 mm tubes) and were mounted into a shaker for 4-7 days at 28 °C and 200 rpm. It was used 40 mL of seed culture to inoculate 1 L of medium. This bacterium was grown in liquid A-medium using Fernbach flasks [5 x (1 L of medium in a flask of 2.8 L + 70 g of HP20)] and placed in shakers for 7 days at 28 °C and 200 rpm. The HP20 was filtered and washed with distilled water and soaked with Acetone. The organic solvent was filtered and dried under vacuum. Then, a partition of EtOAc/H₂O was performed, and the organic phase was separated and dried to give the crude extract (2.3044 g). Extract was purified by SPE-C₁₈ (55 µm, 20 g) using the following gradient: 200 mL (20% MeOH-H₂O, *ICBG311.A1*: 72.80 mg); 200 mL (40% MeOH-H₂O, *ICBG311.A2*: 152.50 mg); 200 mL (60% MeOH-H₂O, *ICBG311.A3*: 132.30 mg); and 200 mL (100% MeOH, *ICBG311.A4*: 1934.90 mg).

Fraction *ICBG311.A3* was purified by semi-preparative HPLC using the column C₁₈ semipreparative (Phenomenex Luna, C₁₈(2), 5 μ m, 250 x 10 mm) and the following gradient at 4 mL/min: min 1-20 (linear gradient from 10% MeOH-H₂O to 100% MeOH); min 20-25 (isocratic flow of 100% MeOH); min 25-25.5 (linear gradient from 100% MeOH to 10% MeOH-H₂O); and min 22.5-30.5 (isocratic flow of 10% MeOH-H₂O) to give 3 fractions [*ICBG311.A3.1* (6.30 mg); *ICBG311.A3.2* (1.90 mg); *ICBG311.A3.3* (3.10 mg, compound **13**, t_R = 18.8 min)].

The fraction *ICBG311.A4* was purified by semi-preparative HPLC using the column C₁₈ semipreparative (Phenomenex Luna, C₁₈(2), 5 μ m, 250 x 10 mm) and the following gradient of MeOH and H₂O (containing 0.1% of acetic acid) at 4 mL/min: min 1-20 (linear gradient from 80% MeOH-H₂O to 100% MeOH); min 20-22 (isocratic flow of 100% MeOH); min 22-22.5 (linear gradient from 100% MeOH to 80% MeOH-H₂O); and min 22.5-27.5 (isocratic flow of 80% MeOH-H₂O) to yield compound **14** (89.0 mg, t_R = 11.3 min), **15** (14.2 mg, t_R = 11.8 min) and **16** (33.7 mg, t_R = 12.3 min). Compounds **13-16** were isolated during the BEPE project (FAPESP 2016/20154-0) at the University of Wisconsin-Madison in 2017.

3.4.8. Large scale culture of *Streptomyces luteogriseus* AB11, *Nocardioides albus* AB12 and *Amycolatopsis orientalis* AC44 and fractionation

Seed cultures were initially grown in 100 mL of ISP-2 (4 falcon tubes of 50 mL) and were mounted into a shaker for 4-7 days at 30 °C and 200 rpm. It was used 0.8 mL of seed culture to inoculate each ISP-2 agar plate (140 x 20 mm, $\approx$ 70 mL of medium). Each bacterium was grown on solid ISP-2 medium in 71 petri plates (5.0 L) for 10 days at 30 °C. The solid agar was cut into small cubes and soaked three times in EtOAc overnight. The solvent was filtered and dried under vacuum to give the crude extract. Extract was purified by SPE-C18 (55 μ m, 20 g) using the following gradient: 200 mL (20% MeOH-H₂O); 200 mL (40% MeOH-H₂O); 200 mL (60% MeOH-H₂O); 200 mL (80% MeOH-H₂O); and 200 mL (100% MeOH).

3.5. Bioassays

3.5.1. Antifungal bioassays

3.5.1.1. Antagonim bioassay against fungi (Method 1)

The bacteria, fractions or pure compounds were tested against one strain of the fungus *Escovopsis* sp (isolated from *Atta* sp. colony) and also against the entomopathogen *Beauveria bassiana*. 150 µL of a spore solution (1×10^6) of the pathogenic fungus was added on ISP-2 agar plates (250 x 20 mm, 25 mL of medium), distributed with Drigalsky's spatula and dried inside laminar flow chamber. The bacteria were placed in the center of the plate (**Figure 8**); and fractions or pure compounds were placed using sterile paper discs. Then, they were incubated during 7-14 days at 30 °C.

3.5.1.2. Antagonim bioassay against fungi (Method 2)

In a second bioassay, bacteria were tested against four different *Escovopsis* strains: *Escovopsis* HH141108-02, LK141126-01, and LK141108-03 isolated from different *Acromyrmex* colonies; and AB150620-3 isolated from a *Trachymyrmex* colony to compare selectivity. ISP-2 agar plates (250 x 20 mm, 25 mL of medium) were used for the bioassays. The agar was cut out to leave four 4-cm wide tracks (**Figure 9**). Three bacteria were placed at the end of each of three tracks in each Petri dish, *Escovopsis* in the center, and one track remains blank as a control. The assays were monitored during 21 days at 30 °C (Gerardo, Jacobs, Currie, & Mueller, 2006; Rodrigues, Cable, Mueller, Bacci, & Pagnocca, 2009).

Figure 8 - Antagonism bioassays of active bacteria against *Escovopsis* sp (isolated from *Atta* colony) and *Beauveria bassiana* using (Method 1): A) Active bacteria against *Escovopsis* sp, B) Negative control of *Escovopsis* sp, C) Positive control with Miconazole (50 μ g) against *Escovopsis* sp. D) Active bacteria against *B. bassiana*, E) Negative control of *Beauveria bassiana*, F) Positive control with Miconazole (50 μ g) against *B. bassiana*.

Figure 9 - Antagonism bioassays against fungi (Method 2): A) bacteria strains I, II and III were placed at the end of each of three tracks, and one track remains blank as a control during 21 days. B) *Escovopsis* was plated alone for 21 days as a control.

3.5.1.3. Antagonism bioassay against fungi (Method 3)

Bacteria or pure compounds were tested against four different *Escovopsis* strains (ICBG711, ICBG740, ICBG741 and ICBG1251, isolated from *Trachymyrmex*, *Acromyrmex*, *Apterostigma* and *Atta* colony, respectively), one *Trichoderma* ICBG1100 (isolated from garden of an Attine colony), and one *Cunninghamella* ICBG752 (isolated from garden of an *Apterostigma* colony). Each bacteria-fungi and compounds-fungi challenge was replicated two times and done on ISP-2 agar. Bacteria were placed in the center of ISP-2 agar petri dishes and grown alone during 7 days; fungal strains were then point-inoculated near the edge of the culture (3 cm from the center for *Escovopsis*, *Trichoderma* and *Cunninghamella* (**Figure 10**). Compounds (100 µg) were placed directly in the center of ISP-2 agar plate. Challenges were monitored during 21 days at 28 °C (Currie, Scott, Summerbell, & Malloch, 1999).

Figure 10 - Antagonism bioassays against *Escovopsis* ICBG741 (Method 3): A) *Streptomyces sioyaensis* ICBG311 vs *Escovopsis* ICBG741; B) Miconazole (100 µg) vs *Escovopsis* ICBG741; C) Control of *Escovopsis* ICBG741.

3.5.1.4. *In vitro* MIC susceptibility testing of compound 14-16 against human pathogenic fungi (assays carried out by Prof. David Andes group – University of Wisconsin-Madison, USA)

Compound **14-16** were tested for antifungal activity against *Candida albicans* K-1, and compound **15** against *Aspergillus fumigatus* 11628 (echinocandin resistant), *C. glabrata* 4720 (triazole resistant), and *C. auris* B11211 (echinocandin, triazole, and amphotericin B resistant). The MIC was determined using a broth microdilution method for yeasts (Rex et al., 2008). Compounds were dissolved in DMSO, serially diluted to 10 concentrations (0.125–64 µg/mL), and tested in a 96-well plate in RPMI medium. Amphotericin B was used as a positive control in bioassay against *C. albicans* K-1 and the MIC was 0.25 µg/mL. Six untreated media

controls were included on each plate. The plates were incubated at 33 °C for 72 h. MIC values were defined as the lowest concentration at which a prominent decrease in growth turbidity (that is, 50% reduction in growth determined spectrophotometrically) relative to the turbidity of the compound-free control at 600 nm was observed.

3.5.1.5. *In vivo* assay of compound 14-16 in murine disseminated candidiasis model (assays carried out by Prof. David Andes group – University of Wisconsin-Madison, USA)

Six-week-old, specific-pathogen-free, female ICR/Swiss mice weighing 23 to 27 g were used for all studies (Harlan Sprague-Dawley, Indianapolis, IN). Animals were maintained in accordance with the criteria of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal studies were approved by the Animal Research Committee of the William S. Middleton Memorial Veterans Hospital.

Mice were rendered neutropenic (neutrophils, $<100/\text{mm}^3$) by injection with cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, IN) subcutaneously 4 days (150 mg/kg) and 1 day (100 mg/kg) before infection and 2 days after infection (100 mg/kg). Previous studies have shown neutropenia (neutrophils, $<100/\text{mm}^3$) in this model for the 96-h study period (Andes, 2005). Organisms were subcultured on SDA 24 h prior to infection. Inoculum was prepared by placing three to five colonies into 5 mL of sterile pyrogen-free 0.9% saline warmed to 35 °C. The final inoculum was adjusted to a 0.6 transmittance at 530 nm. Fungal counts of the inoculum determined by viable counts of *C. albicans* on SDA was $6.29 \pm 0.03 \log_{10}$ CFU/mL.

Disseminated infection with the *Candida* was achieved by injection of 0.1 mL of inoculum via the lateral tail vein 2 h prior to the start of drug therapy. Treatment period was 8 h. Animals were sacrificed by CO₂ asphyxiation. Kidneys of each mouse were removed and placed in sterile 0.9% saline at 4 °C. The homogenate was serially diluted and aliquots were plated on SDA for viable fungal colony counts after incubation for 24 h at 35 °C. The lower limit of detection was 100 CFU/mL. Results were expressed as the mean number of CFU per kidney for three mice. No-treatment and zero-hour controls were included in all experiments (Zhao et al., 2018).

3.5.2. Antiprotozoal bioassays (carried out by Prof. Adriano D. Andricopulo group – Instituto de Física de São Carlos-USP, Brazil)

3.5.2.1. *In vitro* evaluation of crude extract and fractions on *Leishmania donovani* to calculate the growth inhibition percentage (GI%)

The *in vitro* assays are growth inhibition tests using a single concentration of the samples to determine percentage of inhibition values. This is achieved by diluting the samples in an appropriate medium to support the reproduction of the parasites, incubating the cultures and determining inhibition of parasite growth (Gupta, 2011). *L. donovani* promastigotes (strain MHOM/ET/67/HU3) were maintained in M199 medium (pH 7.4) supplemented with 10 % heat-inactivated fetal calf serum and grown in axenic cultures at 28 °C. Stock solutions of the evaluated extracts were prepared in 100% dimethylsulphoxide (DMSO) at 3 mg/mL and kept at -20 °C. Before being added to the assay plates the extracts were diluted to the appropriate concentration using complete culture medium. Fluorimetric assays were performed in 96-well flat-bottom microtiter plates, with each well containing 100 µL of culture medium with an initial population of 1×10^5 *L. donovani* promastigotes obtained from axenic cultures in logarithmic growth. In negative control wells (100% parasite growth) all assay components were added except for the extracts. As positive control, the reference drug miltefosine was used at a concentration of 15 µM. The extracts were assayed in duplicate at a final concentration of 20 µg/mL. After 72 hours of incubation, the plates were inspected using an inverted microscope to assure sterile conditions. Next, 10 µL of Alamar Blue® (Ioset, Brun, Wenzler, Kaiser, & Yardley, 2009) (12.5 mg of resazurin dissolved in 100 mL of distilled water) was added to each well and then the plates were incubated for 3 hours. This indicator of cell viability permeates into viable parasites, where it is reduced by NADPH and NADH enzymes to the highly fluorescent compound resorufin (Shimony & Jaffe, 2008). After this incubation time the plates were read with a microplate fluorometer under an excitation wave length of 536 nm and an emission wave length of 588 nm. Inactive extracts allow the conversion to resorufin, resulting in high fluorescence; active samples impair the reduction of resazurin to resorufin, which results in a decrease of fluorescence (Mikus & Steverding, 2000). Parasite growth inhibition was expressed as a percentage of the fluorescence of the negative control wells (100% parasite growth). Background wells were used for all controls and extracts to compensate for the fluorescence signal emitted by the assay components. Samples showing GI% > 50% were considered active.

3.5.2.2. *In vitro* evaluation of pure compounds on *Leishmania donovani* promastigotes, against intracellular amastigotes and human macrophages

L. donovani axenic cultures (strain MHOM/ET/67/HU3) were maintained as in section 3.5.2.1. Human leukemia cells (THP-1 cell line) were maintained in RPMI-1640 (FCS 10%) and grown at 37 °C and 5% CO₂. Stock solutions of compounds were prepared in 100% DMSO at 10 mM. For the assays, compounds were diluted in culture medium and tested at 10 concentrations (0.125 to 64 µM, 2-fold serial dilutions) in 96-well flat-bottom microtiter plates.

For the promastigote assay, *L. donovani* cells from axenic cultures in logarithmic growth were seeded at 1×10^5 /well (M199, 80 µL) and compounds were added in serial dilutions (20 µL). Fluorimetric assays, negative and positive control preparation, and incubation procedure of plates were done as section 3.5.2.1. The IC₅₀ values were determined using SigmaPlot software. Dose-response curves were fitted using log (inhibitor concentration) vs. normalized response (between 0% and 100%) with variable slope, and IC₅₀ were automatically calculated.

In the intracellular amastigote assay, THP-1 cells were seeded at 2×10^4 /well (RPMI-1640, 100 µL) with phorbol 12-myristate 13-acetate (PMA) at 20 ng/mL for differentiation into macrophages. After incubation for 72 hours (5% CO₂, 37°C), medium was aspirated and late-stage promastigotes were added (2×10^5 /well, 100 µL). After 24 hours of incubation, medium was aspirated to clear extracellular parasites, compounds were added in serial dilutions (100 µL) and the plates were incubated for 120 hours. All plates included negative controls and miltefosine as a positive control. Following incubation, medium was removed and the cells were fixed in methanol and stained with Giemsa. The average number of intracellular amastigotes per THP-1 cell was determined using an inverted microscope and a cell counter (Zulfiqar, Shelper, & Avery, 2017). Growth inhibition was expressed as a percentage of the average number of amastigotes per macrophage in the negative control wells. IC₅₀ values were determined as described above for the promastigote assay.

For the selectivity assay, THP-1 cells were seeded at 2×10^4 /well (RPMI-1640, 100 µL) with PMA at 20 ng/mL for differentiation into macrophages (Karwaciak, Gorzkiewicz, Bartosz, & Pulaski, 2017). After incubation for 72 hours (5% CO₂, 37°C), medium was aspirated, compounds were added in serial dilutions (100 µL) and the plates were incubated for 120 h. All plates included negative controls and doxorubicin as a positive control. Following incubation, 10 µL of Alamar Blue® was added to each well and then the plates were incubated for 3 hours. Next, the plates were read with a microplate fluorometer under an excitation wave length of 536 nm and an emission wave length of 588 nm. Growth inhibition was expressed as a

percentage of the fluorescence of the negative control wells. IC₅₀ values were determined as described above for the promastigote and amastigote assays.

3.5.2.3. Screening against β -galactosidase-expressing strains of *Trypanosoma cruzi* (Evaluation of crude extracts and fractions to calculate the GI%)

Assays are performed using amastigotes of *T. cruzi* strain Tulahuen parasites genetically engineered to express the *Escherichia coli* β -galactosidase gene, lacZ, which catalyzes a colorimetric reaction with chlorophenol red β -D-galactopyranoside as the substrate. The assays are performed in 96-well tissue culture plates in duplicates. Samples were tested in final concentration of 20 μ g/mL. In wells with β -galactosidase activity, the media changed color from yellow to red, and the color change is measured at 570 nm. Benznidazole is used as a positive standard, and untreated parasite cultures are used as negative controls (Buckner, Verlinde, La Flamme, & Van Voorhis, 1996; Ferreira et al., 2014). Samples showing GI% > 50% were considered active. This bioassay was part of the prescreening of extracts from ICBG-Brazil project (FAPESP/FIC-NIH Thematic Project 2013/50954-0), but in this PhD Thesis a major focus was directed to the anti-*L. donovani* extracts for isolation of active compounds.

3.5.3. Agar-based antibiotic assay

The extracts, fractions or compounds were tested against *B. subtilis* (NRS 231), *E. coli* (ATCC 25922), Methicillin-sensitive *S. aureus* (MSSA) (Wichita), *C. albicans* (K1), and *P. aeruginosa* (Boston 41501) using an agar-based antibiotic assay at Bugni's laboratory (University of Wisconsin-Madison). Seed cultures of each screening strain were grown overnight in 10 mL in LB media (25 $\times$ 150 mm tubes). To prepare uniform lawns for screening, 4 mL of overnight culture was inoculated into 200 mL of Cation-adjusted Mueller-Hinton Agar (CAMHA) maintained at 50–55 °C. For each screening plate, 30 mL of inoculated agar was poured into OmniTray (Thermo Scientific, Waltham, MA, USA) or plates of 250 x 20 mm and cooled for 30 min. Sample (1–5 μ L) was spotted onto the agar. Screening plates were incubated at 37 °C overnight and subsequently visually observed for zones of inhibition (Adnani et al., 2015).

3.6. Computational methods for absolute configuration analysis by Vibrational Circular Dichroism (VCD)

3.6.1. For griseorhodin A (1) and griseorhodin C (2)

All DFT calculations were carried out at 298 K in DMSO solution using the polarizable continuum model (PCM) in its integral equation formalism version (IEFPCM) incorporated in Gaussian 09 software (Frisch et al., 2009). Calculations were performed for the arbitrarily chosen (6*S*,6*aS*,7*S*,8*S*)-**1** and (6*R*,6*aS*,7*S*,8*R*)-**2**. The conformational search was carried out at the molecular mechanics level of theory with the Monte Carlo algorithm employing the MM+ force field, incorporated in HyperChem 8.0.10 software. For both griseorhodins A (**1**) and C (**2**) a single conformer with a relative energy (rel E.) within 6 kcal mol⁻¹ of the lowest-energy conformer was selected and geometry optimized at the B3LYP/PCM(DMSO)/6-31G(d) level. IR and VCD spectra were created using dipole and rotational strengths from Gaussian, which were calculated at the B3LYP/PCM(DMSO)/6-31G(d) level, and converted into molar absorptivities (M⁻¹ cm⁻¹). Each spectrum was plotted as a sum of Lorentzian bands with half-widths at half-maximum of 6 cm⁻¹. The calculated wavenumbers were multiplied with a scaling factor of 0.975 and the final spectra were plotted using Origin 8 software.

3.6.2. For strepchazolin A (5) and strepchazolin B (6)

All DFT calculations were carried out at 298 K in methanol solution using the polarizable continuum model (PCM) in its integral equation formalism version (IEFPCM) incorporated in Gaussian 09 software (Frisch et al., 2009). Calculations were performed for the arbitrarily chosen (5*S*,6*S*,9*R*)-**5** and (5*S*,6*S*,9*S*)-**6**. The conformational search was carried out at the molecular mechanics level of theory with the Monte Carlo algorithm employing the MM+ force field, incorporated in HyperChem 8.0.10 software. Initially, for strepchazolin A (**5**), nine conformers with a relative energy (rel E.) within 6 kcal mol⁻¹ of the lowest-energy conformer were selected and geometry optimized at the B3LYP/PCM(MeOH)/6-31G(d) level. The conformers for strepchazolin B (**6**) were generated by inverting the configuration at C-9 for all conformers identified in the previous step for strepchazolin A (**5**). These conformers were then geometry optimized at the B3LYP/PCM(MeOH)/6-31G(d) level. Before IR and VCD calculations all conformers obtained for both diastereoisomers were further geometry optimized at the B3PW91/PCM(MeOH)/6-311G(d,p) level. The four conformers identified for each

configuration with rel E. $<1.6 \text{ kcal mol}^{-1}$, which corresponded to more than 97% of the total Boltzmann distributions, were considered for IR and VCD spectral calculations. In order to better reproduce experimental data recorded in methanol- d_4 , the input structures were created with exchangeable hydrogens replaced with deuterium atoms (Sprenger, Thomasi, Ferreira, Cass, & Batista, 2016). IR and VCD spectra were created using dipole and rotational strengths from Gaussian, which were calculated at the B3PW91/PCM(MeOH)/6-311G(d,p) level, and converted into molar absorptivities ($\text{M}^{-1} \text{ cm}^{-1}$). The Boltzmann factor for each conformer was calculated based on Gibbs free energies. Each spectrum was plotted as a sum of Lorentzian bands with half-widths at half-maximum of 6 cm^{-1} . The calculated wavenumbers were multiplied with a scaling factor of 0.98 and the final Boltzmann-population-weighted composite spectra were plotted using Origin 8 software.

3.7. Molecular properties predictions of compounds 4 and 10-16

The computational predictions were run using SwissADME (Daina, Michielin, & Zoete, 2017) and Stardrop (Optibrium[®]) (Segall, 2014) by Dr. Leonardo Ferreira from Laboratório de Química Medicinal e Computacional, Centro de Pesquisa e Inovação em Biodiversidade e Fármacos, Instituto de Física de São Carlos, Universidade de São Paulo.

4. RESULTS AND DISCUSSION

4.1. Selection of bacteria for the isolation of active compounds

The strains isolation procedure resulted in 59 bacteria isolated from exoskeleton of workers ants and 8 from fungal garden of the *Acromyrmex rugosus rugosus* colony, and 10 bacteria from the exoskeleton of workers of *Acromyrmex subterraneus brunneus* (**Table 1**). Considering media used for isolation, 41 bacteria were recovered using IPS-2 and 36 using chitin (**Table 1**). In the preliminary antifungal bioassay of 77 bacteria (**Method 1**), seven bacteria showed activity against *Escovopsis* sp. (isolated from *Atta* colony) and two against *Beauveria bassiana* (**Table 1**). In the second antifungal bioassay (**Method 2**) 27 bacteria were active against *Escovopsis* HH141108-02; none were active against *Escovopsis* LK141126-01; 10 were active against *Escovopsis* LK141108-03; and 55 were active against *Escovopsis* AB150620-3 (**Table 1**). These results show that several bacteria have a selective inhibition against some *Escovopsis*

strains. All bacteria of **table 1** were isolated from *Acromyrmex* colonies but not all showed activity against *Escovopsis* isolated from the same genus of the ants studied. Almost all of the active bacteria against *Escovopsis* were inactive against *Beauveria bassiana*. So, most of the 77 bacteria were more selective against fungus *Escovopsis* than *B. bassiana*, a general entomopathogen.

Eight EtOAc crude extracts of 77 bacteria showed a GI% > 50% against *Trypanosoma cruzi*; thirty showed a GI% > 50% against *Leishmania donovani*; and four against both parasites (**Table 1**). Twenty bacteria showed activity against *Escovopsis* and parasites. The bacteria could produce antifungal compounds that inhibit the growth of parasites or produce a wide variety of secondary metabolites with different biological activities.

Table 1 - Bioassay results of bacteria isolated from *Acromyrmex rugosus rugosus* and *Acromyrmex subterraneus brunneus* colonies against five *Escovopsis* strains and one *Beauveria bassiana* strain; and of their crude extracts against *Trypanosoma cruzi* and *Leishmania donovani* parasites.

Bacteria*	Medium used for isolation	Source	<i>Escovopsis</i> sp**					<i>Beauveria bassiana</i>	GI% of crude extracts against <i>T. cruzi</i>	GI% of crude extracts against <i>L. donovani</i>
			A	B	C	D	E			
AC1	ISP-2	Ant	-	-	-	-	-	-	39	83
AC2	ISP-2	Ant	-	-	-	+	-	-	14	59
AC3	ISP-2	Ant	+	-	-	+	-	-	30	75
AC4	ISP-2	Ant	+	-	+	-	-	-	26	99
AC6	Chitin	Ant	+	-	-	+	-	-	NI	NI
AC7	Chitin	Ant	-	-	-	+	-	-	7	NI
AC8	ISP-2	Ant	-	-	-	+	-	-	24	53
AC9	ISP-2	Ant	-	-	-	+	-	-	24	65
AC10	ISP-2	Ant	-	-	-	+	-	-	22	58
AC11	Chitin	Ant	+	-	-	+	-	-	23	63
AC12	Chitin	Ant	-	-	-	-	-	-	4	53
AC13	Chitin	Ant	-	-	-	+	-	-	30	NI
AC14	Chitin	Ant	-	-	-	+	-	-	29	98
AC15	ISP-2	Ant	-	-	-	-	-	-	18	9
AC17	ISP-2	Ant	-	-	-	-	-	-	29	34
AC18	Chitin	Ant	+	-	-	+	-	-	NI	66
AC20	Chitin	Ant	-	-	-	+	-	-	24	99
AC21	ISP-2	Ant	-	-	-	-	-	-	21	64
AC22	ISP-2	Ant	+	-	-	+	-	-	21	54
AC23	ISP-2	Ant	+	-	-	+	-	-	26	NI

*"AC" means that bacteria were isolated from *Acromyrmex subterraneus brunneus* colony and

"AB" from *Acromyrmex rugosus* colony

***Escovopsis*: A: HH141108-02 (*Acromyrmex*), B: LK141126-01 (*Acromyrmex*),

C: LK141108-03 (*Acromyrmex*), D: AB150620-3 (*Trachymyrmex*), E: *Escovopsis* (*Atta*)

The symbol "+" means active against fungi and "NI" no inhibition against parasite

Table 1 (cont.) - Bioassay results of bacteria isolated from *Acromyrmex rugosus rugosus* and *Acromyrmex subterraneus brunneus* colonies against five *Escovopsis* strains and one *Beauveria bassiana* strain; and of their crude extracts against *Trypanosoma cruzi* and *Leishmania donovani* parasites.

Bacteria*	Medium used for isolation	Source	<i>Escovopsis</i> sp**					<i>Beauveria bassiana</i>	GI% of crude extracts against <i>T. cruzi</i>	GI% of crude extracts against <i>L. donovani</i>
			A	B	C	D	E			
AC26	Chitin	Ant	-	-	-	+	-	-	19	98
AC27	ISP-2	Ant	+	-	+	-	-	-	16	76
AC28	ISP-2	Ant	+	-	-	+	+	-	18	30
AC29	ISP-2	Ant	-	-	-	-	-	-	24	28
AC30	ISP-2	Garden	-	-	-	-	-	-	20	4
AC31	ISP-2	Garden	-	-	-	-	-	-	14	NI
AC32	ISP-2	Garden	-	-	-	+	-	-	26	69
AC34	Chitin	Ant	-	-	-	-	-	-	27	NI
AC35	ISP-2	Garden	-	-	-	+	-	-	23	30
AC36	ISP-2	Garden	+	-	-	+	-	-	22	11
AC37	ISP-2	Garden	-	-	-	-	-	-	15	5
AC38	Chitin	Ant	-	-	-	-	-	-	10	NI
AC39	Chitin	Ant	-	-	+	+	-	-	NI	NI
AC40	Chitin	Ant	-	-	-	-	-	-	12	40
AC41	Chitin	Ant	-	-	-	-	-	-	33	NI
AC43	ISP-2	Ant	-	-	-	-	-	-	9	NI
AC44	ISP-2	Ant	+	-	-	+	+	-	33	NI
AC45	ISP-2	Ant	-	-	-	+	-	+	20	NI
AC46	Chitin	Ant	-	-	-	-	-	-	11	NI
AC47	Chitin	Ant	+	-	+	+	-	-	41	27
AC48	Chitin	Ant	+	-	+	+	+	-	52	80
AC49	ISP-2	Ant	-	-	-	+	-	-	45	NI
AC50	ISP-2	Ant	-	-	-	-	-	-	45	63
AC51	Chitin	Ant	-	-	-	+	-	-	49	65
AC52	Chitin	Ant	-	-	-	-	-	-	18	67
AC53	ISP-2	Ant	+	-	-	+	-	+	40	31
AC54	ISP-2	Ant	+	-	+	+	+	-	53	NI
AC55	ISP-2	Ant	-	-	-	+	-	-	39	5
AC56	ISP-2	Ant	-	-	-	+	-	-	54	81
AC57	Chitin	Ant	-	-	-	-	-	-	50	75
AC58	Chitin	Ant	-	-	-	+	-	-	19	79
AC59	Chitin	Ant	+	-	+	+	-	-	30	NI
AC60	Chitin	Ant	-	-	-	+	-	-	64	91
AC61	Chitin	Ant	-	-	-	+	-	-	45	33
AC62	ISP-2	Ant	-	-	-	+	-	-	41	NI

*"AC" means that bacteria were isolated from *Acromyrmex subterraneus brunneus* colony and

"AB" from *Acromyrmex rugosus rugosus* colony

***Escovopsis*: A: HH141108-02 (*Acromyrmex*), B: LK141126-01 (*Acromyrmex*),

C: LK141108-03 (*Acromyrmex*), D: AB150620-3 (*Trachymyrmex*), E: *Escovopsis* (*Atta*)

The symbol "+" means active against fungi and "NI" no inhibition against parasite

Table 1 (cont.) - Bioassay results of bacteria isolated from *Acromyrmex rugosus rugosus* and *Acromyrmex subterraneus brunneus* colonies against five *Escovopsis* strains and one *Beauveria bassiana* strain; and of their crude extracts against *Trypanosoma cruzi* and *Leishmania donovani* parasites.

Bacteria*	Medium used for isolation	Source	<i>Escovopsis</i> sp**					<i>Beauveria bassiana</i>	GI% of crude extracts against <i>T. cruzi</i>	GI% of crude extracts against <i>L. donovani</i>
			A	B	C	D	E			
AC63	ISP-2	Ant	+	-	-	-	-	-	36	51
AC64	Chitin	Ant	-	-	-	+	-	-	NI	42
AC65	Chitin	Ant	-	-	-	-	-	-	31	28
AC66	Chitin	Ant	-	-	-	+	-	-	51	96
AC67	Chitin	Ant	-	-	-	+	-	-	41	70
AC68	Chitin	Ant	-	-	-	+	-	-	45	17
AC69	Chitin	Garden	+	-	-	+	-	-	26	29
AC70	Chitin	Garden	+	-	-	+	+	-	60	6
AC71	ISP-2	Ant	+	-	+	+	-	-	51	NI
AC72	ISP-2	Ant	+	-	-	+	-	-	44	72
AC73	ISP-2	Ant	-	-	-	+	-	-	38	NI
AC74	ISP-2	Ant	-	-	-	+	-	-	39	43
AB4	ISP-2	Ant	-	-	-	+	-	-	25	26
AB5	ISP-2	Ant	-	-	-	+	-	-	20	21
AB6	ISP-2	Ant	+	-	-	+	-	-	45	NI
AB7	ISP-2	Ant	-	-	-	+	-	-	33	NI
AB8	ISP-2	Ant	+	-	-	+	-	-	30	NI
AB9	Chitin	Ant	-	-	-	+	-	-	16	10
AB10	Chitin	Ant	+	-	+	+	+	-	17	99
AB11	Chitin	Ant	+	-	+	+	+	-	NI	NI
AB12	Chitin	Ant	+	-	-	+	-	-	37	50
AB13	Chitin	Ant	+	-	-	+	-	-	69	NI

*“AC” means that bacteria were isolated from *Acromyrmex subterraneus brunneus* colony and “AB” from *Acromyrmex rugosus rugosus* colony

***Escovopsis*: A: HH141108-02 (*Acromyrmex*), B: LK141126-01 (*Acromyrmex*), C: LK141108-03 (*Acromyrmex*), D: AB150620-3 (*Trachymyrmex*), E: *Escovopsis* (*Atta*)
The symbol “+” means active against fungi and “NI” no inhibition against parasite

Some of the 77 bacteria strains were identified as actinobacteria by morphology and 16S rRNA gene sequencing. Five strains of the total 77 bacteria isolated initially (*Streptomyces puniceus* AB10, *Streptomyces luteogriseus* AB11, *Nocardioideus albus* AB12, *Amycolatopsis orientalis* AC44, and *Streptomyces chartreusis* AC70) were selected for further chemical studies based on the bioactivity results and 16S rRNA gene sequence (**Table 2**). Actinobacteria AB10, AB11 and AB12 were isolated from the exoskeleton of workers of *Acromyrmex rugosus rugosus* colony. Bacterium AC70 was isolated from the fungal garden, and AC44 from workers of the *Acromyrmex subterraneus brunneus* colony. The five actinobacteria showed antagonist

activity against *Escovopsis*. The crude extract of AB10 was active against *L. donovani* (GI% = 99%), of AC70 against *T. cruzi* (GI% = 60%); and of AB11, AB12 and AC44 were inactive against both parasites (**Table 1**). The media chosen for large scale culture of these five actinobacteria was ISP-2.

Table 2 - Identification of actinobacterial strains

Code	Max. Ident.	P.B.	Gene seq.	Description	GB Closest relative	Ez Taxon closest relative
AB10	100%	1367	16S rRNA	<i>Streptomyces puniceus</i>	KP209438	AB184163
AB11	99%	1388	16S rRNA	<i>Streptomyces luteogriseus</i>	GQ985454	NBRC 13402(T)
AB12	99%	1347	16S rRNA	<i>Nocardioides albus</i>	KM186616	AF004988
AC44	99%	1364	16S rRNA	<i>Amycolatopsis orientalis</i>	HM368600	DSM 43134(T)
AC70	98%	830	16S rRNA	<i>Streptomyces chartreusis</i>	FJ481059	AB184839
ICBG292	100%	1355	16S rRNA	<i>Candidatus Streptomyces philanthi</i> bv. <i>triangulum</i>	KC607728	DQ375802
ICBG311	99%	1400	16S rRNA	<i>Streptomyces sioyaensis</i>	KF475886	NRRL B-5408(T)

The two additional bacteria (ICBG292 and ICBG311) isolated from *Cyphomyrmex* colony were identified as *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292 and *Streptomyces sioyaensis* ICBG311, respectively (**Table 2**). The actinobacterium ICBG292 was isolated from the exoskeleton of a worker of a *Cyphomyrmex* sp. colony; and the actinobacterium ICBG311 was isolated from a winged male ant of the same colony. Both actinobacteria showed antagonist activity against *Escovopsis* and their crude extracts obtained in three different media (ISP-2, YPM and A-medium) were tested against *T. cruzi* and *L. donovani* (**Table 3**). The three crude extracts of ICBG292 showed a GI% > 90% against *L. donovani* and only one (obtained in A-medium) was active against *T. cruzi* (GI% = 52%). The crude extract of ICBG311 obtained in A-medium showed a GI% = 94%, in ISP-2 a GI% = 52%, and in YPM a GI% = 17%, and all were inactive against *T. cruzi*. Thus, the selectivity of compounds produced by both actinobacteria was higher against *L. donovani* than *T. cruzi*. The media chosen for large scale culture of bacterium ICBG292 was ISP-2 and for ICBG311 was A-medium based on the activity against *L. donovani*.

Table 3 - Anti-parasitic activity of crude extract of actinobacteria ICBG292 and ICBG311 in three culture media

Bacteria	GI% against <i>T. cruzi</i>			GI% against <i>L. donovani</i>		
	A-medium	ISP-2	YPM	A-medium	ISP-2	YPM
ICBG292	52	29.7	29.1	93	96	94
ICBG311	NI	18.7	18.2	94	52	17

*Data obtained from ICBG-Brazil project (Pupo et al., 2017)

4.2. Compounds produced by *Streptomyces puniceus* AB10

4.2.1. Structure elucidation of compounds 1-4

Figure 11 - Compounds produced by *Streptomyces puniceus* AB10.

Compounds **1** was obtained as purple solid and **2** and **3** as red solids. The ^1H NMR and gHSQC of compound **2** in acetone- d_6 (**Appendix D** and **F**) showed the presence of a methoxyl (δ_{H} 3.98), a methyl (δ_{H} 2.25), three phenolic hydroxyls (δ_{H} 11.07, 12.08 and 13.29), two alkene hydrogens (δ_{H} 6.35 and 6.51), one aromatic hydrogen (δ_{H} 7.15) and three oxymethine hydrogens (δ_{H} 4.64, 4.78 and 5.54). The position of each hydrogen were established by gHMBC correlations (**Appendix G-J**). The molecular formula of **2** was determined to be $\text{C}_{25}\text{H}_{18}\text{O}_{13}$ based on negative ion HRESIMS ($[\text{M}-\text{H}]^-$ m/z 525.0663, err 2.2 ppm) (**Appendix E**), suggesting that **2** is griseorhodin C (Eckardt, Tresselt, & Ihn, 1978) (**Figure 11**). The ^1H NMR and gHMBC data of this compound were also acquired in DMSO- d_6 (**Appendix K** and **L**) to compare with NMR data of literature using same solvent. We obtained almost identical chemical shift values and some HMBC correlations not observed using acetone- d_6 , supporting

the structure of griseorhodin C (**2**). The main difference observed in the ^1H NMR spectra in acetone- d_6 of compounds **1** (**Appendix A**) and **2** (**Appendix D**) are the chemical shifts of H-7 and H-8 [δ_{H} 4.36 and 4.40 for **1** and δ_{H} 4.64 and 4.78 for **2**, respectively]. Such differences arise from the fact that **1** presents an oxirane between C-7 and C-8 while **2** is a dihydroxy derivative. The molecular formula of **1** was determined to be $\text{C}_{25}\text{H}_{16}\text{O}_{12}$ based on negative ion HRESIMS ($[\text{M}-\text{H}]^-$ m/z 507.0551, err 3.5 ppm) (**Appendix B**), suggesting that **1** is griseorhodin A (Stroshane et al., 1979) (**Figure 11**). The main difference observed in the ^1H NMR spectra in acetone- d_6 of compound **3** (**Appendix O**) and **2** was the presence of methylene hydrogens (δ_{H} 3.09 and 3.42). The molecular formula of **3** was determined to be $\text{C}_{25}\text{H}_{18}\text{O}_{12}$ based on negative ion HRESIMS ($[\text{M}-\text{H}]^-$ m/z 509.0701, err 4.8 ppm) (**Appendix P**), showing one oxygen atom less than **2**, supporting the presence of methylene group in place of an oxymethine, suggesting that **3** is griseorhodin G (Stroshane et al., 1979) (**Figure 11**).

The compound **4** was obtained as colorless oil. The ^1H NMR spectrum in CDCl_3 (**Appendix Q**) showed a group of signals at δ_{H} (4.91, m), (4.86, m), (3.99, m), (3.81, m), (2.48, m), (1.96, m), (1.89, m), (1.72, m), (1.58, m), (1.41-1.53, m); methyl triplet at δ_{H} 0.83; and methyl doublets between δ_{H} 1.02-1.08 and δ_{H} 1.16-1.21. The ^{13}C NMR spectrum (**Appendix R**) showed signals for carbonyl groups at δ_{C} 174.6 and 174.3; oxymethine carbons at δ_{C} 80.1, 79.9, 76.5, 76.3, 73.3 and 69.1; methine carbons at δ_{C} 45.3 and 45.1; methylene carbons at δ_{C} 42.3, 40.0, 31.5, 31.4, 28.2, 28.1, and 27.5; and methyl carbons at δ_{C} 20.5, 13.2, 12.7 and 9.4. The molecular formula of **4** was determined to be $\text{C}_{42}\text{H}_{68}\text{O}_{12}$ based on positive ion HRESIMS ($[\text{M}+\text{H}]^+$ m/z 765.47713, err 1.6 ppm) (**Appendix S**), suggesting that **4** is dinactin (Hussain et al., 2018) (**Figure 11**).

Streptomyces puniceus AB10 was also cultured in three liquid media (ISP-2, YPM and A-medium) to compare the chemical profile of their crude extracts. When cultured in ISP-2 and YPM it was possible to observe the ^1H NMR signals of griseorhodin A (**1**) (**Figure 12**). The ^1H NMR spectra of fractions from ISP-2 medium were more complex, indicating other possible analogues of **1**. When cultured in A-medium the bacteria produced one class of compounds represented by **4** (major compound) and no ^1H NMR signals of compounds **1-3** were observed. The extract obtained from A-medium culture was a dark oil while extract from YPM and ISP-2 culture were a dark red solid (characteristic color of griseorhodins).

Figure 12 - ^1H NMR of fractions of crude extract obtained from culture of actinobacterium *Streptomyces puniceus* AB10 in ISP-2, YPM and A-medium. The fractions were obtained using SPE-ENV+ and a gradient of MeOH (25, 50, 75 and 100 %). The signals highlighted are from griseorhodin A (**1**).

4.2.2. Relative and absolute configuration determination of griseorhodin A (**1**) and griseorhodin C (**2**)

The relative and absolute configurations of most of the rubromycin family members have yet to be assigned. As unambiguous stereochemical information is very important for biological and biosynthetic studies, therefore in this work we decided to reinvestigate the absolute stereochemistry of griseorhodin A (**1**) and also assign that of griseorhodin C (**2**), for the first time, using vibrational circular dichroism (VCD) spectroscopy. VCD is a well-established and reliable method for stereochemical studies of natural molecules directly in solution-phase (Bringmann, Kraus, Schmitt, Puder, & Zeeck, 2000).

The 1 and 2 D NMR analyses of griseorhodin C (**2**) were carried out in both DMSO- d_6 and Acetone- d_6 . NOESY experiments in acetone- d_6 showed NOE correlations between H-6 and H-7, H-7 and H-8, H-8 and H-9 as well as H-9 and H-10 using NOESY-1D and NOEDIFF experiments (See NOE correlation in **Appendix M** and **N**). The ^1H NMR spectrum of **2** showed broad singlets assigned to hydrogens H-7 (δ_{H} 4.64) and H-8 (δ_{H} 4.78). So, they could be in pseudo-equatorial position in the ring of six members of spiroketal moiety. These results allowed us to determine its relative configuration as $6R^*, 6aS^*, 7S^*, 8R^*$. The ^1H NMR spectrum of **1** showed doublets assigned to H-7 (δ_{H} 4.36) and H-8 (δ_{H} 4.40). The NOE correlation of H-7/H-8 and coupling constant $^3J_{\text{H-7,H-8}}$ (4.02 Hz), supported the *cis* configuration of epoxide moiety in **1**. It was not observed the NOE correlation of H-6/H-7 suggesting that relative configuration of **1** could be $6S^*, 6aS^*, 7S^*, 8S^*$ as was published previously (Yunt et al., 2009). The key NOE correlations of **1** and **2** are shown in **Figure 13**.

Griseorhodin A (**1**) was reported with the configuration $6S, 6aS, 7S, 8S$ based on TD-DFT calculations of its ECD spectrum (Yunt et al., 2009). Our experimental and theoretical VCD investigation on this molecule (**Figure 14**) revealed exactly the same spatial arrangement of the atoms and same absolute configuration (Ortega, Batista, Melo, Clardy, & Pupo, 2018). Experimental and calculated IR and VCD spectra of griseorhodin C (**2**) were compared in order to assign its absolute configuration. Due to the polycyclic nature of the molecule a single conformer was identified after molecular mechanics conformational search and B3LYP/PCM(DMSO)/6-31G(d) geometry optimization steps. The good agreement between observed and DFT-predicted IR/VCD spectra led to the assignment of **2** as (-)-($6R, 6aS, 7S, 8R$) (Ortega et al., 2018) (**Figure 15**).

Interestingly, griseorhodins A (**1**) and C (**2**) showed a different configuration at C-6. Other related compounds, such as heliquinomycin and γ -rubromycin, have been reported (Bringmann

et al., 2000) from *Streptomyces* sp. with opposite configuration at their spiro centers (C-6a) (R and S configuration, respectively), showing the complex biosynthesis of this family of secondary metabolites.

Figure 13 - Key NOE correlations of griseorhodin A (1) and C (2).

Figure 14 - (Left) Comparison of the observed IR and VCD spectra of **1** with the calculated (B3LYP/PCM(DMSO)/6-31G(d)) IR and VCD spectra of the lowest-energy conformer identified for (6S,6aS,7S,8S)-griseorhodin A (right)

Figure 15 - (Left) Comparison of the observed IR and VCD spectra of (-)-2 with the calculated (B3LYP/PCM(DMSO)/6-31G(d)) IR and VCD spectra of the lowest-energy conformer identified for (6*R*,6*aS*,7*S*,8*R*)-griseorhodin C (right). Numbers on spectra represent selected vibrational modes.

4.2.3 Description and biological evaluation of compounds 1-4

The actinobacterium *Streptomyces puniceus* AB10 showed antagonist activity against *Escovopsis* sp. and its crude extract was active against the parasite *Leishmania donovani* (GI% = 99%). Therefore, it was selected to isolate active compounds.

Compounds **1-3** (Figure 11) belong to the rubromycin family and represent an interesting group of polyketide pigments with wide spectrum of biological activities (Atkinson & Brimble, 2015). They possess a naphthoquinone ring and an isocoumarin ring connected through a 5,6-spiroketal system (Atkinson & Brimble, 2015). This family of compounds have been reported from Actinobacteria of the genera *Streptomyces*, *Dactylosporangium*, and *Actinoplanes* (Atkinson & Brimble, 2015). Griseorhodins A, C and G have been described with antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Penicillium notatum* and others; and with cytotoxic activity against cells derived from human carcinoma of nasopharynx (KB) (Stroshane et al., 1979; Suetsuna, Seino, Kudo, & Osajima, 1989). Griseorhodin A also inhibits human leukocyte elastase at micromolar concentrations (Yunt et al., 2009). Griseorhodins A and C inhibit the Moloney murine leukemia virus reverse transcriptase (M-MLV RT) with IC₅₀ values of 7.38 and 9.38 μM, respectively (Ueno et al., 2000). Griseorhodins A and C also show

similar levels of activity against human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) with IC_{50} values between 20–33 μ M, as well as inhibition of the telomerase enzyme (IC_{50} = 6–12 μ M) (Ueno et al., 2000). Compound **1-3** were identified from an active fraction against *Escovopsis* (**Figure 16A**), but they were inactive individually against this fungus. Compounds **1-3** were also inactive against *L. donovani* (GI% < 50%).

Figure 16 - Antagonism bioassay of two fractions of crude extract of *Streptomyces puniceus* AB10 against *Escovopsis* sp (isolated from *Atta* colony) (Method 1): A) Fraction AB10.A5, and B) AB10.A6.

Dinactin (**4**) was published with strong antitumor and antibacterial activity (Hussain et al., 2018). This compound is considered an ionophore, compounds that reversibly binds and transport ions across biological membranes (Bakker, Bühlmann, & Pretsch, 1997). Dinactin (**4**) was isolated from an active fraction against *Escovopsis* sp. (**Figure 16B**) and *L. donovani* with GI% = 99%. **4** displayed moderate antagonist activity against *Escovopsis* sp ICBG741 (**Figure 17A**) than miconazole; and showed a higher activity against *L. donovani* in promastigote form and on intracellular amastigotes compared to the positive control miltefosine (**Table 4**). The high activity and good selectivity index obtained for dinactin (**4**) in our experiments (**Table 4**) are in agreement with previous data for a related compound of **4** as nonactin, using *ex-vivo* splenic explant culture system from hamsters infected with *L. donovani* (Osorio, Travi, Renslo, Peniche, & Melby, 2011). Dinactin (**4**) is one member of the family of macrotetrolide nactins with ability to selectively complex a wide variety of cations (Asher, Phillies, Kim, & Stanley, 1977). Some ionophore compounds have been described to inhibit *L. donovani* by affecting the cell membrane and metabolism. One example is the ionophore A23187 that binds Ca^{2+} and kills intracellular *Leishmania* in the presence of lipopolysaccharide (LPS), mediated by generation of L-arginine-dependent nitrogen oxidation products (Buchmuller-Rouiller, Corradin, & Mauel, 1992). Other ionophore, named calcimycin, has been described to kill *Leishmania* promastigotes by activating parasite nitric oxide synthase (Grekov, Pombinho, Kobets, Bartůňek, & Lipoldová, 2017). The *Leishmania* cell death is accompanied by the loss of mitochondrial polarization and plasma membrane integrity and can be blocked by specific

inhibitors of constitutive Ca^{2+} /calmodulin-dependent nitric oxide synthase (Grekov et al., 2017). The most recognized mechanism of action of miltefosine against *L. donovani* is the inhibition of phospholipid synthesis and cytochrome *c* oxidase, but recently another mechanism has been described based in the abrupt increase in the intracellular Ca^{2+} concentration in the *L. donovani* (Pinto-Martinez, Rodriguez-Durán, Serrano-Martin, Hernandez-Rodriguez, & Benaim, 2018), a similar property of ionophores. This the first report of the antileishmanial activity of antibiotic dinactin (**4**). The mechanism of action of **4** against *L. donovani* has not been described.

Figure 17 - A) Antagonist activity of dinactin (**4**) (100 μg) against *Escovopsis* sp ICBG741 (Method 3), B) Miconazole (100 μg) vs *Escovopsis* sp ICBG741, C) Negative control against *Escovopsis* sp ICBG741.

Table 4 - Activity of compound **4** against *L. donovani* intracellular amastigotes, promastigotes and THP-1 cells

Compounds	IC_{50} (μM) Intracellular amastigotes	IC_{50} (μM) Promastigotes	CC_{50} (μM) THP-1*	Selectivity Index**
4	0.018 ± 0.003	0.032 ± 0.005	11.81 ± 1.57	656.11
Doxorubicin	---	---	0.571 ± 0.068	---
Miltefosine	5.80 ± 0.59	4.74 ± 0.25	---	---

Data are shown as mean $\pm$ SD ($n = 2$ biological replicates)

*THP-1 human leukemia macrophages (host cells of *L. donovani*)

**Selective index = CC_{50} THP-1/ IC_{50} intracellular amastigotes

Compound **4** did not show drug-like properties according to several rules such as Lipinski and Veber filters (Lipinski, Lombardo, Dominy, & Feeney, 2001; Veber et al., 2002). Compound **4** exceed the ideal molecular weight, number of hydrogen-bond acceptors (HBA) and Topological surface area (TPSA) values (Table 5). Moreover, compound **4** was predicted to have a good Log *P* value, inhibition of human ether-a-go-go-related gene (hERG) potassium

ion channels value, human intestinal absorption (HIA), no hydrogen-bond donors (HBD) and two rotatable bonds.

Table 5 - Molecular properties of compound **4**

Code	MW	LogP	hERG pIC ₅₀	HIA	HBD	HBA	TPSA	nrotb	Drug-like
4	764.98	4.70	4.43	+	0	12	142.10	2	no
Desired Value	≤ 500	< 5	< 6.3	+	≤ 5	≤ 10	≤ 140 Å	≤ 10	

LogP: octanol/water partition coefficient; hERG pIC₅₀: -logIC₅₀ on human ether-a-go-go-related gene potassium ion channels; HIA: human intestinal absorption; HBD: hydrogen-bond donors; HBA: hydrogen-bond acceptors; TPSA: topological surface area; nrotb: number of rotatable bonds. Drug-likeness according to Lipinski and Veber filters.

Several natural products that do not comply with Lipinski's rules have been approved as drugs regardless, such as taxol, rapamycin, cyclosporine A, and others. In general, natural products are considered as an exception to Lipinski's rules. However, the properties Log *P* and hydrogen-bond donors are very important for predicting bioavailability. A possible explanation is that nature can maintain low hydrophobicity and intermolecular hydrogen-bond donating potential when it needs to produce active compounds with high molecular weight and rotatable bonds; and natural products could also take advantage of active transport mechanisms since they contain biosynthetic moieties that resemble endogenous metabolites (Ganesan, 2008). So, dinactin (**4**) could be an interesting compound for further pharmacological studies in the treatment of leishmaniasis based on the high selectivity index against *L. donovani* and on Log *P* and HBD values that comply with Lipinsky's rules.

4.3. Compounds produced by *Streptomyces chartreusis* AC70

4.3.1. Structure elucidation of compounds 5-9

Figure 18 - Compounds produced by *Streptomyces chartreusis* AC70.

Compounds **7** and **8** were obtained as colorless oils. The ^1H NMR spectrum of **7** showed hydrogens signal at δ_{H} 1.91 (3H, d, $J = 7.3$ Hz), 2.14-2.55 (2H, m), 3.35-3.50 (2H, m), 4.28 (1H, d, $J = 6.7$ Hz), 4.74 (1H, d, $J = 6.7$ Hz), 4.88 (1H, br s), 6.04 (1H, m) and 6.16 (1H, q, $J = 7.3$ Hz) (**Appendix GG**). The molecular formula of **7** was determined to be $\text{C}_{11}\text{H}_{13}\text{NO}_3$ based on positive ion HRESIMS ($[\text{M}+\text{H}]^+$ m/z 208.0972, err -1.8 ppm) (**Appendix HH**), suggesting that **7** is the streptazolin (Drautz, Zähler, Kupfer, & Keller-Schierlein, 1981) (**Figure 18**). The ^1H NMR of **8** was similar as **7**, with hydrogens signals at δ_{H} 1.91 (3H, d, $J = 7.3$ Hz), 2.25-2.60 (2H, m), 3.38-3.53 (2H, m), 4.27 (1H, d, $J = 7.4$ Hz), 4.58 (1H, br s), 4.69 (1H, d, $J = 7.4$ Hz), 5.95 (1H, q, $J = 7.3$ Hz) and 6.11 (1H, m) (**Appendix II**). The molecular formula of **8** was identical as **7** based on positive ion HRESIMS ($[\text{M}+\text{H}]^+$ m/z 208.0973, err -2.3 ppm) (**Appendix JJ**), suggesting that **8** is the *E*-isomer of streptazolin (Li, Warshakoon, & Miller, 2004) (**Figure 18**). The difference in hydrogen chemical shifts of compound **7** and **8** are produced by the configuration of the double bond between C-8 and C-9. The hydrogen H-9 in **7** is more deshielded due to the anisotropic effect produced by the double bond $\Delta^{3,4}$, and H-7 by steric compression of the methyl group.

We established the planar structures of streptchazolin A (**5**) and streptchazolin B (**6**) (**Figure 18**) by NMR using chloroform-*d*, and their absolute configuration in methanol using vibrational circular dichroism (VCD) and density functional theory (DFT) calculations in collaboration

with Dr. João M. Batista (UNIFESP) previously of the stereochemistry published by Yang et al. (Yang et al., 2017). The planar structures of **5** and **6** were described by NMR using methanol-*d*₄ and their absolute configuration by X-ray and Mosher's method, respectively (Yang et al., 2017). Therefore, in this Thesis the complete structural analysis determination of **5** and **6** is described.

Compound **5** gave a peak in the HRESIMS consistent with a molecular formula C₁₂H₁₇NO₃ (**Appendix U**). The NMR data (**Table 6** and **Appendix T, V-Y**) indicated that it is an analogue of streptazolin (**7**). The ¹³C NMR spectrum of **5** showed twelve carbons [δ_C 45.1 (C-1), 25.2 (C-2), 115.8 (C-3), 140.2 (C-4), 67.5 (C-5), 79.8 (C-6), 131.0 (C-7), 146.2 (C-8), 64.5 (C-9), 21.9 (C-10), 173.0 (C-11), and 23.1 (C-12)]. DEPT-135 spectrum confirmed carbon multiplicities. The ¹H NMR spectrum showed two methyl groups [δ_H 1.44 (H-10), 2.19 (H-12)]; two methylene groups [δ_H 3.17 and 3.72 (H-1); and 2.31 (H-2)]; five methine groups [δ_H 6.01 (H-3), 4.35 (H-5), 4.61 (H-6), 5.96 (H-7) and 4.58 (H-9)]; and a hydrogen bond to heteroatom [δ_H 5.86 (C6-OH)]. Hydrogen and carbon chemical shifts were unequivocally assigned by gHSQC and gHMBC spectra (**Appendix V** and **W**). The gHMBC spectrum showed strong correlations of H₃-10 to C-8 and C-9; H₃-12 to C-1 and C-11; H-7 to C-4, C-5, C-6, C-8 and C-9; the hydroxyl hydrogen at C-6 to C-5, C-6 and C-7; and H-1 to C-2, C-3, C-5 and C-11 (**Appendix W**). The gCOSY spectrum (**Appendix X**) showed strong correlations of H₂-1 to H₂-2; H₂-2 to H₂-1 and H-3; H-3 to H₂-2; H-5 to H-6, H-6 to H-5 and H-7; H-7 to H-6; and between H₃-10 and H-9 that allow the assignment of the bicyclic spin systems. Compound **5** does not contain the cyclourethane ring as observed in **7**. Instead, compound **5** has an acetyl group bound to the nitrogen atom, two hydroxyl groups at C-6 and C-9, and a double bond between C-7 and C-8. Both compounds **5** and **7** have a double bond between C-3 and C-4.

Compound **6** gave the same peak in the HRESIMS as observed for **5** (**Appendix AA**). The NMR data obtained for **6** are very similar to that of **5** (see **Table 6**, **Appendix Z, BB-EE**). There are small differences in hydrogen and carbon chemical shifts between **5** and **6**: H-3 (δ_H 6.01 for **5** and δ_H 5.90 for **6**), C-3 (δ_C 115.8 for **5** and δ_C 115.3 for **6**), C-7 (δ_C 131.0 for **5** and δ_C 129.8 for **6**), C-8 (δ_C 146.2 for **5** and δ_C 146.7 for **6**) and C-10 (δ_C 21.9 for **5** and δ_C 22.6 for **6**). gCOSY and gHMBC correlations observed for **6** were identical to those observed for **5** (**Figure 19**), allowing the establishment of the same planar structure for **5** and **6**. The chemical shifts of these atoms could be affected by the configuration of the chiral center at C-9.

Cyclooctasulfur (**9**) (**Figure 18**) was identified by Gas Chromatography-Mass Spectrometry (GC-MS) (**Appendix KK**), comparing the fragmentation patterns with the data base.

Figure 19 - Key gCOSY and gHMBC correlations of compounds **5** and **6**

Table 6 - ^1H (500 MHz) and ^{13}C NMR (100 MHz) data for strepchazolin A (**5**) and strepchazolin B (**6**) vs ^1H (400 MHz) and ^{13}C NMR (100.6 MHz) data for streptazolin (**7**) in CDCl_3

No.	strepchazolin A (5)		strepchazolin B (6)		streptazolin (7) *	
	δ_{C} , type	δ_{H} , mult. (J in Hz)	δ_{C} , type	δ_{H} , mult. (J in Hz)	δ_{C} , type	δ_{H} , mult. (J in Hz)
1	45.1, CH_2	3.17, td (11.3; 3.9) 3.72, dt (12.0; 3.6)	45.1, CH_2	3.16, td (11.2; 3.8) 3.71, dt (12.0; 3.6)	39.9, CH_2	3.36-3.51, m
2	25.2, CH_2	2.31, m	25.2, CH_2	2.28, m	22.8, CH_2	2.13-2.25, m 2.51, dtd (16.7, 7.2, 3.3)
3	115.8, CH	6.01, quint (3.4)	115.3, CH	5.90, m	118.9, CH	6.02-6.07, m
4	140.2, C	---	140.5, C	---	142.8, C	---
5	67.5, CH	4.35, br s	67.5, CH	4.34, br s	59.1, CH	4.26-4.31, m
6	79.8, CH	4.61, br s	79.8, CH	4.60, br s	81.5, CH	4.73, d (6.8)
7	131.0, CH	5.96, br s	129.8, CH	5.98, br s	74.5, CH	4.88, br s
8	146.2, C	---	146.7, C	---	139.1, C	---
9	64.5, CH	4.58, m	64.7, CH	4.58, m	123.7, CH	6.15, q (7.3)
10	21.9, CH_3	1.44, d (6.42)	22.6, CH_3	1.41, d (6.56)	14.9, CH_3	1.91, d (7.3)
11	173.0, C	---	172.9, C	---	159.3, C	---
12	23.1, CH_3	2.19, s	23.2, CH_3	2.18, s	---	---
6-O-H	---	5.86, s	---	5.86, s	---	---

* Yamada, H.; Aoyagi, S.; Kibayashi, C. *Journal of the American Chemical Society* **1996**, *118*, 1054-1059

4.3.2. Relative and absolute configuration determination of strepchazolin A (**5**) and strepchazolin B (**6**)

The relative configurations of compounds **5** and **6** were determined by NOESY experiments. The key NOE correlations are presented in **Figure 20**. Hydrogen H-5 presents a correlation with the hydrogen of the hydroxyl group at C-6 in both compounds, indicating that they are on the same face of the molecule. Therefore, their absolute configuration could be only *5S,6S* or *5R,6R*. Compounds **5** and **6** also share other common NOE correlations: H-3 and H-9, H-3 and H-10, as well as between H-10 and H-7. Based on that, compounds **5** and **6** are concluded to be epimers at C-9. The different arrangement of the substituents at C-9 accounts for the distinct chemical shifts of H-3, C-3, C-7, C-8 and C-10 in both compounds. Compounds **5** and **6** presented different retention times in HPLC analysis (**Figure 23**), reinforcing they are stereoisomers

Figure 20 - Key NOE correlations of compounds **5** and **6**

Once the relative configurations of **5** and **6** were established, we proceeded with attempts to assign their absolute configurations. To that end, experimental IR/VCD spectra were recorded for both compounds in methanol-*d*₄, which were then compared to the theoretical data obtained for both diastereoisomers possible, reported as strepchazolin A (**5**) and strepchazolin B (**6**) (Yang et al., 2017). The comparison between observed and calculated spectra at the B3PW91/PCM(MeOH)/6-311G(d,p) level (**Figure 21**) allowed for the unambiguous determination of the absolute configuration of compound (–)-**5** as (*5S,6S,9R*) and (–)-**6** as (*5S,6S,9S*). Despite the high degree of similarity between the IR and VCD spectra of **5** and **6**, the VCD band at around 1385 cm^{–1}, which presented opposite signs for both epimers and was consistently reproduced by the calculations, represented the key spectral marker considered for these assignments. This band arises predominantly from the in-plane bending of H-9 coupled with the symmetrical bending of the methyl group at C-10, as well as with the C-C stretches between C-4/C-8 and C-8/C-9. The fact that these particular vibrations involve the atoms

directly linked to chiral center at C-9 provides a higher level of confidence for this assignment. The lowest-energy conformers identified for both configurations at C-9 (**Figure 22**) also help to explain most of the NMR data, such as the NOE correlations that are common to both **5** and **6**. Interestingly, for strepchazolin A (**5**) the OH group points predominantly towards H-3, while for strepchazolin B (**6**) the same group at C-9 points away from H-3 for most conformers. These findings may be responsible for the small difference in the chemical shift observed for H-3 in both epimers due to the deshielding anisotropic effect of the oxygen lone-pairs.

The wealth of structural and conformational information obtained from vibrational spectroscopy combined with the extra sensitivity to molecular chirality provided by VCD has made the latter a valuable new addition to the natural product chemistry toolbox (Batista, Blanch, & Da Silva Bolzani, 2015).

Figure 21 - Comparison of the observed IR and VCD spectra of (–)-**5** (black trace) and (–)-**6** (red trace) with the calculated (B3PW91/PCM(MeOH)/6-311G(d,p)) IR and VCD spectra of the Boltzmann average of the four lowest-energy conformers identified for strepchazolin A (black trace) and strepchazolin B (red trace). The asterisks represent the key VCD bands considered for the assignment of (–)-**5** as (5*S*,6*S*,9*R*) and (–)-**6** as (5*S*,6*S*,9*S*).

Figure 22 - (A) Structures, relative Gibbs free energies (referenced to Conf1, $G = -469127.091$ kcal/mol), and Boltzmann population (%) of the four lowest-energy conformers of streptazolin A at the B3PW91/PCM(MeOH)/6-311G(d,p) level. (B) Structures, relative Gibbs free energies (referenced to Conf1, $G = -469126.203$ kcal/mol), and Boltzmann population (%) of the four lowest-energy conformers of streptazolin B at the B3PW91/PCM(MeOH)/6-311G(d,p) level.

4.3.3. Description and biological evaluation of compounds 5-9

Streptomyces chartreusis AC70 showed antagonist activity against different *Escovopsis* strains and its crude extract presented $GI\% = 60\%$ against *T. cruzi* (**Table 1**). Therefore, it was selected to isolate compounds. The two major compounds detected by HPLC UV detector (**Figure 23**) from the EtOAc extract of this strain were streptazolin (**7**) and its *E*-isomer (**8**). Compound **7** has only been isolated from the genus *Streptomyces*. It is an unusual antibiotic and antifungal compound (Drautz et al., 1981), which possesses major structural features composed by a 1,2,5,6-tetrahydropyridine ring system, a cyclourethane, and an exocyclic ethylidene side chain (Yamada, Aoyagi, & Kibayashi, 1996). Structural variants of streptazolin (**7**) such as 3,9-dihydrostreptazolin and Diels-Alder adducts with naphthoquinones show enhanced antimicrobial and cytotoxic activities (Grabley, Kluge, & Hoppe, 1987). Recent studies have found that streptazolin increases bacterial killing and the elaboration of immunostimulatory cytokines by macrophages *in vitro* (Perry et al., 2015). However, it is not suitable for therapeutic use due to its polymerization tendency (Drautz et al., 1981). The *E*-

isomer of streptazolin (**8**) has been reported as an undesired side product in the synthesis of the streptazolin (Yamada et al., 1996). It has not been previously reported as natural product.

Figure 23 - Chromatogram of fractions AC70.A1-AC70.A5 containing strepchazolin A (**5**), strepchazolin B (**6**), streptazolin (**7**) and its *E*-isomer (**8**). Column: C₆-Phenyl column (Phenomenex Luna, 5 μ m, 250 x 10 mm). HPLC method: isocratic flow of 5% CH₃CN at 6 mL/min. Note: strepchazolin A (**5**) eluted after strepchazolin B (**6**) using C₆-Phenyl column, while in C₁₈-column **5** elutes first than **6**.

Strepchazolin A (**5**) and strepchazolin B (**6**) were reported from *Streptomyces chartreusis* NA02069, collected in the Coast of Hainan Island, China (Yang et al., 2017). Compound **5** was published with weak anti-*Bacillus subtilis* activity with the MIC value of 64.0 μ M, and weak inhibitory activity against acetylcholinesterase (AChE) *in vitro* with IC₅₀ value of 50.6 μ M, while compound **6** was almost inactive (Yang et al., 2017).

All fractions containing compounds **5-9** were inactive against *T. cruzi*. Compounds **7** and **8** were also inactive against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* at 50 μ g using agar diffusion test.

Cyclooctasulfur (**9**) was identified as the major compound by Gas Chromatography-Mass Spectrometry (GC-MS) (**Appendix KK**) from the bioactive fraction against *Escovopsis* sp (**Figure 24A**). Compound **9** has been previously reported from the North Sea *Streptomyces* GWS-BW-H5 (Dickschat, Martens, Brinkhoff, Simon, & Schulz, 2005). Under normal conditions, sulfur atoms form cyclic octatomic molecules. The element sulfur has been used as antimicrobial agent, insecticide, fumigant, etc (Deshpande et al., 2008). So, *Streptomyces chartreusis* AC70 could use **9** to control the growth of the pathogenic fungus *Escovopsis*.

Figure 24 - A) Antagonist activity of fraction AC70.B1.2 (10 μ L of 0.1 mg) [containing cyclooctasulfur (**9**)] against *Escovopsis* sp. from *Atta* colony (Method 3 modified, using paper disks). B) Miconazole (50 μ g) vs *Escovopsis* sp. C) Negative control.

4.4. Compounds produced by *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292

4.4.1. Structure elucidation of compounds 10-12

Mer-A2026B (10): R = H

Piericidin-A₁ (11): R = OCH₃

Nigericin (12)

Figure 25 - Compounds produced by *Candidatus Streptomyces philanthi* bv. *triangulum* ICBG292

Compounds **10-12** were obtained as pale yellow oils. The ¹H NMR spectrum of **11** showed six methyl hydrogens at δ_H 0.80, 1.62, 1.63, 1.75, 1.80 and 2.09; two methoxy hydrogens at δ_H 3.85 and 3.94; two methylene hydrogens at δ_H 2.79 and 3.37; one oxymethine hydrogen at δ_H 3.62; one methine sp³ hydrogen at δ_H 2.68; and five alkene hydrogens at δ_H 5.21, 5.41, 5.48, 5.60 and 6.09 (**Appendix NN**). The ¹³C NMR spectrum of **11** showed the six methyl carbons

at δ_C 10.4, 10.5, 13.1, 13.2, 16.6 and 17.4; two methoxy carbons at δ_C 53.0 and 60.6; two methylene carbons at δ_C 34.4 and 43.1; one oxymethine carbon at δ_C 82.8; one methine sp^3 carbon at δ_C 36.8; and thirteen alkene carbons at δ_C 111.9, 122.2, 123.5, 126.7, 127.8, 133.0, 134.8, 135.5, 135.7, 136.0, 150.8, 153.5 and 154.0 (**Appendix OO**). The molecular formula of **11** was determined to be $C_{25}H_{37}NO_4$ based on positive ion HRESIMS ($[M+H]^+$ m/z 416.2812, err -4.1 ppm) (**Appendix PP**), suggesting that compound **11** is piericidin-A_I (Yoshida, Yoneyama, Shiraishi, Watanabe, & Takahashi, 1977) (**Figure 25**). The 1H NMR spectrum of **10** was similar to **11**. The main difference observed was an additional alkene hydrogen at δ_H 5.89 and only one methoxy hydrogen at δ_H 3.81 (**Appendix LL**). The molecular formula of **10** was determined to be $C_{24}H_{35}NO_3$ based on positive ion HRESIMS ($[M+H]^+$ m/z 386.26752, err 3.8 ppm) (**Appendix MM**), suggesting that compound **10** is Mer-A2026B (Kominato et al., 1995a) (**Figure 25**). The 1H NMR spectrum of **12** showed several methyl hydrogen signals at δ_H 0.8-1.2, 1.41, 2.17; one methoxy hydrogen at δ_H 3.35; and group of signals at δ_H 1.2-2.5 and 3.4-4.4 (**Appendix QQ**). The ^{13}C NMR spectrum of **12** showed nine methyl carbons at δ_C 10.8, 13.1, 13.1, 13.2, 15.6, 16.3, 17.3, 22.7 and 27.9; one methoxyl carbon at δ_C 57.5; ten methylene carbons at δ_C 23.4, 25.8, 26.1, 30.8, 31.8, 32.3, 35.4, 37.4, 42.5 and 68.3; eight oxymethine carbons at δ_C 60.3, 69.0, 72.9, 74.5, 77.2, 78.0, 82.4 and 85.8; seven methine sp^3 carbons at δ_C 27.5, 32.6, 35.8, 37.2, 35.2, 39.0 and 44.2; four quaternary carbon sp^3 at δ_C 81.6, 83.5, 97.0 and 108.2; and one carbonyl carbon at δ_C 177.4 (**Appendix RR-TT**). The molecular formula of **12** was determined to be $C_{40}H_{68}O_{11}$ based on positive ion HRESIMS ($[M+Na]^+$ m/z 747.4667, err -1.8 ppm) (**Appendix UU**), suggesting that compound **12** is nigericin (Berrada, Dauphin, & David, 1987; Seto et al., 1979; Steinrauf, Pinkerton, & Chamberlin, 1968; Wu, Bai, Wang, & Shen, 2009) (**Figure 25**).

4.4.2. Description and biological evaluation of compounds 10-12

Candidatus Streptomyces philanthi bv. *triangulum* ICBG292 showed antagonist activity against *Escovopsis* sp. and its crude extract, obtained in ISP-2 medium, was active against the parasite *L. donovani* (GI% = 96%) (**Table 3**). Compounds **10-12** were isolated from active fractions (ICBG292.4 and ICBG292.5) against *Escovopsis* (**Figure 26**). **Figure 27** shows the peaks of compounds **9-11** in ELSD chromatogram of the mixture of these fractions. Nigericin (**12**) does not have absorption in the UV due to the absence of chromophores.

Figure 26 - Antagonist activity of fractions *ICBG292.4* and *ICBG292.5* (50 μ g) against *Escovopsis* sp of *Atta* colony (Method 3).

Figure 27 - UV and ELSD chromatogram of the mixture of fractions *ICBG292.4* and *ICBG292.5* containing compounds **10-12**. HPLC column: C₆-Phenyl (Phenomenex Luna, 5 μ m, 250 x 4.6 mm). Method: gradient at 1 mL/min; 1-20 min, linear gradient from 70% MeOH-H₂O to 100% MeOH; 20-25 min, isocratic flow of 100% MeOH; 25-25.5 min, linear gradient from 100% MeOH to 70% MeOH-H₂O; 25.5-30.5 min, isocratic flow of 70% MeOH-H₂O.

The piericidin family of compounds is mainly produced by genus *Streptomyces* and has a 4-pyridinol core linked to a methylated polyketide side chain (Zhou & Fenical, 2016). A potent vasodilating activity has been reported for mer-A2026B (**10**) (Kominato et al., 1995b); and insecticidal, antimicrobial and cytotoxic activities for piericidin-A₁ (**11**) (Schnermann et al., 2006; Tamura, Takahashi, Miyamoto, & Mori, 1963; Urakawa et al., 1996); while strong antibacterial and anticancer activities have been found for nigericin (**12**) (Liu et al., 2018; Wu et al., 2009).

The three compounds showed activity against *Escovopsis* sp. ICBG741 (**Figure 28**). Compound **10** was the only one active against the fungus *Trichoderma* sp. ICBG1100 (**Figure 29**) and other three different *Escovopsis* strains with similar or higher inhibition than miconazole, while **11** and **12** showed selectivity against some *Escovopsis* strains (**Figure 28, 30-32**). Compound **10-12** were inactive against fungus *Cunninghamella* ICBG752. Compound **12** showed higher activity against *L. donovani* promastigotes and intracellular amastigotes than **10-11** and positive control miltefosine (**Table 7**). The high activity and good selectivity index obtained for nigericin (**12**) in our experiments (**Table 7**) are in agreement with previous data for nigericin monosodium salt, using *ex-vivo* splenic explant culture system from hamsters infected with *L. donovani* (Osorio et al., 2011).

Table 7 - Activity of compounds **10-12** against *L. donovani* intracellular amastigotes, promastigotes and THP-1 cells

Compounds	IC ₅₀ (μM) Intracellular amastigotes	IC ₅₀ (μM) Promastigotes	CC ₅₀ (μM) THP-1*	Selectivity Index**
10	49.85 ± 7.01	35.86 ± 1.83	> 64	---
11	> 64	38.41 ± 4.63	> 64	---
12	0.129 ± 0.008	0.284 ± 0.072	11.47 ± 0.68	88.91
Doxorubicin	---	---	0.571 ± 0.068	---
Miltefosine	5.80 ± 0.59	4.74 ± 0.25	---	---

Data are shown as mean ± SD (n = 2 biological replicates)

*THP-1 human leukemia macrophages (host cells of *L. donovani*)

**Selective index = CC₅₀ THP-1/IC₅₀ intracellular amastigotes

Nigericin (**12**) is an ionophore, such as dinactin (**4**). Nigericin (**12**) has been proved to possess the ability to carry sodium and potassium ions through membranes (Riddell et al., 1988). *L. donovani* lives in the sandfly gut as a promastigote and in mammalian macrophages as an amastigote (Jiang, Anderson, Winget, & Mukkada, 1994). This protozoan extrudes protons through H⁺-ATPase to regulate intracellular pH and to facilitate nutrient uptake (Jiang

et al., 1994). This proton extrusion property is enhanced by the addition of K^+ (Jiang et al., 1994). This could be one mechanism by which nigericin controls the growth of *Leishmania* parasite. The mechanism of action of nigericin (**12**) against *L. donovani* has not been described. This the first report of the antileishmanial activity of antibiotics Mer-A2026B (**10**) and piericidin-A₁ (**11**).

Compounds **10-12** can join the chemical cocktail used by actinobacteria to control the growing of the pathogenic fungus *Escovopsis* sp. and other opportunistic fungi such as *Trichoderma* sp. in fungus-growing ant colonies. Compound **11** and other piericidin derivatives together with nigericin (**12**) have also been reported from *Candidatus Streptomyces philanthi* symbiont of solitary beewolf digger wasps (*Philanthus triangulum*, Hymenoptera, Crabronidae) as antibiotic protectors of their larval offspring against pathogens (Engl et al., 2018; Kroiss et al., 2010). Authors argue that the mixture of these antibiotics could help in the evolutionary stable defense against different pathogens (Engl et al., 2018; Kroiss et al., 2010), and the current identification of the same compounds in bacterial symbionts of attine ants reinforces this hypothesis. Therefore, the ecological function of natural products from actinobacteria associated to insects as attine ants can be aligned to potential pharmacological activities.

Compounds **10** and **11** were also identified from *Streptomyces* sp. ICBG233 associated to workers of *Atta sexdens* and from the organic extract of these ants by mass spectrometry (**Figure 33-38**), highlighting their production in the natural environment. In this experiment it was used a total of 25 *A. sexdens* individuals, and then they were mechanically cleaned using small forceps and extracted with 50 mL of methanol to obtain the organic extract. This analysis was carried by Dr. Ana Ligia L. de Oliveira during his postdoctoral research at LQMo.

Figure 28 - Antagonist activity of compounds **10-12** (100 µg) against *Escovopsis* sp ICBG741 (Method 3): A) mer-A2026B (**10**), B) piericidin-A₁ (**11**), C) nigericin (**12**), D) miconazole (100 µg), and E) Negative control.

Figure 29 - Antagonist activity of compound **10** (100 µg) against *Trichoderma* sp ICBG1100 (21 days of growth at 28 °C): A) mer-A2026B (**10**), B) miconazole (100 µg), C) Negative control.

Figure 30 - Antagonist activity of compounds **10-12** (100 μ g) against *Escovopsis* sp ICBG711 (21 days of growth at 28 °C): A) mer-A2026B (**10**), B) piericidin-A₁ (**11**), C) nigericin (**12**), D) miconazole, and E) Negative control.

Figure 31 - Antagonist activity of compounds **10-12** (100 μ g) against *Escovopsis* sp ICBG740 (21 days of growth at 28 °C): A) mer-A2026B (**10**), B) piericidin-A₁ (**11**), C) nigericin (**12**), D) miconazole, and E) Negative control.

Figure 32 - Antagonist activity of compounds **10-12** (100 μ g) against *Escovopsis* sp ICBG1251 (21 days of growth at 28 $^{\circ}$ C): A) mer-A2026B (**10**), B) piericidin-A₁ (**11**), C) nigericin (**12**), D) miconazole, and E) Negative control.

Compounds **10** and **11** showed drug-like properties according to Lipinski's rules and Veber filters (Lipinski et al., 2001; Veber et al., 2002) (**Table 8**). Moreover, the drug-like compounds were predicted to have human intestinal absorption (HIA) and low inhibition of human ether-a-go-go-related gene (hERG) potassium ion channels. Compound **12** exceed the ideal molecular weight and number of hydrogen-bond acceptors (HBA), but comply with the important properties Log *P* and hydrogen-bond donor (HBD) value. So, nigericin (**12**) could be other interesting compound for further studies in the treatment of leishmaniasis based in the high selectivity index against *L. donovani* and on Log *P* and HBD values that comply with Lipinsky's rules.

Table 8 - Molecular properties of compound **10-12**

Code	MW	LogP	hERG pIC ₅₀	HIA	HBD	HBA	TPSA	nrotb	Drug-like
10	385.54	4.10	5.93	+	2	4	62.58	9	yes
11	415.56	3.93	5.93	+	2	5	71.81	10	yes
12	724.96	3.77	3.96	-	3	11	142.37	9	no
Desired Value	≤ 500	< 5	< 6.3	+	≤ 5	≤ 10	$\leq 140 \text{ \AA}$	≤ 10	

LogP: octanol/water partition coefficient; hERG pIC₅₀: -logIC₅₀ on human ether-a-go-go-related gene potassium ion channels; HIA: human intestinal absorption; HBD: hydrogen-bond donors; HBA: hydrogen-bond acceptors; TPSA: topological surface area; nrotb: number of rotatable bonds. Drug-likeness according to Lipinski and Veber filters.

Figure 33 - LC-HRESIMS of *Atta sexdens* methanolic extract (MRM m/z 386, positive mode)

Figure 34 - MS/MS spectra of compound eluted at 32.7 minutes (m/z 386), identified as mer-A2026B (**10**) (positive mode)

Figure 35 - MS/MS spectra of mer-A2026B (**10**) (positive mode)

Figure 36 - LC-HRESIMS of *Atta sexdens* methanolic extract (MRM m/z 416, positive mode)

Figure 37 - MS/MS spectra of compound eluted at 40.1 minutes (m/z 416), identified as piericidin-A₁ (**11**) (positive mode)

Figure 38 - MS/MS spectra of piericidin-A₁ (**11**) (positive mode)

4.5. Compounds produced by *Streptomyces sioyaensis* ICBG311

4.5.1. Structure elucidation of compounds 13-16

Figure 39 - Compounds isolated from actinobacterium *Streptomyces sioyaensis* ICBG311

Compound **13** gave a peak in the HRESIMS consistent with a molecular formula $C_{16}H_{16}O_5$ (**Appendix BBB**). The 1H and ^{13}C NMR spectral data of **13** are shown in **Table 9**. The ^{13}C NMR spectrum of **13** showed sixteen carbons [δ_C 176.6 (C-1), 48.7 (C-2), 76.4 (C-3), 150.2 (C-4), 125.2 (C-5), 133.6 (C-6), 186.3 (C-7), 136.5 (C-8), 149.9 (C-9), 186.3 (C-10), 133.0 (C-11), 127.6 (C-12), 133.2 (C-13), 52.3 (C-14), 14.0 (C-15), and 16.3 (C-16)] (**Appendix WW**). The 1H NMR and gHSQC spectra showed three methyl groups [δ_H 3.71 (H-14), 0.95 (H-15) and 2.17 (H-16)]; and six methine groups [δ_H 2.84 (H-2), 4.90 (H-3), 8.00 (H-5), 6.88 (H-8), 8.07 (H-12) and 7.77 (H-13)] (**Appendix VV** and **XX**). The gHSQC suggested the presence of one ester carbonyl carbon C-1 at δ_C 176.6; two ketone carbonyl carbons C-7 and C-10 at δ_C 186.3; and four tertiary carbons sp^2 C-4, C-6, C-9 and C-11 at δ_C 150.2, 133.6, 149.9 and 133.0, respectively (**Appendix XX**). The HMBC correlation of H-14 (δ_H 3.71) to C-1 indicated the presence of a methoxy group at C-14 (**Appendix ZZ**). The 1H NMR signals for H-5 (δ_H 8.00),

H-12 (δ_{H} 8.07) and H-13 (δ_{H} 7.77); ^1H - ^1H coupling constants $^3J_{\text{H-5,H-13}}$ (1.6 Hz) and $^3J_{\text{H-12,H-13}}$ (8 Hz); and ^1H - ^1H COSY correlations (**Appendix YY**) of H-5 to H-13 and H-13 to H-12 indicated the presence of a trisubstituted aromatic ring moiety. The HMBC correlations of H-5 to C-7; H-12 to C-6 and C-10; H-13 to C-11; H-8 to C-6, C-10 and C-16; and H-16 to C-8, C-9 and C-10 indicated the presence of a methylated quinone moiety in C-9 and connected to the aromatic ring (Appendix ZZ). The HMBC correlation of H-15 to C-1, C-2 and C-3; H-3 to C-1 and C-4; H-5 and H-13 to C-3 evidence the connection of C-1 to C-4, C-14 and C-15 to aromatic ring (Appendix ZZ). The Key HMBC and COSY correlations of **13** is in **Figure 40**. Compound **13** was elucidated as a new naphthoquinone named cyphoquinone (**Figure 39**).

Figure 40- Key HMBC and COSY correlations of cyphoquinone (**13**).

The configuration of C-2 as *anti* with C-3 was determined by a large coupling constant $^3J_{\text{H-2,H-3}}$ (8.3 Hz) and ^1H - ^1H NOESY correlations between H-2/H-13, H-2/H-5, H-5/H-15 and H-13/H-15 (**Figure 41** and **Appendix AAA**).

Figure 41 - Newman projection and NOESY correlations in cyphoquinone (**13**).

Table 9 - ^1H and ^{13}C NMR (MeOH- d_4 , 500/125 MHz) data of cyphoquinone (**13**)

No.	δ_{C}	δ_{H} , mult. (J in Hz)
1	176.6	
2	48.7	2.84, dq (7.1, 8.3 Hz)
3	76.4	4.90, d (8.3 Hz)
4	150.2	
5	125.2	8.00, d (1.6 Hz)
6	133.6	
7	186.3	
8	136.5	6.88, d (1.4 Hz)
9	149.9	
10	186.3	
11	133.0	
12	127.6	8.07, d (8.0 Hz)
13	133.2	7.77, dd (1.6, 8.0 Hz)
14	52.3	3.71, s
15	14.0	0.95, d (7.1 Hz)
16	16.3	2.17, d (1.3 Hz)

The molecular formula of **14** was determined to be $\text{C}_{77}\text{H}_{122}\text{O}_{26}$ based on positive ion HRESIMS ($[\text{M} + \text{H}]^+$ m/z 1463.8282, err 1.0 ppm) (**Appendix DDDD**). The ^1H and ^{13}C NMR spectral data of **14** are showed in **Table 10**. The ^{13}C NMR spectrum (**Appendix HHH-LLL**) showed 77 signals assigned to 12 methyl, 18 methylene, 36 methine, 5 carbonyl carbons, 4 tertiary carbons sp^2 and 2 quaternary carbon groups by multiplicity-edited gHSQC experiment (**Appendix MMM**). The COSY and HSQC-TOCSY spectra revealed connectivities from H-2 to H-15 and H-18 to H-43 (**Appendix WWW-CCCC**). The connection of these two spin systems was revealed with the HMBC correlation of H-18 (δ_{H} 2.09 and 1.33) to C-16 and C-17; H-15 (δ_{H} 4.69) to C-16; and H-35 (δ_{H} 5.14) to C-1 (**Appendix RRR** and **VVV**). The geometry of the two double bonds of the macrolide moiety were determine to be *E* configured by the large coupling constants of $^3J_{\text{H-2,H-3}}$ (15.8 Hz) and $^3J_{\text{H-32,H-33}}$ (15.6 Hz). The position of methine groups H-4, H-14, H-34, H-36, H-38 and H-40 were assigned by ^1H - ^1H COSY correlations to methyl groups H-44, H-45, H-46, H-47, H-48 and H-49, respectively (**Appendix XXX**). The methyl group H-43 was assigned as terminal group of side chain by its triplet multiplicity in ^1H NMR spectrum and HMBC correlations to C-41 and C-42 (**Appendix PPP** and **QQQ**). The positions of oxygenated methine and methylene groups in macrolactone moiety were established by HMBC, COSY and HSQC-TOCSY correlations, and comparison of NMR data with those reported for compounds PM100117 and PM100118 (Pérez et al., 2016) (**Figure 42**). The difference in macrolactone moiety of compound **14** versus PM100117 and PM100118 is the additional methyl group H-44 attached to C-4. Other similar macrolactones also have been described, such as compound axenomycin B (Arcamone, Barbieri, Franceschi, Penco, &

Vigevani, 1973), GT-35 (Takahashi et al. 1997), and deplelides A and B (Takeuchi et al., 2017) (Figure 42).

Table 10 - ^1H and ^{13}C NMR (MeOH- d_4 , 500/125 MHz) data of cyphomycin (**14**), epoxycyphomycin (**15**) and GT-35 (**16**)

No.	14 δ_{C}	14 δ_{H} , mult. (<i>J</i> in Hz)	15 δ_{C}	15 δ_{H} , mult. (<i>J</i> in Hz)	16 δ_{C}	16 δ_{H} , mult. (<i>J</i> in Hz)
1	168.7	---	168.6	---	168.6	---
2	122.1	5.86, br d (15.8)	122.3	5.92, d (15.8)	122.3	5.93, d (15.8)
3	153.5	7.06, dd (15.8, 6.8)	154.0	7.10, dd (15.8, 6.7)	154.0	7.09, dd (15.8, 6.9)
4	43.5	2.47, m	43.4	2.49, m	43.3	2.50, m
5	74.2	3.78, m	74.2	3.82, m	74.1	3.84, m
6	41.7	1.60, m	41.7	1.50-1.65	41.6	1.53-1.67, m
		1.54, m				
7	71.8	3.78, m	71.9	3.79, m	71.8	3.81, m
8	38.9	1.32-1.62, m	39.0	1.36-1.54, m	38.8	1.33-1.60, m
9	23.0	1.45-1.68, m	23.1	1.46-1.56, m	22.9	1.46-1.56, m
10	38.5	1.32-1.62, m	38.5	1.35-1.56	38.4	1.31-1.65, m
11	72.9	3.51, m	72.4	3.52, m	72.8	3.51, m
12	36.4	1.38-1.47, m	36.4	1.40-1.46, m	36.2	1.35-1.45, m
		1.53-1.63 m		1.52-1.61, m		1.52-1.69, m
13	31.9	1.69, m	32.1	1.31, m	31.6	1.19-1.27, m
		1.30, m		1.69, m		1.45-1.76, m
14	36.2	2.25, m	36.3	2.23, m	36.7	2.17, m
15	75.8	4.69, d (2.2)	75.8	4.67, d (2.1)	75.4	4.59, d (3.9)
16	210.6	---	210.6	---	208.7	---
17	99.4	---	99.4	---	99.7	---
18	41.1	2.09, m	41.2	2.08, m	75.2	3.49, d (9.2)
		1.33, m		1.33, m		
19	64.9	4.08, m	64.9	4.07, m	69.6	3.87, m
20	42.0	1.96, m	42.0	1.94, m	40.9	1.98, m
		1.21, m		1.22, m		1.43, m
21	67.7	4.20, m	67.8	4.20, m	67.4	4.24, m
22	45.6	1.45-1.74, m	45.6	1.51-1.68, m	45.1	1.47-1.72, m
23	65.4	4.08, m	65.4	4.07, m	65.5	4.00, m
24	46.3	1.43-1.67, m	46.3	1.53, m	46.2	1.40-1.69, m
25	68.2	4.04, m	68.4	4.06, m	68.5	4.05, m
26	46.0	1.40-1.76, m	45.7	1.58, m	45.6	1.39-1.75, m
27	71.3	3.79, m	71.6	3.79, m	71.7	3.76, m
28	38.4	1.32-1.62, m	38.5	1.35-1.56	38.5	1.31-1.66, m
29	22.7	1.51, m	22.4	1.34-1.40, m	22.5	1.30-1.39, m
				1.60-1.66, m		1.61-1.70, m
30	38.4	1.32-1.62, m	33.8	1.28-1.39, m	33.8	1.28-1.38, m
				1.40-1.44, m		1.40-1.49, m
31	72.4	3.94, m	70.9	3.43, m	71.0	3.42, m
32	135.2	5.53, dd (15.6, 4.5)	63.7	2.78, m	63.7	2.78, dd (4.1, 2.0)
33	133.1	5.49, dd (15.6, 7.2)	60.6	2.73, br d (8.2)	60.6	2.73, dd (8.4, 2.0)
34	40.4	2.54, m	40.5	1.58, m	40.5	1.59, m
35	77.3	5.14, br d (9.5)	75.1	5.33, br d (10.4)	75.1	5.33, br d (10.4)
36	38.7	1.97, m	38.5	1.94, m	38.6	1.94, m
37	78.5	3.39, br d (9.5)	78.0	3.37, m	78.0	3.38, br d (9.5)
38	36.0	1.81, m	36.1	1.81, m	36.1	1.81, m
39	79.4	3.52, m	79.5	3.51, br d (7.5)	79.5	3.52, dd (9.2, 2.3)
40	38.5	2.00, m	38.5	2.00, m	38.5	1.99, m
41	80.5	3.88, m	80.5	3.87, m	80.5	3.87, m

Table 10 (Cont.) - ^1H and ^{13}C NMR (MeOH- d_4 , 500/125 MHz) data of cyphomycin (**14**), epoxycyphomycin (**15**) and GT-35 (**16**)

No.	14 δ_{C}	14 δ_{H} , mult. (<i>J</i> in Hz)	15 δ_{C}	15 δ_{H} , mult. (<i>J</i> in Hz)	16 δ_{C}	16 δ_{H} , mult. (<i>J</i> in Hz)
42	22.9	1.64, m 1.36, m	23.0	1.62, m 1.40, m	22.9	1.63, m 1.40, m
43	11.5	0.98, t (7.2)	11.5	0.99, t (7.5)	11.5	0.99, t (7.4)
44	14.1	1.09, d (6.7)	13.6	1.11, d (6.5)	13.5	1.11, d (6.8)
45	13.6	0.79, d (6.4)	13.5	0.79, d (6.5)	13.8	0.84, d (6.8)
46	17.3	1.03, d (6.5)	14.5	1.07, d (6.5)	14.4	1.07, d (6.8)
47	9.7	0.90, d (6.8)	9.4	0.86, d (6.7)	9.4	0.86, d (6.9)
48	5.0	0.90, d (6.8)	4.9	0.89, d (6.7)	4.9	0.89, d (6.9)
49	10.6	0.78, d (6.4)	10.6	0.78, d (6.5)	10.6	0.78, d (6.9)
1'	96.4	5.01, br s	96.4	5.00, br s	96.4	5.01, br s
2'	37.9	1.93, dd (3.70, 14.0) 1.55, d (14.0)	38.0	1.92, br d (13.8) 1.55, br d (13.8)	38.0	1.91, dd (3.0, 14.0) 1.56, d (14.0)
3'	71.2	---	71.2	---	71.2	---
4'	81.7	3.31, m	81.7	3.31, br s	81.7	3.31, br s
5'	64.7	4.44, m	64.7	4.44, m	64.7	4.44, m
6'	17.3	1.21, d (6.6)	17.3	1.21, d (5.7)	17.3	1.21, d (5.7)
7'	27.5	1.24, s	27.5	1.24, s	27.5	1.24, s
1''	104.0	4.58, br d (7.6)	104.0	4.58, br d (7.7)	104.0	4.58, br d (7.0)
2''	31.3	2.01, m 1.56, m	31.4	2.01, m 1.56, m	31.4	2.02, m 1.57, m
3''	28.6	2.10, m 1.56, m	28.6	2.10, m 1.55, m	28.6	2.10, m 1.56, m
4''	74.4	4.45, m	74.4	4.44, m	74.4	4.44, m
5''	74.5	3.54, dq (6.1, 9.3)	74.5	3.54, m	74.5	3.52, dq (6.3, 9.6)
6''	18.5	1.19, d (6.1)	18.5	1.19, d (5.7)	18.5	1.19, d (6.3)
50	175.5	---	175.6	---	175.6	---
51	48.9	2.78, dq (8.5, 7.1)	49.0	2.78, dq (7.0, 8.6)	49.0	2.79, dq (8.6, 7.0)
52	76.4	4.86, d (8.5)	76.5	4.85, d (8.6)	76.5	4.85, d (8.6)
53	150.3	---	150.4	---	150.4	---
54	125.3	8.01, br s	125.3	8.02, br s	125.3	8.02, d (1.5)
55	133.5	---	133.6	---	133.6	---
56	186.2	---	186.3	---	186.2	---
57	136.5	6.88, br d (1.4)	136.5	6.89, d (1.1)	136.5	6.89, br d (1.3)
58	149.8	---	149.9	---	149.9	---
59	186.3	---	186.3	---	186.3	---
60	132.9	---	133.0	---	133.0	---
61	127.6	8.07, d (8.0)	127.6	8.08, d (8.0)	127.6	8.08, d (8.0)
62	133.2	7.79, dd (8.0, 1.3)	133.2	7.79, br d (8.0)	133.2	7.79, dd (8.1, 1.2)
63	14.3	0.94, d (7.1)	14.3	0.94, d (7.0)	14.3	0.94, d (7.0)
64	16.4	2.17, s	16.4	2.17, br s	16.4	2.17, d (1.3)

Figure 42 - Chemical structures of axenomycin B, GT-35, PM100117, PM100118, and deplelide A and B.

The presence of two sugar units was evidenced by ^1H NMR and ^{13}C NMR data, mainly signals of two anomeric protons and carbons at δ_{H} 5.01 (H-1') and δ_{H} 4.58 (H-1''); and δ_{C} 96.4 (C-1') and δ_{C} 104.0 (C-1''), respectively (**Appendix DDD, EEE and KKK**). The first sugar unit was established as α -axenose by HMBC, COSY, ROESY correlations (**Figure 44A** and **Appendix PPP, QQQ, TTT, UUU, XXX, YYY and NNNN-PPPP**) and NMR data comparison with literature (Perez et al., 2016). There are two ^1H spin systems from H-1' to H-2' and from H-4' to H-6'; and HMBC correlations from methyl H-7' to C-2', C-3' and C4'. The connection of this sugar to side chain of macrolactone was observed with HMBC correlations of H-1' to C-41 (**Appendix TTT**). The small coupling constant of H-1' and H-4' suggested they should be in equatorial orientation. The ROE correlation of H-41 to H-5' supported the axial orientation of H-5'.

The second sugar unit was established as β -amicetose. The chemical shifts of β -amicetose were compared to the natural product langkolide (Helaly et al., 2012). This sugar was assigned by the ^1H spin system from H-1'' to H-6'' by HMBC and COSY spectra (**Appendix PPP, TTT, UUU, XXX and YYY**). The large coupling constants $^3J_{\text{H-1'',H-2''ax}}$ (7.6 Hz) and $^3J_{\text{H-4'',H-5''}}$ (9.3 Hz); and ROESY/NOESY correlation between H-1'' and H-5'' (**Appendix PPPP and QQQQ**) suggested the axial orientation of H-1'', H-4'' and H-5''. The connection of both sugars was observed by the HMBC correlation of H-1'' to C-4' (**Appendix TTT**). The HMBC correlation of H-4'' to C-50 connected the β -amicetose with naphthoquinone moiety (**Appendix RRR**). The naphthoquinone derivative moiety in **14** has identical NMR data as observed for compound **13**. So, configuration of C-51 and C-52 is also *anti* (**Figure 44B**). The UV spectrum of **14** was similar than for **13** (**Appendix GGG**) supporting the presence of same naphthoquinone derivative in compound **14**. The key COSY, HSQC-TOCSY and HMBC correlations of **14** are shown in **Figure 43**. An extensive NMR and MS analysis suggested that compound **14** is a new macrolide natural product named cyphomycin (**Figure 39**).

Figure 43 - Key COSY, HSQC-TOCSY and HMBC correlations of cyphomycin (**14**).

Figure 44 - Relative configuration proposed for fragment: A) disaccharide and B) naphthoquinone units.

The partial relative configuration of macrolactone moiety of **14** was determined using ROESY, NOESY-1D, E.COSY, *J*-based configuration analysis and Kishi's universal NMR database method (Kobayashi et al. 1999; Kobayashi et al. 2000). The coupling constants were measured by ^1H NMR and E.COSY spectra. The ^1H - ^1H ROESY correlation of H-5/H-44 (**Appendix GGGG**), large coupling constant $^3J_{\text{H-4,H-5}}$ (8.8 Hz) (**Appendix SSSS-UUUU**) and NMR data comparison with deplide A (Takeuchi et al., 2017) indicated a 5,44-*syn* configuration (**Figure 45A**). The configuration of C-5 as *syn* with C-7 was proposed based in shift of C-7 ($\delta_{\text{C-7}}$ 71.8) and data set I of Kishi's universal NMR data base (**Appendix VVVV**). The ^1H - ^1H ROESY correlation of H-14/H-15; the no ROE correlation of H-15/H-45; and small coupling constant $^3J_{\text{H-14,H-15}}$ (2.2 Hz) suggested the *syn* configuration of H-14 with H-15 (**Figure 45B** and **Appendix FFFF**). The ^1H - ^1H ROESY correlation of H-18ax/H-15, H-18eq/H-19, H-19/H-21 and H-21/H-23 inferred a chair conformation of the tetrahydropyran moiety with equatorial substituents, C-16, 19-OH and C-22, and axial hydroxyl group at C-17 (**Figure 45C**, **Appendix KKKK** and **LLLL**). The relative configurations of oxymethine carbons C-23, C-25 and C-27 were assigned as *anti/syn* using ^{13}C NMR signals (δ_{C} 65.4, 68.2 and 71.3, respectively) and data set I and II of Kishi's universal NMR data base (**Appendix VVVV**).

Figure 45 - Relative configuration proposed for fragments: A) C-4 to C-7, B) C-14 to C-15, and C) C-17 to C-27.

The ^1H - ^1H ROESY correlation of H-33/H-35, H-35/H-46, H-35/H-36, H-34/H-47, H-37/H-47, H-37/H-38, H-38/H-39, H-38/H-49, H-39/H-49, H-40/H-48, H-40/H-41 and H-41/H-1' (**Appendix EEEE-JJJJ** and **Figure 48**); the absence of ^1H - ^1H ROESY correlation of H-35/H-47, H-39/H-48 and H-41/H-49; the large coupling constants $^3J_{\text{H-34,H-35}}$ (9.5 Hz), $^3J_{\text{H-36,H-37}}$ (9.5 Hz) and $^3J_{\text{H-39,H-40}}$ (7.4 Hz); small coupling constants $^3J_{\text{H-35,H-36}}$, $^3J_{\text{H-37,H-38}}$ and $^3J_{\text{H-38,H-39}}$; data set III of Kishi's universal NMR data base for configuration of C-37 to C-39 [$\delta_{\text{C-48}}$ 5.0, 37,38,39-*syn/syn*] (**Appendix VVVV**); and NMR data comparison with deplelide A (Takeuchi et al., 2017) suggested that relative configuration of C-34 to C-41 in cyphomycin (**14**) is identical as observed for deplelide A (**Figure 42**).

Figure 46 - Relative configuration proposed for fragment of C-32 to C-43.

The molecular formula of **15** was determined to be $C_{77}H_{122}O_{27}$ based on positive ion HRESIMS ($[M + H]^+$ m/z 1479.8212, err 2.3 ppm) (**Appendix IIIII**). Compound **15** has one additional oxygen atom than compound **14**. The differences between compound **14** and **15** are in carbons C-32 and C-33, based on the g COSY, g HSQC, g HMBC, g ROESY, NOESY-1D and UV spectra (**Table 10** and **Appendix WWW-HHHHH** and **JJJJJ-LLLLL**). Compound **15** has an epoxide in C-32 (δ_C 63.7) and C-33 (δ_C 60.6); while **14** has an alkene in C-32 (δ_C 135.2) and C-33 (δ_C 133.1). The small coupling constant $^3J_{H-32,H-33} < 3$ Hz, large coupling constant $^3J_{H-33,H-34}$ (8.2 Hz) and $^3J_{H-34,H-35}$ (10.4 Hz), and 1H - 1H ROESY correlations of H-33/H-35, H-32/H-34 and H-2/H-32 suggested that geometry of hydrogens H-32 and H-33 of epoxide ring should be *trans* (**Figure 47** and **Appendix KKKKK**). Compound **15** has an epoxide group in the same position in macrolactone moiety as in GT-35 (Takahashi et al. 1997), and deplelides A and B (Takeuchi et al., 2017) (**Figure 42**). An extensive NMR and MS analysis suggested that compound **15** is a new macrolide natural product named epoxycyphomycin (**Figure 39**).

Figure 47 - Relative configuration proposed for fragment of C-32 to C-35 of compound **15**.

The molecular formula of **16** was determined to be $C_{77}H_{122}O_{28}$ based on positive ion HRESIMS ($[M + H]^+$ m/z 1495.8189, err 0.4 ppm) (**Appendix BBBBBB**). Compound **16** has one additional oxygen atom than **15**. The different between compound **15** and **16** should be in carbon C-18, based in the 1 and 2 D NMR data and UV spectrum (**Table 10** and **Appendix MMMMM-AAAAAA**). Compound **16** has a hydroxyl group at C-18 (δ_C 75.2) while **15** has a methylene in C-18 (δ_C 41.2). The large coupling constant $^3J_{H-18,H-19}$ (9.2 Hz), and 1H - 1H ROESY correlation between H-15/H-18 and H-19/H-21 suggested the configuration of H-18 as anti with H-19, and H-19 and H-21 have axial orientation in the tetrahydropyran moiety (**Figure 48** and **Appendix ZZZZZ-AAAAAA**). Extensive NMR and MS analyses suggested that compound **16** is the known macrolide GT-35 (**Figure 42**), previously isolated from *Streptomyces* sp. (Takahashi et al. 1997).

Figure 48 - Relative configuration proposed for fragment of C-15 to C-21 of compound **16**.

4.5.2. Physicochemical data for compounds 13-16

Cyphoquinone (**13**): yellow amorphous solid; $[\alpha]_D^{23} = +35.1$ (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ) 205 (5.3), 249 (5.4), 254 (5.4), 334 (4.6); ^1H and ^{13}C NMR (CD_3OD , 500/125 MHz) refer to **Table 9**; HRESIMS m/z 311.08801 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{16}\text{H}_{16}\text{NaO}_5$, 311.08899, err 3.2 ppm).

Cyphomycin (**14**): yellow amorphous solid; $[\alpha]_D^{26} = +25.5$ (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (5.2), 248 (5.0), 254 (5.0), 334 (4.2); ^1H and ^{13}C NMR (CD_3OD , 500/125 MHz) refer to **Table 10**; HRESIMS m/z 1463.8282 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{77}\text{H}_{123}\text{O}_{26}$, 1463.8297, err 1.0 ppm).

Epoxyxycyphomycin (**15**): yellow amorphous solid; $[\alpha]_D^{26} = +14.5$ (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (5.0), 248 (4.8), 254 (4.8), 333 (4.0); ^1H and ^{13}C NMR (CD_3OD , 500/125 MHz) refer to **Table 10**; HRESIMS m/z 1479.8212 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{77}\text{H}_{123}\text{O}_{27}$, 1479.8246, err 2.3 ppm).

GT-35 (**16**): yellow amorphous solid; $[\alpha]_D^{26} = +8.5$ (*c* 0.18, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (4.8), 248 (4.6), 254 (4.6), 333 (3.7); ^1H and ^{13}C NMR (CD_3OD , 500/125 MHz) refer to **Table 10**; HRESIMS m/z 1495.8189 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{77}\text{H}_{123}\text{O}_{28}$, 1495.8195, err 0.4 ppm).

4.5.3. Description and biological evaluation of compounds 13-16

Streptomyces sioyaensis ICBG311 showed antagonist activity against *Escovopsis* sp. and its crude extract, obtained in A-medium, was active against the parasite *Leishmania donovani* (GI% = 94%) (**Table 3**). Compounds **14-16** were isolated from an active fraction (*ICBG311.A4*) against *Escovopsis* sp. ICBG1251 (**Figure 49**). **Figure 50** shows the peaks in the UV

chromatogram of this fraction. Compound **14** was slightly more polar than **15** and **16** due the presence of double bond in carbon C-32 and C-33 in place of an epoxide group. Compound **15** eluted before **16** although the additional hydroxyl group in carbon C-18 in **16**. Different intramolecular interactions in the three analogues (**14-16**) could account for the observed behavior during HPLC purification.

Figure 49 - Antagonist activity of fraction *ICBG311.A4* (100 μ g) against *Escovopsis* sp ICBG1251 (Method 3).

Figure 50 - UV chromatogram of fraction *ICBG311.A4* containing compounds **14-16**. HPLC column: C_{18} semipreparative (Phenomenex Luna, $C_{18}(2)$, 5 μ m, 250 x 10 mm). Method: gradient of MeOH and H_2O (containing 0.1% of acetic acid) at 4 mL/min; min 1-20 (linear gradient from 80% MeOH- H_2O to 100% MeOH); min 20-22 (isocratic flow of 100% MeOH); min 22-22.5 (linear gradient from 100% MeOH to 80% MeOH- H_2O); and min 22.5-27.5 (isocratic flow of 80% MeOH- H_2O).

Compounds **14-16** have a similar macrolactone with sugar units and a methylnaphthoquinone as the natural products axenomycin B (Arcamone et al., 1973), PM100117 and PM100118 (Pérez et al., 2016), and deplelides A and B (Takeuchi et al., 2017) (**Figure 42**). These compounds have interesting antifungal, antibiotic, antitumor and immunosuppressive properties (Arcamone et al. 1973; Pérez et al. 2016; Takahashi et al. 1997; Takeuchi et al. 2017). This explains the antifungal activity of **14-16** against four different *Escovopsis* sp. strains (**Figure 51**). They showed inhibition zones similar to the positive control miconazole. Compounds **14-16** did not inhibit the opportunistic fungi *Trichoderma* ICBG1100 and *Cunninghamella* ICBG752, showing more selectivity against *Escovopsis*. Compound **13** was inactive against *Escovopsis* sp., highlighting the importance of macrolactone moiety and sugar units in **14-16** for the antifungal activity.

Compounds **14-16** displayed *in vitro* activity against *Candida albicans* K1 with MIC of 1.0, 0.5 and 0.25 µg/mL, respectively; and were more active against both *L. donovani* forms than the positive control miltefosine, while **13** showed lower activity (**Table 11**). Compound **14** showed lower selectivity index than **15-16** in antileishmanial bioassay (**Table 11**). The presence of the epoxide group in C-32 and C-33 in macrolactone moiety of **15** and **16** (**Figure 39**) increased the anti-*Candida*, antileishmanial activity and selectivity index (**Table 11**).

Table 11 - Activity of compounds **13-16** on *L. donovani* intracellular amastigotes, against promastigotes and THP-1 cells

Compounds	IC ₅₀ (µM) Intracellular amastigotes	IC ₅₀ (µM) Promastigotes	CC ₅₀ (µM) THP-1*	Selectivity Index**
13	60.90 ± 0.01	16.63 ± 1.55	19.84 ± 1.30	0.33
14	2.25 ± 0.15	0.112 ± 0.015	10.95 ± 1.41	4.87
15	0.087 ± 0.015	0.033 ± 0.006	6.54 ± 0.73	75.17
16	0.068 ± 0.013	0.040 ± 0.006	5.76 ± 1.20	84.71
Doxorubicin	---	---	0.571 ± 0.068	---
Miltefosine	5.80 ± 0.59	4.74 ± 0.25	---	---

Data are shown as mean ± SD (*n* = 2 biological replicates)

*THP-1 human leukemia macrophages (host cells of *L. donovani*)

**Selective index = CC₅₀ THP-1/IC₅₀ intracellular amastigotes

Figure 51 - Antagonist activity of compounds **14-16** (100 μ) against four different *Escovopsis* strains (21 days at 28 °C) (Method 3): A) **14** vs *Escovopsis* sp ICBG711, B) **14** vs *Escovopsis* sp ICBG740, C) **14** vs *Escovopsis* sp ICBG741, D) **14** vs *Escovopsis* sp ICBG1251, E) **15** vs *Escovopsis* sp ICBG711, F) **15** vs *Escovopsis* sp ICBG740, G) **15** vs *Escovopsis* sp ICBG741, H) **15** vs *Escovopsis* sp ICBG1251, I) **16** vs *Escovopsis* sp ICBG711, J) **16** vs *Escovopsis* sp ICBG740, K) **16** vs *Escovopsis* sp ICBG741, L) **16** vs *Escovopsis* sp ICBG1251, M) Miconazole vs *Escovopsis* sp ICBG711, N) Miconazole vs *Escovopsis* sp ICBG740, O) Miconazole vs *Escovopsis* sp ICBG741, P) Miconazole vs *Escovopsis* sp ICBG1251, Q) Control of *Escovopsis* sp ICBG711, R) Control of *Escovopsis* sp ICBG740, S) Control of *Escovopsis* sp ICBG741, T) Control of *Escovopsis* sp ICBG1251.

Compounds **14-16** were inactive against *B. subtilis* (NRS 231), *E. coli* (ATCC 25922), methicillin-sensitive *S. aureus* (MSSA) (Wichita) and *P. aeruginosa* (Boston 41501) using an agar-based antibiotic assay (section 3.5.3.), while **13** was active against *B. subtilis* and MSSA (Figure 52). So, compounds **14-16** showed higher selectivity against fungi than bacteria.

Figure 52 - Agar-based antibiotic assay of compound **13** (10 µg) against bacteria: A) *B. subtilis* and B) MSSA.

Cyphomycin (**14**) showed potent *in vitro* activity against the resistant human pathogens *Aspergillus fumigatus* 11628 (echinocandin resistant), *C. glabrata* 4720 (triazole resistant), and *C. auris* B11211 (echinocandin, triazole, and amphotericin B resistant), with MIC of 0.5, 0.5 and 4 µg/mL, respectively. A single-dose study of **14** in a neutropenic mouse disseminated candidiasis model (Zhao et al., 2018) exhibited a dose-like response with 0.56 and 0.66 log reduction of infectious burden when treated with 20 and 40 mg/kg, respectively, and **15** exhibited 0.53 log reduction with 40 mg/kg, demonstrating clinical relevance and effectiveness of **14** and **15** in this industry-standard model of *Candida* infection. On the other hand, compound **16** killed the mice 1 hr post dose at 40 mg/kg. Antifungal bioassays were carried out in the laboratory of Dr. David R. Andes at the Department of Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA.

Compounds **13** showed drug-like properties according to Lipinski's rules and Veber filters (Lipinski et al., 2001; Veber et al., 2002). Compounds **14-16** exceeded several properties as ideal molecular weight, number of hydrogen-bond acceptors (HBA) and hydrogen-bonds donor (HBD), and others, but they comply with the log *P* and hERG pIC₅₀ values (Table 12).

Table 12. Molecular properties of compound **13-16**

Code	MW	LogP	hERG pIC ₅₀	HIA	HBD	HBA	TPSA	nrotb	Drug- likeness
13	288.29	2.05	3.88	+	1	5	80.67	4	yes
14	1463.78	2.46	4.05	-	14	26	433.18	16	no
15	1479.78	2.28	3.73	-	14	27	445.71	16	no
16	1495.78	1.77	3.41	-	15	28	465.94	16	no
Desired Value	≤ 500	< 5	< 6.3	+	≤ 5	≤ 10	≤ 140 Å	≤ 10	

LogP: octanol/water partition coefficient; hERG pIC₅₀: -logIC₅₀ on human ether-a-go-go-related gene potassium ion channels; HIA: human intestinal absorption; HBD: hydrogen-bond donors; HBA: hydrogen-bond acceptors; TPSA: topological surface area; nrotb: number of rotatable bonds. Drug-likeness according to Lipinski and Veber filters.

4.6. Cultivation of actinobacteria *Streptomyces luteogriseus* AB11, *Nocardioides albus* AB12 and *Amycolatopsis orientalis* AC44

The three actinobacteria were active against *Escovopsis* in antagonism bioassays (**Figure 53**). Their crude extracts obtained in ISP-2 agar were inactive against parasites *T. cruzi* and *L. donovani* (**Table 1**). These actinobacteria were cultured in 5 L of ISP-2 agar to isolate antifungal compounds. The crude extracts were fractionated by SPE-C18. All fractions were inactive against *Escovopsis*. There are some possible explanations for this: the actinobacteria did not produce active compounds after re-cultivation, they produced active volatile compounds not detected with the employed methods, the active compounds have low stability or the production of antifungal compounds is only elicited in co-culture with *Escovopsis* sp.

Streptomyces luteogriseus AB11 also produced the antibiotic streptazolin (**7**) as *Streptomyces chartreusis* AC70, but not the *E*-isomer (**8**).

Figure 53 - Antagonist activity of actinobacteria against *Escovopsis*: A) *Streptomyces luteogriseus* AB11 vs *Escovopsis* (Method 1), B) *Amycolatopsis orientalis* AC44 vs *Escovopsis* (Method 1), C) *Nocardioides albus* AB12 vs *Escovopsis* (Method 2).

5. CONCLUSIONS

The class of antibiotics produced by *Streptomyces puniceus* AB10 change drastically in A-medium versus in medium ISP-2 and YPM. This bacterium produced in more abundance the antibiotic dinactin (**4**) and analogues using A-medium, and not the griseorhodin compounds (**1-3**). Antibiotics griseorhodins A (**1**), C (**2**) and G (**3**) were inactive against *L. donovani* and *Escovopsis* sp. On the other hand, dinactin (**4**) was active against *Escovopsis* sp ICBG741 and showed more potent activity against *L. donovani* than the positive control miltefosine. The antiprotozoal activity of dinactin (**4**) has not been published previously reported.

Streptomyces chartreusis AC70 can produce the inorganic compound cyclooctasulfur (**9**) to control the growth of *Escovopsis* sp. Compound **9** has been previously reported from a marine *Streptomyces* and is the most common allotrope of sulfur found in nature. The element sulfur has been used as antimicrobial agent so this explains the antifungal activity of **9** against *Escovopsis*.

Candidatus Streptomyces philanthi bv. *triangulum* ICBG292 could control the growth of *Escovopsis* and other opportunistic fungus as *Trichoderma* sp with antibiotics Mer-A2026B (**10**), piericidin-A₁ (**11**) and nigericin (**12**). Compounds **10-12** also showed antileishmanial activity, and **12** was more potent than miltefosine. Antileishmanial activity has not been previously reported for compounds **10** and **11**.

Streptomyces sioyaensis ICBG311 produced a naphthoquinone derivative **13** and three potent antifungal compounds **14-16** that possess an aglycone macrolide system, a disaccharide moiety and a naphthoquinone unit. Compounds **13-15** are new natural products. Compounds **14-16** showed activity against *Escovopsis* sp. and higher activity against *L. donovani* in both forms than miltefosine. The presence of the epoxide group in C-32 and C-33 in the macrolactone moiety of **15** and **16** increased the anti-*Candida*, antileishmanial activity and selectivity index versus compound **14**. Compounds **14-16** showed higher selectivity against fungi than bacteria. Cyphomycin (**14**) and epoxycyphomycin (**15**) showed clinical relevance and effectiveness in this industry-standard model of *in vivo* *Candida* infection.

The actinobacteria *Streptomyces luteogriseus* AB11, *Nocardioides albus* AB12 and *Amycolatopsis orientalis* AC44 also produced secondary metabolites that control the growing of *Escovopsis* sp.

Compounds **10-12** and **14-16** showed antifungal and antiprotozoal activity. This mixed biological activity has been found for some compounds such as azoles and amphotericin B.

Dinactin (**4**) and nigericin (**12**) could be interesting compounds for further pharmacological studies in the treatment of leishmaniasis based in the high selectivity index against *L. donovani* and on Log *P* and HBD values that comply with Lipinsky's rules.

This work supports that ecological function of natural products from actinobacteria associated to insects as attine ants can be aligned to pharmacological activities.

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APPENDICES

Appendix A - ^1H NMR spectrum of griseorhodin A (1) (Acetone- d_6 , 500 MHz)

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdB	e ⁻	Conf	N-Rule
507.0551	1	C23H3N14O2	507.0569	3.5	31.3	1	100.00	29.5	even		ok
	2	C38H7N2O	507.0564	2.5	402.0	2	0.00	36.5	even		ok
	3	C32H11O7	507.0510	-8.1	406.2	3	0.00	27.5	even		ok
	4	C33H7N4O3	507.0524	-5.5	406.9	4	0.00	32.5	even		ok
	5	C25H15O12	507.0569	3.5	412.8	5	0.00	18.5	even		ok
	6	C26H11N4O8	507.0582	6.1	413.2	6	0.00	23.5	even		ok
	7	C27H7N8O4	507.0596	8.8	413.6	7	0.00	28.5	even		ok

Appendix B - HRESIMS of griseorhodin A (1)

Appendix C - NOESY-1D of griseorhodin A (**1**) (Acetone- d_6 , 500 MHz)

Appendix D - ^1H NMR spectrum of griseorhodin C (2) (Acetone- d_6 , 500 MHz)

Appendix E - HRESIMS of griseorhodin C (2)

Appendix F - gHSQC of griseorhodin C (2) (Acetone- d_6 , 500 MHz)

Appendix G - gHMBC-1 of griseorhodin C (2) (Acetone- d_6 , 500 MHz)

Appendix H - gHMBC-2 of griseorhodin C (**2**) (Acetone-*d*₆, 500 MHz)

Appendix I - gHMBC-3 of griseorhodin C (**2**) (Acetone-*d*₆, 500 MHz)

Chemical Structure 10: COc1cc(=O)c2c(c1)c(O)c3c2O[C@H]4[C@@H](O)[C@H](O)[C@@H](OC5=CC(=C(C=C5)C(=O)OC)C(=C5)O)C4

¹H NMR Data (DMSO-d₆):

Chemical Shift (ppm)	Integration
7.09	1.00
6.99	1.00
6.58	1.00
6.33	1.00
5.78	1.00
5.20	1.00
5.19	1.00
4.57	1.00
4.37	1.00
3.88	3.00
3.68	1.00
2.23	1.00

Appendix K - ^1H NMR spectrum of griseorhodin C (**2**) (DMSO- d_6 , 500 MHz)

Appendix L - gHMBC correlations observed for griseorhodin C (**2**) only in DMSO- d_6 and not in Acetone- d_6

Appendix M - NOESY-1D of griseorhodin C (2) (Acetone- d_6 , 500 MHz)

H-6 5.53 ppm

Current Data Parameters
 NAME he01_39_2
 EXPRO 600
 PROCNO 6000

F2 - Acquisition Parameters
 Date_ 20161104
 Time 15.08
 INSTRUM spect
 PROBHD 5 mm BBI 1H-BB
 PULPROG noedif
 TD 65536
 SOLVENT Acetone
 NS 142
 DS 2
 SWH 8503.401 Hz
 FIDRES 0.129752 Hz
 AQ 3.8536255 sec
 RC 90.3
 DW 58.800 usec
 DE 6.00 usec
 TR 296.2 K
 D1 1.00000000 sec
 d11 0.03000000 sec
 d13 0.00000300 sec
 D20 2.00000000 sec
 L4 16
 NBL 2

===== CHANNEL f1 =====
 NUC1 1H
 P1 8.25 usec
 PL1 3.00 dB
 SFO1 500.1335010 MHz

===== CHANNEL f2 =====
 NUC2 1H
 PL2 3.00 dB
 PL14 70.00 dB
 SFO2 500.1335010 MHz

F1 - Acquisition parameters
 ND0 1
 TD 2
 SFO1 500.1335 MHz
 FIDRES 500.000000 Hz
 SW 1.999 ppm
 FMODE undefined

F2 - Processing parameters
 SI 32768
 SF 500.1300148 MHz
 WDW EM
 SSB 0
 LB 0.00 Hz
 GB 0
 PC 1.00

Appendix N - NOEDIFF of griseorhodin C (2) (Acetone- d_6 , 500 MHz)

Appendix O - ¹H NMR spectrum of fraction with Griseorhodin G (3) (Acetone-*d*₆, 500 MHz)

Appendix P - HRESIMS of griseorhodin G (3)

[illegible]

Appendix R - ¹³C NMR spectrum of dinactin (4) (CDCl₃, 125 MHz)

Appendix S - HRESIMS of dinactin (4)

Appendix T - ¹H NMR spectrum of strepazolin A (5) (CDCl₃, 500 MHz)

Appendix U - HRESIMS of strepchazolin A (**5**)

Appendix V - gHSQC of strepchazolin A (**5**) (CDCl₃, 500 MHz)

Appendix W - gHMBC of strepchazolin A (5) (CDCl₃, 500 MHz)

Appendix X - gCOSY of strepchazolin A (5) (CDCl₃, 500 MHz)

Appendix Y - NOESY 1D spectrum of strepchazolin A (5) (CDCl₃, 500 MHz)

Appendix Z - ¹H NMR spectrum of strepchazolin B (6) (CDCl₃, 500 MHz)

Appendix AA - HRESIMS of strepchazolin B (6)

Appendix BB - gHSQC of strepchazolin B (6) ($CDCl_3$, 500 MHz)

Appendix CC – gHMBC-1 of strepchazolin B (6) (CDCl₃, 500 MHz)

Appendix DD – gHMBC-2 of strepchazolin B (6) (CDCl₃, 500 MHz)

Appendix EE - gCOSY of strepchazolin B (6) (CDCl₃, 500 MHz)

Appendix FF - NOESY 1D spectra of strepachazolin B (**6**) (CDCl_3 , 500 MHz)

Appendix GG - ^1H NMR spectrum of streptazolin (**7**) (CDCl_3 , 500 MHz)

Appendix HH - HRESIMS of streptazolin (7)

Appendix II - 1H NMR spectrum of *E*-isomer of streptazolin (8) ($CDCl_3$, 500 MHz)

Appendix JJ - HRESIMS of *E*-isomer of streptazolin (8)

Appendix KK - GC-MS spectrum of octasulfur (9)

Appendix LL - ^1H NMR spectrum of mer-A2026B (10) (CDCl_3 , 500 MHz)

Appendix MM - HRESIMS of mer-A2026B (10)

Appendix NN - ¹H NMR spectrum of piericidin-A₁ (11) (CDCl₃, 500 MHz) Appendix OO - ¹³C NMR spectrum of piericidin-A₁ (11) (CDCl₃, 125 MHz)

Appendix PP - HRESIMS of piericidin-A₁ (11)

Appendix QQ - ¹H NMR spectrum of nigericin (12) (CDCl₃, 500 MHz)

Spectrum View - 20170812-HO-34-3_1-A_2_01_10070.d

Intensity $\times 10^6$

m/z

MS, 12.79min @ 1537

Measured m/z: 747.4667 Tolerance: 2 mDa Charge: 1

Meas. m/z	#	Ion Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e ⁻	Co
747.4667	1	C ₃₈ H ₆₃ N ₆ O ₉	59.19	747.4651	-1.6	-2.1	31.0	11.0	even	
747.4667	1	C ₄₀ H ₆₈ N ₈ O ₁₁	67.25	747.4654	-1.3	-1.8	32.8	7.0	even	
747.4667	2	C ₃₉ H ₅₉ N ₁₀ O ₅	94.78	747.4664	-0.3	-0.4	41.6	16.0	even	
747.4667	2	C ₃₈ H ₅₆ N ₁₄ NaO	53.95	747.4654	-1.3	-1.8	40.9	18.0	even	
747.4667	3	C ₄₂ H ₆₇ O ₁₁	57.69	747.4678	1.1	1.4	44.5	10.0	even	
747.4667	3	C ₄₁ H ₆₄ N ₄ NaO ₇	100.00	747.4667	0.0	0.0	43.2	12.0	even	
747.4667	4	C ₄₀ H ₅₅ N ₁₄ O	45.13	747.4678	1.1	1.4	52.9	21.0	even	
747.4667	4	C ₄₂ H ₆₀ N ₈ NaO ₃	35.99	747.4681	1.3	1.8	54.3	17.0	even	
747.4667	5	C ₅₅ H ₅₉ N ₂	3.33	747.4673	0.6	0.8	120.1	28.0	even	
747.4667	5	C ₅₃ H ₆₀ N ₂ Na	2.93	747.4649	-1.8	-2.5	107.5	25.0	even	

Appendix UU - HRESIMS of nigericin (12)

Appendix VV - ^1H NMR spectrum of cyphoquinone (13) (CD_3OD , 500 MHz)

Appendix WW - ^{13}C NMR spectrum of cyphoquinone (13) (CD_3OD , 125 MHz)

Appendix XX - gHSQC of cyphoquinone (13) (CD₃OD, 500 MHz)

Appendix YY - gCOSY of cyphoquinone (13) (CD₃OD, 500 MHz)

Appendix ZZ - gHMBC of cyphoquinone (13) (CD₃OD, 500 MHz)

Appendix AAA - gNOESY of cyphoquinone (13) (CD₃OD, 500 MHz)

Appendix BBB - HRESMS of cyphoquinone (13)

Appendix CCC - ^1H NMR spectrum of cyphomycin (**14**) (CD_3OD , 500 MHz)

Appendix DDD - ^1H NMR spectrum of cyphomycin (**14**) (CD_3OD , 500 MHz). Region of 4.8 to 8.2 ppm

Appendix EEE - ¹H NMR spectrum of cyphomycin (**14**) (CD₃OD, 500 MHz). Region of 2.4 to 4.8 ppm

Appendix FFF - ¹H NMR spectrum of cyphomycin (**14**) (CD₃OD, 500 MHz). Region of 0.7 to 2.3 ppm

Appendix GGG - UV spectra of cyphoquinone (**13**) and cyphomycin (**14**) in MeOH

Appendix HHH - ¹³C NMR spectrum of cyphomycin (**14**) (CD₃OD, 125 MHz)

Appendix III - ^{13}C NMR spectrum of cyphomycin (14) (CD₃OD, 125 MHz). Region of 4 to 41.2 ppm

Appendix JJJ - ^{13}C NMR spectrum of cyphomycin (14) (CD_3OD , 125 MHz). Region of 41 to 89 ppm

Appendix NNN - gHMBC of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix OOO - gHMBC-1 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix PPP - gHMBC-2 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix QQQ - gHMBC-3 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix RRR - gHMBC-4 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix SSS - gHMBC-5 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix VVV - gHMBC-8 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix WWW - gCOSY of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix XXX - gCOSY-1 of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix YYY - gCOSY-2 of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix ZZZ - gCOSY-3 of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix AAAA - gHSQC-TOCSY of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix BBBB - gHSQC-TOCSY-1 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix CCCC - gHSQC-TOCSY-2 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix DDDD - HRESMS spectrum of cyphomycin (14)

Appendix EEEE - gROESY of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix FFFF - gROESY-1 of cyphomycin (**14**) (CD_3OD , 500 MHz)

Appendix GGGG - gROESY-2 of cyphomycin (**14**) (CD_3OD , 500 MHz)

Appendix HHHH - gROESY-3 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix IIII - gROESY-4 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix JJJJ - gROESY-5 of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix KKKK - gROESY-6 of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix LLLL - gROESY-7 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix MMMM - gROESY-8 of cyphomycin (14) (CD₃OD, 500 MHz)

Appendix NNNN - gROESY-9 of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix OOOO - gROESY-10 of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix PPPP - gROESY-11 of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix QQQQ - NOESY-1D of cyphomycin (**14**) (CD₃OD, 500 MHz)

Appendix RRRR - E.COSY of cyphomycin (**14**) (CD₃OD, 600 MHz)

Appendix SSSS - E.COSY of cyphomycin (**14**) (CD₃OD, 600 MHz). Calculation of $^3J_{H-4,H-5}$

Appendix TTTT - E.COSY of cyphomycin (**14**) (CD₃OD, 600 MHz). Calculation of $^3J_{H-3,H-4} = 6.8 \text{ Hz}$

Appendix UUUU - E.COSY of cyphomycin (**14**) (CD₃OD, 600 MHz). Calculation of $^3J_{H-4,H-44} = 6.7 \text{ Hz}$

Appendix VVVV - Data sets of Kishi's universal NMR database method for elucidation of relative configuration of 1,3-diol (I), 1,3,5-triol (II) and 2-methyl-1,3-diol (III)

Appendix WWWW - ^1H NMR spectrum of epoxycyphomycin (**15**) (CD₃OD, 500 MHz)

Appendix XXXX - ^1H NMR shifts of epoxycyphomycin (**15**)

Appendix YYYY - UV spectrum of epoxycyphomycin (**15**) in MeOH

Appendix ZZZZ - ¹³C NMR spectrum of epoxycyphomycin (**15**) (CD₃OD, 125 MHz)

Appendix AAAAA - ^{13}C NMR spectrum of epoxycyphomycin (15) (CD_3OD , 125 MHz). Region of 3 to 50 ppm

Appendix BBBBB - ^{13}C NMR spectrum of epoxycyphomycin (15) (CD_3OD , 125 MHz). Region of 58 to 82 ppm

Appendix CCCCC - ^{13}C NMR spectrum of epoxycyphomycin (15) (CD_3OD , 125 MHz). Region of 91 to 177 ppm

Appendix DDDDD - ^{13}C NMR spectrum of epoxycyphomycin (**15**) (CD_3OD , 125 MHz). Region of 185 to 212 ppm

Appendix EEEEE - Multiplicity-edited gHSQC of epoxycyphomycin (**15**) (CD_3OD , 500 MHz)

Appendix FFFFFF - gCOSY of epoxycyphomycin (**15**) (CD₃OD, 500 MHz)

Appendix GGGGGG - gHMBC of epoxycyphomycin (**15**) (CD₃OD, 500 MHz)

Appendix HHHHHH - Key COSY and HMBC correlation of epoxycyphomycin (**15**)

Appendix IIII - HRESMS spectrum of epoxycyphomycin (15)

Appendix JJJJ - gROESY of epoxycyphomycin (15) (CD₃OD, 500 MHz)

Appendix KKKKK - gROESY-1 of epoxycyphomycin (15) (CD₃OD, 500 MHz)

Appendix LLLLL - NOESY-1D of epoxycyphomycin (15) (CD₃OD, 500 MHz)

Appendix M - ^1H NMR spectrum of GT-35 (**16**) (CD_3OD , 500 MHz)

Appendix N - ^1H NMR shifts of GT-35 (**16**) (CD_3OD , 500 MHz)

Appendix O - UV spectrum of GT-35 (**16**) in MeOH

Appendix P P P P P - ^{13}C NMR spectrum of GT-35 (16) (CD_3OD , 125 MHz)

Appendix Q Q Q Q Q - ^{13}C NMR spectrum of GT-35 (16) (CD_3OD , 125 MHz). Region of 4.5 to 36 ppm

Appendix RRRRR - ¹³C NMR spectrum of GT-35 (16) (CD₃OD, 125 MHz). Region of 35 to 93 ppm

Appendix TTTTT - ^{13}C NMR spectrum of GT-35 (16) (CD_3OD , 125 MHz). Region of 186 to 210 ppm

Appendix UUUUU - Multiplicity-edited gHSQC of GT-35 (16) (CD_3OD , 500 MHz)

Appendix VVVVV - gCOSY of GT-35 (16) (CD₃OD, 500 MHz)

Appendix WWWW - gHMBC of GT-35 (16) (CD₃OD, 500 MHz)

Appendix XXXXX - Key COSY and HMBC correlations of GT-35 (16) (CD₃OD, 500 MHz)

Appendix YYYYY - gROESY of GT-35 (16) (CD₃OD, 500 MHz)

Appendix ZZZZZ - gROESY-1 of GT-35 (16) (CD₃OD, 500 MHz)

Appendix AAAAAA - gROESY-2 of GT-35 (16) (CD₃OD, 500 MHz)

Appendix BBBBBB - HRESMS spectrum of GT-35 (16)