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Identification of Antibiotic GE37468A from Pseudonocardia Symbionts of Trachymyrmex Septentrionalis Ants

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Identification of antibiotic GE37468A from *Pseudonocardia* symbionts of *Trachymyrmex septentrionalis* ants

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ABSTRACT

In response to the growing rates of antibiotic resistance in human bacterial pathogens, this study explores the natural products involved in the defensive symbiosis between actinobacteria and fungus-growing ants to uncover new potential antibiotics. This study also seeks to understand the function of natural antibiotics in their ecological contexts, especially those involved in defensive symbioses. Defensive symbiosis can be a beneficial platform for discovering useful antibiotics, because antibiotics in these relationships must be able to selectively inhibit enemies without harming hosts, and are therefore likely more specific and less toxic. *Pseudonocardia* sp. associated with *Trachymyrmex septentrionalis* ants demonstrated antibiotic activity against several gram-positive bacteria. Therefore, the natural products from this strain were extracted and purified through activity-guided fractionation. Using mass spectrometry, the structure of the active compound was elucidated as GE37468A, an antibiotic that has been previously identified from *Streptomyces* sp. ATCC 55365 from Italy. This compound had never before been characterized in a defensive symbiosis, which demonstrates the use of the molecule in a new context. Antibiotic GE37468A is a thiopeptide, which is a group of antibiotics that has previously demonstrated strong activity against many gram-positive bacteria, including bacterial human pathogens. Due to its potency against dangerous bacteria and its likely low toxicity, this antibiotic could therefore hold potential pharmacological uses.

INTRODUCTION

Antibiotic resistance in human pathogens has been an ongoing challenge for the past several decades.[1, 2] Since the 1960s, the pharmaceutical industry has struggled to keep up with the fast pace of resistance developing for each new antibiotic introduced in medicine.[2] ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pose the greatest risk to antibacterial resistance, as these bacteria are responsible for a breadth of serious infections and are also increasingly resistant to most antibacterial medications.[3,4] Methicillin-resistant *Staphylococcus aureus* (MRSA) has caused ongoing concern, as it is responsible for approximately 46,000 infections annually.[5,6] For all common bacterial and fungal pathogens, resistance has been identified for most antibiotics developed and used in medicine.[2,7,8]

The recent resurgence of antibiotic resistance has made the need for new antibiotics much more acute. The volume of novel antibiotics introduced by pharmaceutical companies has markedly reduced since the early 1980s.[1, 2] In the search for novel antibiotics, natural products have served as a crucial starting point due to their breadth of chemical structures and wide range of antibiotic activities.[9, 10] In fact, two of the most widely used antibiotics (penicillin and streptomycin) are both natural products, the former isolated from the fungus *Penicillium notatum*, and the latter from the bacteria *Streptomyces griseus*.[11,12] The prospect of discovering novel natural products with high potency and low host toxicity is encouraging because several antibiotics have recently been identified against a variety of human pathogens, including teixobactin, ulleungamides A and B, copsin, and albicidin.[13,14]

Commonly, organisms produce antibiotics as a form of defense. Many plant and

fungal species use natural products to protect themselves from predators or parasites. Other species use the antibiotics to defend their specialized niches from invasion by similar organisms.[15] Researchers are currently exploring the use of natural products in defensive symbioses. In microbial defensive symbiosis, the microbe produces antibiotics to protect a host species from an enemy species; in return, the host promotes the fitness of the microbe, typically through nutrition-provision or protection from other enemies.[16] The natural products from defensive symbiosis offer an underexplored context for natural product discovery, and are therefore a promising source for novel antibiotic development.

This study specifically analyzes the natural products produced by actinobacteria that are involved in defensive symbiosis with a host organism: fungus-growing ants. Natural products from actinomycetes are one of the most promising sources of novel antibiotics because they are predicted to encode for a large diversity of antimicrobial drug classes that are yet to be explored.[13, 14] Most antibiotics developed for pharmaceutical uses are actinomycete metabolites[17] or chemical modifications of existing actinomycete products.[15] Considering previous successes in isolating novel antibiotics from bacteria involved in defensive symbiosis, this study focuses on actinobacteria of genus *Pseudonocardia*, which create antibiotics as part of a symbiotic relationship with fungus-growing ants.

Fungus-Growing Ants

Fungus-farming ants have origins that trace back 45–65 million years. The ants, from the monophyletic tribe Attini, cultivate gardens of symbiotic fungi in their nests that serve as a primary food source for the ants.[18] This cultivar fungus is transferred to new ant nests across generations.[19] In return, the ants feed the cultivar with leaves, flowers, and vegetative

matter. There are over 200 described species of attine ants.[18] Although found exclusively in the New World, fungus-farming ants are widely distributed across North, Central and South America, extending as far south as Chubut, Argentina and as far north as Long Island, New York.[20] Common genera of fungus-farming ants include *Acromyrmex*, *Apterostigma*, *Atta*, *Cyphomyrmex*, and *Trachymyrmex*.[17]

While this mutualistic relationship between the ants and cultivar fungus has been established for several decades, recent studies have uncovered more complex relationships within this symbiosis. In studying the ant nests from several different species of attine ants, researchers observed the presence of a pathogenic fungus in addition to the cultivar.[17] The pathogenic fungus, of genus *Escovopsis*, competes with the ants by consuming the cultivar fungus and overgrowing the fungal gardens.[21] If allowed to grow freely, the *Escovopsis* will therefore eventually deplete the food source for the ant colony.[22]

To combat the presence of *Escovopsis* in fungal gardens, fungus-farming ants of several different genera were found to participate in a symbiotic relationship with specialized actinomycete bacteria. The actinomycetes live on the underside of the ants on an anatomical structure termed the propleural plates, resembling a white, powdery coating on the ant bodies.[17] The ants secrete a nutrient-rich food source from specialized exocrine glands for the bacteria, and in exchange, the actinomycetes produce antifungal compounds that specifically target *Escovopsis* (Figure 1).[17, 22] When new ant colonies are formed, the actinomycetes are transferred across generations such that all of the worker ants within a colony share the same strain of actinomycetes that have co-evolved with their ant hosts.[23] Actinomycetes of genera *Pseudonocardia*, *Streptomyces*, and *Amycolatopsis* have been most commonly observed in this symbiotic relationship with ants.[18]

In addition to the production of antifungal compounds, ant-associated *Pseudonocardia* have been found to produce antibacterial compounds as well. These antibacterial compounds allow the *Pseudonocardia* to defend its niche from invasion and occupation by other bacteria. Although the host bacterial strain is resistant to the produced antibiotic, these specialized antibacterial compounds target other closely related actinomycetes.[19]

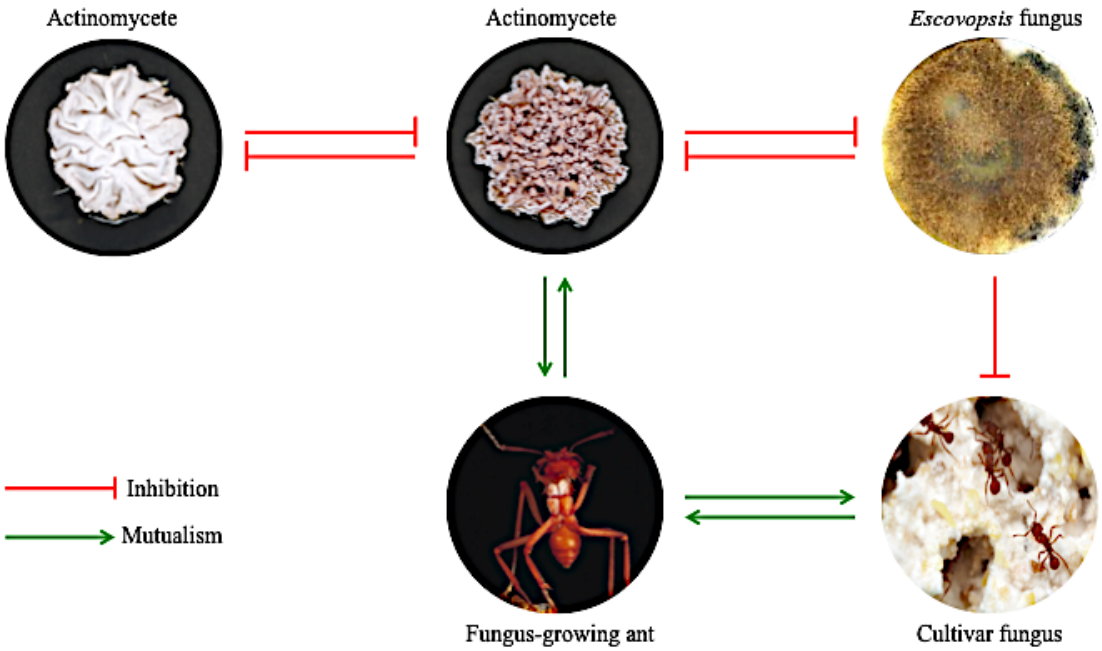


FIG 1. Overview of interactions between fungus farming ants, symbiotic cultivar fungus, symbiotic actinomycetes, and pathogenic *Escovopsis* fungus. Actinomycetes also produce antibacterial compounds to defend niche from other competitive actinomycetes. Green arrows represent a mutualistic interaction, whereas red bars indicate inhibition. Photos: Currie et al, 1999; Pagnocca et al., 2011; www.alexanderwild.com

Studies of ant-associated have already identified a large variety of antifungal and antibacterial compounds. For example, *Streptomyces* strains isolated from *Acromyrmex octospinosus* ants in Panama were found to produce candicidins that can inhibit the human pathogenic fungus, *Candida albicans*.[24, 25] Similarly, antifungal actinomycins D, X2, and X0Bβ were purified from *Streptomyces* associated with *Acromyrmex echinator* ants.[26] From

Pseudonocardia associated with *Apterostigma dentigerum* ants in Panama, two antifungal compounds have been identified: dentigerumycin[27] and selvamicin.[28] Antibacterial compounds isolated from ant-associated bacteria include 9-methoxyrebeccamycin from *Apterostigma dentigerum*-associated *Pseudonocardia,*[29] as well as elaiophylin, efomycin A and efomycin G produced by *Streptomyces* associated with *Acromyrmex echinator*.[26]

Trachymyrmex septentrionalis

In this study, the natural products of bacterial strains associated with *Trachymyrmex septentrionalis* ants from Long Island, NY were investigated to identify associated antibiotics. *Trachymyrmex* are higher attine ants that use dead vegetative matter to feed cultivar fungi.[18] *Trachymyrmex* is the most abundant attine genera in the United States. *Trachymyrmex* nests are also found in a large variety of climates, ranging from tropical Central and South America to the temperate zones of the American Southwest and the cold environments of Long Island, NY.[30]

Trachymyrmex septentrionalis nests are usually found in oak- or pine-dominated habitats with very sandy soils.[30] *Trachymyrmex* ants typically store cultivar in nests consisting of multiple chambers that range from 0.5 to 4 meters deep.[31] Although *Escovopsis* has never been isolated from the fungal gardens of North American *Trachymyrmex* ants,[32, 33] *Trachymyrmex* nests contain a large diversity of other fungal genera, including *Cunninghamella*, *Fusarium*, *Mortierella*, *Penicillium*, and *Trichoderma.*[32] It is therefore believed that *Trachymyrmex*-associated actinomycetes may have some other specialized roles in the ant-cultivar symbiosis.[33] According to one study, *Pseudonocardia* were the most prevalent actinomycetes found on *Trachymyrmex* ants.[33] Prior studies of actinomycetes from

Trachymyrmex in Brazil identified two antifungal analogs that were both active against *Candida albicans*: urauchimycin A and urauchimycin B.[34]

Considering prior successes in isolating antibiotics from ant-associated bacteria, the minimally studied *Trachymyrmex* actinobacteria hold promise as a source for a wide range of novel antibacterial and antifungal compounds. Furthermore, from an ecological context, studying the chemicals involved in the defensive symbiosis between *Pseudonocardia* and *Trachymyrmex* ants can help gain a better understanding how the two species interact with each other. Therefore, in this exploratory study, *Trachymyrmex septentrionalis* ants were collected from Long Island, NY, and *Pseudonocardia* were isolated from the propleural plates of the ants, and natural products were isolated from the bacteria. The active compound was then purified through activity-guided fractionation, and the structure of the antibiotic compound was elucidated through mass spectrometry techniques.

METHODS

Initial "Resident-Intruder" Assay with Bacterial Intruders

Pseudonocardia strains EV170527-10 (17SE-10), EV170527-11 (17SE-11), EV170708-06 (17SM-6), EV170708-14 (17SM-14), and EV170708-15 (17SM-15) were collected and cultured by Dr. Ethan Van Arnam from *Trachymyrmex septentrionalis* (17SE-10 and 17SE-11) ants from Long Island, NY and *Trachymyrmex smithi* (17SM-6, 17SM-14, and 17SM-15) ants from Las Cruces, NM. Spores were cultured, collected, and stored in a 25% glycerol solution at –20 °C. The concentrated spore suspension of each of these "resident" *Pseudonocardia* strains (5 μL) was spotted to the center of a 60 x 15 mm Petri dish with 8 mL yeast malt extract agar (YMEA, 1.5% agar). Three "resident" plates were

prepared per strain of *Pseudonocardia*. One of these plates per strain had no intruders plated (positive control). These plates were incubated for 14 days at 30 °C.

Concentrated spore suspensions of "intruder" strains of *Pseudonocardia* (5 µL) were spread around the center colony from the plates above and around the remainder of the plate surface area with a wetted sterile cotton swab, using 200 µL of sterile water per plate to assist in spreading. Intruder strains used included EC080529-05 (PLR-2) and EV170708-01 (17SM-1). As a negative control, intruders were also spread evenly across plates with no resident colonies. These plates were incubated at 30 °C for an additional 7 days. Zones of inhibition were measured as the difference between the diameter of inhibition and the diameter of resident colony growth (Figure 2).

To test 17SE-10 against even more intruders, a concentrated spore suspension of 17SE-10 (5 µL) was spotted to the center of each of 10 Petri dishes (60 x 15 mm) filled with YMEA 1.5% media (8 mL). These plates were incubated for 14 days at 30 °C. Concentrated spore suspensions of "intruder" strains of *Pseudonocardia* (5 µL) were spread around the remainder of the plate with a wetted sterile cotton swab, using 200 µL of sterile water per plate to assist in spreading. Intruder strains tested included PLR-2, EV170527-09 (17SE-9), and EV170527-03 (17SE-3). Two replicates were performed for each resident and intruder pair. Intruders were also spread evenly across plates with no resident colonies (negative control). Two of these plates had no intruders plated, and only sterile water was spread instead (negative control). All cultures were incubated at 30 °C for an additional 7 days. Zones of inhibition were measured as the distance from the center of the resident colony to the closest edge of fungal growth (Figure 2).

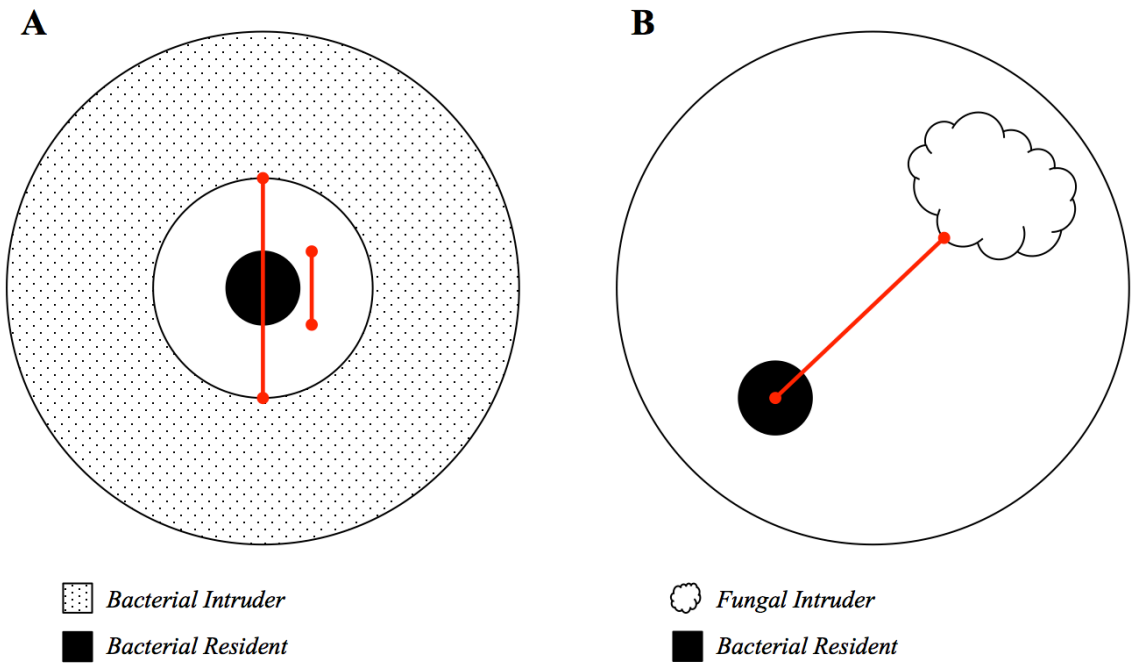


FIG 2. Diagram of Resident-Intruder assays for bacterial (A) and fungal (B) intruders. To bacterial intruders, the zone of inhibition was measured as the difference between the diameter of the zone and the diameter of the resident colony. For the *Escovopsis* fungal intruder, the zone of inhibition was measured as the distance from the center of the resident colony to the closest edge of fungal growth.

Initial "Resident-Intruder" Activity Assay with Fungal Intruders

Concentrated spore suspension of each 17SE-10, 17SE-11, 17SM-6, 17SM-14, and 17SM-15 (5 µL) was spotted 1 cm away from the edge of a 60 x 15 mm Petri dish filled with YMEA (1.5% agar) (8 mL). Two of these "resident" plates were prepared per *Pseudonocardia* strain tested. One of these plates per strain had no intruders plated (positive control). These plates were incubated for 14 days at 30 °C.

Escovopsis sp. (EV151027-02-2) was passaged onto a Petri dish filled with YMEA (2% agar) (25 mL). This plate was sealed with parafilm to maintain humidity and incubated at room temperature for 7 days. After incubation, a punch tool was used to punch holes into the agar of the plate along the periphery of the fungal growth. Then, one plug of fungus was

placed on each of the "resident" plates, directly across the plate from the resident-colony and 1 cm away from the edge of the plate. These plates were sealed with parafilm to maintain humidity, and incubated at room temperature for 7 days. Zones of inhibition were measured as the distance from the center of the resident colony to the closest edge of fungal growth (Figure 2).

Initial Spot-on-Lawn Activity Testing of *Pseudonocardia* Extracts

A concentrated spore suspension of each 17SE-10, 17SE-11, 17SM-6, 17SM-14, and 17SM-15 (5 µL) was spread evenly across a Petri dish (100 x 15 mm) filled with YMEA (2% agar) (25 mL), using 200 µL of sterile water per plate to assist with spreading. These plates were incubated at 30 ºC for 14 days.

Agar from each plate was cut into 1cm x 1cm squares. The squares from each plate were then divided between two 50-mL centrifuge tubes. For each strain, 25 mL of ethyl acetate was added to one tube, and 25 mL of methanol was added to the other. Cultures were soaked in solvent for 24 hours, then decanted and fully evaporated. Each extract was subsequently rehydrated in 500 µL of methanol.

Liquid cultures were prepared of each *Bacillus subtilis* and *Escherichia coli*. Luria broth (LB) media were added (4 mL) to each of two 13-mL snap-top culture tubes. Each were inoculated with a single colony of bacteria. Liquid cultures were incubated at 30 ºC and 250 RPM on a shaking incubator for 24 hours. For each of strain of bacteria, 50 µL of liquid culture was combined with molten low-density LB (0.75 % agar) (4 mL) and quickly poured over a Petri dish (100 x 15 mm) filled with LB (2% agar) (25 mL). Simultaneously, concentrated spore suspension of PLR-2 (5 µL) was combined with molten low density

YMEA (0.75% agar) (4 mL) and quickly poured over a Petri dish with YMEA (2% agar) (25 mL). Each extract was spotted onto the different plates (5 µL), spaced at approximately 2-cm intervals. An extract of EV151025-09-1 (GA6-1) was used as a positive control, and pure methanol was used as a negative control. All spotted plates were incubated at 30 ºC for 72 hours. The diameter of each zone of inhibition was then measured.

Culturing and Creation of Crude Extract

Concentrated spore stock of 17SE-10 (20 µL) was spread evenly on each of 10 Petri dishes (150 x 15 mm) with YMEA 2% media (60 mL), using 200 µL of sterile water per plate to assist with spreading. These plates were incubated for 14 days at 30 °C. Agar was then cut into 1cm x 1cm squares and combined with 350 mL of ethyl acetate. Cultures were soaked in solvent for 24 hours, then decanted and fully evaporated. The dry extract was massed and rehydrated in 10 mL of methanol.

Fractionation Through Reverse-Phase Column Chromatography

Celite® powder was added to the methanol solution of crude extract in a three-fold greater proportion by mass than the dry extract and dried with a rotary evaporator. A 5 g C_{18} SepPak® column was attached to a vacuum manifold and preconditioned with water followed by 100% acetonitrile + 0.1% formic acid, followed by 20% acetonitrile and 0.1% formic acid in water. The Celite® + extract mixture was transferred to the top of the column using approximately 3 mL of a 20% acetonitrile + 0.1% formic acid solution. 10 fractions were eluted by running 10 mL of each of the following solvents 2 times each through the column: 20%, 40%, 60%, 80%, and 100% acetonitrile + 0.1% formic acid in water. Fractions were evaporated and rehydrated in methanol (500 µL).

Spot-on-Lawn Activity Testing of Fractions

A soft lawn of *Pseudonocardia* on agar was prepared by combining 4 mL molten low-density yeast malt extract agar (YMEA, 0.75 % agar) with PLR-2 spore stock (5 µL). This mixture was quickly and evenly poured over petri dishes (100 x 15 mm) filled with YMEA (2% agar) (25 mL). Fractions were spotted (5 µL) on top of the lawn along with the unfractionated extract (positive control) and methanol (negative control). The plates were incubated for 72 hours at 30 °C. The diameter of each zone of inhibition was then measured.

Preparative Fractionation with Reverse-Phase HPLC

The fraction from SepPak chromatography that was most active against the *Pseudonocardia* strain PLR-2 (fraction 6) was further fractionated by preparative reverse-phase high performance liquid chromatography (Column: Kinetex® 5 µm EVO C_{18} 100 Å LC Column 250 x 10.0 mm) using a gradient concentration of acetonitrile + 0.1% formic acid in water from 30% to 100% over 20 minutes (flow rate: 2.500 mL/min, injection volume: 50 µL) to elute. Fractions were collected in one-minute intervals. UV/Vis absorbances were measured across the full spectrum of wavelengths. Preparative fractionation was performed with four different injections, and corresponding fractions from each injection were pooled together. Fractions were then evaporated and rehydrated in methanol (200 µL). Activity of fractions was assessed through spot-on-lawn testing against PLR-2. The active fraction from the initial separation by SepPak chromatography was used as a positive control in activity testing.

High-Resolution Mass Spectrometry and Initial Identification

The active fraction purified through one round of reverse-phase HPLC fractionation

was sent to University of Illinois, Urbana-Champaign for high-resolution liquid-chromatography mass spectrometry analysis (Waters Synapt G2-Si ESI MS, solvent: 10-100% acetonitrile in water, collected over 10 min). High-resolution ESI-TOF mass spectrometry data was collected in both positive and negative ion modes. The active compound eluted at 4.798 minutes.

Extraction and Fractionation of *Streptomyces* sp. ATCC 55365

Streptomyces sp. strain ATCC 55365 (*Streptomyces* sp. Waksman and Henrici ATCC® 55365™) was purchased as a lyophilized solid. The solid was rehydrated in YMEA liquid media (1.000 mL). This culture was streaked across a 100 x 15 mm petri dish filled with (YMEA 2% agar) (25 mL) to isolate individual colonies, and incubated for 14 days at 30ºC. A vial with 1000 µL of sterile water was inoculated with a single colony of *Streptomyces*. The spores were suspended, and evenly spread (200 µL) across each of 5 Petri dishes (150 x 15 mm) filled with YMEA (2% agar) (60 mL). These plates were incubated at 30 ºC for 14 days. Agar from all plates was then cut into 1cm x 1cm squares and combined with 200 mL of ethyl acetate total. Cultures were soaked in solvent for 24 hours, and the solvent was decanted and fully evaporated to produce a dry extract that was then massed and rehydrated in methanol (10 mL). Celite® powder was added to the methanol solution of crude extract in a three-fold greater proportion by mass than the dry extract. The crude extract was separated into 10 fractions using reverse-phase SepPak column chromatography using the same procedure as above. Fractions were then assessed for activity using the spot-on-lawn assay.

HR-LCMS/MS Comparison of *Streptomyces* and 17SE-10 Active Compounds

Because the eighth fraction to elute from reverse-phase column chromatography of the *Streptomyces* sp. extract appeared to contain the most pure antibiotic through UV/Vis spectrometric analysis at 300 nm, this fraction was used for high resolution-liquid chromatography tandem mass spectrometry (HR-LCMS/MS) analysis and compared against a fraction of the 17SE-10 extract purified through preparative HPLC. A small volume (200 μL) of each active fraction was dried with a rotary evaporator and sent to University of Illinois, Urbana-Champaign for analysis (Waters Synapt G2-Si ESI MS, solvent: 10-100% acetonitrile in water, collected over 10 min, CE = 28-33). Elutions were also analyzed for UV/Vis absorbance at 305 nm.

RESULTS

Initial "Resident-Intruder" Assay

"Resident-intruder" assays were completed to assess the antibiotic abilities of various *Pseudonocardia* symbionts isolated from *Trachymyrmex* ants. 17SE-10 and 17SE-11 were isolated from *Trachymyrmex septentrionalis*, and 17SM-6, 17SM-14, and 17SM-15 were isolated from *Trachymyrmex smithi*. All strains were tested for antibiotic activity against two bacterial strains (PLR-2 and 17SM-1), as well as the fungus *Escovopsis*. For 17SE-11 and 17SM-14, zones of inhibition were irregular and non-uniform against 17SM-1. However, the inside of the inhibition zones also contained a clear, shiny film often associated with bacterial contamination and thus these zones could not be measured. Of all strains assessed, only 17SE-10 demonstrated antibacterial activity, and activity against PLR-2 was almost twice the activity against 17SM-1. All strains demonstrated antifungal activity with respect to the

negative control. Antifungal activity was strongest for 17SM-6 and 17SM-15. The antifungal abilities for 17SE-10, 17SE-11, and 17SM-14 were all moderate and similar. Only 17SE-10 showed activity against both fungi and bacteria (Table 1).

TAB 1. Size of zone of inhibition (centimeters) for activity testing of various *Pseudonocardia* strains against three different intruders. 17SM-1 and PLR-2 are *Pseudonocardia* and *Escovopsis* is a type of fungus. The negative control condition only contained intruders (no residents were plated). Blank fields indicate no collected data due to contamination.

	Intruder Strain		
Resident Strain	17SM-1	PLR-2	Escovopsis sp.
17SE-10	1.20	2.10	2.45
17SE-11	--	0.00	2.25
17SM-6	0.00	0.00	3.45
17SM-14	--	0.00	2.30
17SM-15	0.00	0.00	3.25
Neg. Control	0.00	0.00	1.30

Initial Spot-on-Lawn Activity Testing of *Pseudonocardia* Extracts

Extracts of 17SE-10, 17SE-11, 17SM-6, 17SM-14, and 17SM-15 were prepared using ethyl acetate and methanol as the extracting solvents. All extracts were all then evaporated and rehydrated in methanol to use for activity testing. Antibacterial activity against two different gram-positive bacterial strains (*B. subtilis* and *Pseudonocardia*-PLR-2) was assessed as well as one gram-negative strain (*E. coli*). Methanol and ethyl acetate extracts of *Pseudonocardia* strain GA6-1 were used as positive controls. This strain had been previously demonstrated to have strong antibiotic activity against gram-positive bacteria.

The positive control was an extract from a *Pseudonocardia* strain that had been known to produce antibiotics against gram-positive bacteria. This control created zones of inhibition against the PLR-2 and the *B. subtilis*, as expected. The negative control, methanol,

did not demonstrate any inhibition.

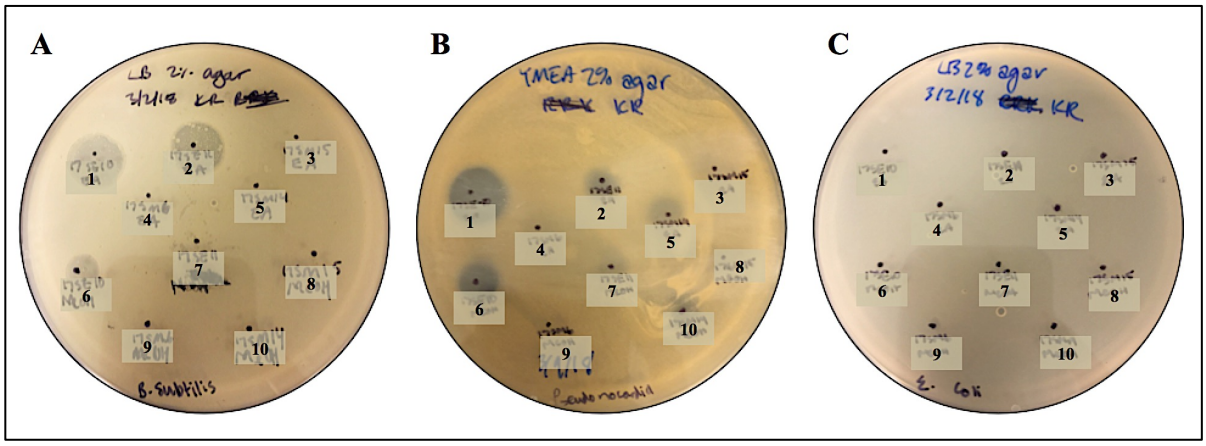


FIG 3. Initial spot-on-lawn activity testing of extracts of all strains against *B. subtilis* (A), *Pseudonocardia* PLR-2 (B), and *E. coli* (C). Strains were extracted in each ethyl acetate and methanol. Spots on each plate: *1*: 17SE-10 in ethyl acetate, *2*: 17SE-11 in ethyl acetate, *3*: 17SM-15 in ethyl acetate, *4*: 17SM-6 in ethyl acetate, *5*: 17SM-14 in ethyl acetate, *6*: 17SE-10 in methanol, *7*: 17SE-11 in methanol, *8*: 17SM-15 in methanol, *9*: 17SM-6 in methanol, *10*: 17SM-14 in methanol.

None of the extracts demonstrated inhibition against *E. coli*. Extracts had similar activity against PLR-2 versus *B. subtilis* (Figure 3). The 17SE-10 extract prepared with ethyl acetate had the strongest inhibition against both strains of bacteria compared with other extracts (1.25 cm for PLR-2, 1.35 cm for *B. subtilis*). 17SE-11 extracts in ethyl acetate had much stronger activity against *B. subtilis* (1.30 cm) than against PLR-2 (0.73 cm); however, the 17SE-11 extract in methanol had no activity against *B. subtilis*. Both extracts from 17SM-6, 17SM-14, and 17SM-15 had minimal activity against the two strains of gram-positive bacteria. Since the 17SE-10 extract in ethyl acetate had the strongest antibacterial activity against both strains of gram-positive bacteria, this strain was prioritized for further study.

Activity Assessment of 17SE-10

Multiple "resident-intruder" assays were conducted to assess the activity of 17SE-10 against other types of *Pseudonocardia*. Assays were conducted with 17SE-10 as the resident

and each 17SE-3 and 17SE-9 as intruders. These strains were selected because both are *Pseudonocardia* collected from *T. septentrionalis* ants from different colonies. 17SE-10 was also assayed against PLR-2 as a positive control.

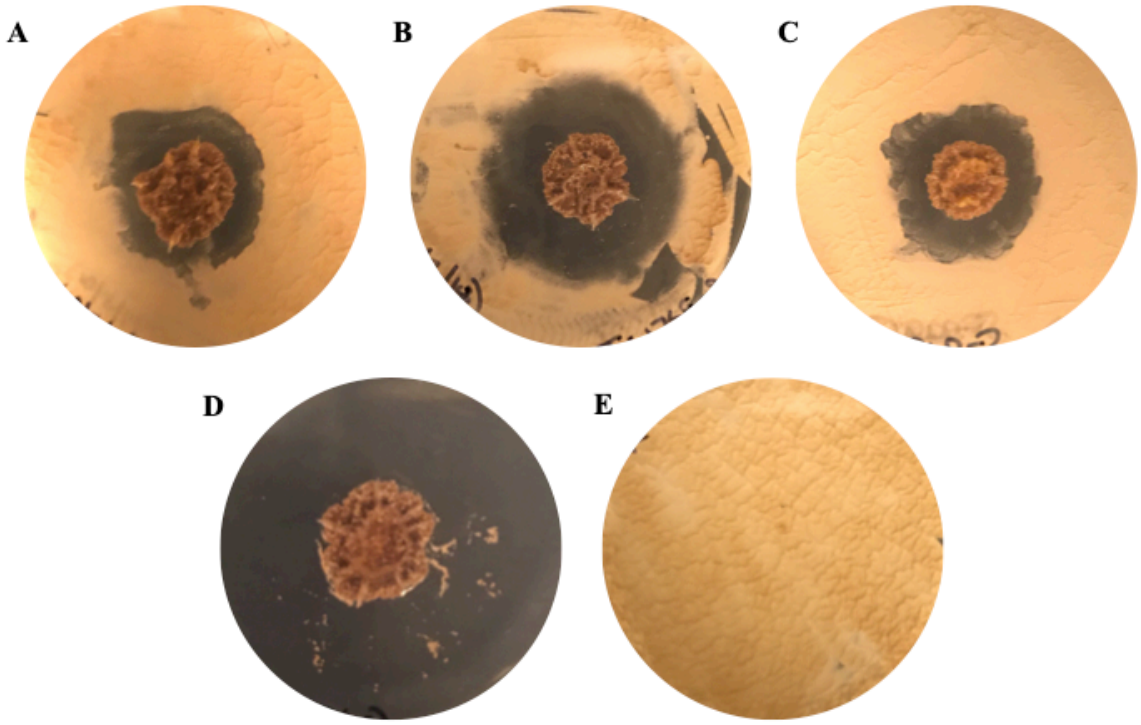
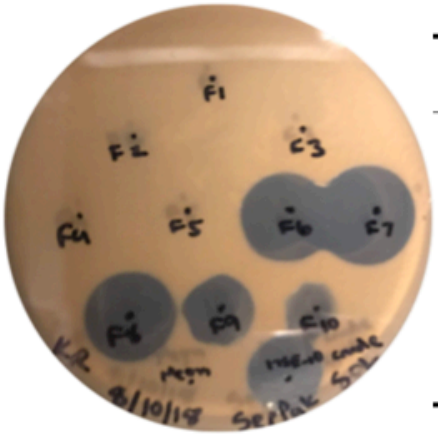


FIG 4. Resident-intruder assays with17SE-10 as the resident and 17SE-3 (a), 17SE-9 (b), and PLR-2 (c) as the intruders. *Fig 4d* shows the growth of 17SE-10 in the absence of any intruders, and *Fig 4e* shows the growth of PLR-2 in the absence of any residents.

Based on preliminary resident-intruder data (n = 1 for each), 17SE-10 had potent activity against all strains tested (Figure 4). The activity against 17SE-9 (zone of inhibition = 1.85 cm) was stronger than that against PLR-2 (1.00 cm) and 17SE-3 (1.50 cm). Activity against 17SE-3 was also stronger than that against PLR-2. This demonstrates that 17SE-10 is active against many types of *Pseudonocardia* bacteria.

Activity-Guided Fractionation

Since 17SE-10 demonstrated antibiotic abilities against multiple strains of gram-positive bacteria, the active compound was extracted from 17SE-10 and purified through activity-guided fractionation. To begin, a concentrated extract of 17SE-10 was prepared in ethyl acetate. This extract was then fractionated through reverse-phase column chromatography, using acetonitrile + 0.1% formic acid in varying concentrations as the mobile phase. Activity of these fractions was tested against PLR-2 in a spot-on-lawn assay. Activity was strongest in fractions 6 (1.75 cm), 7 (2.00 cm), and 8 (1.80 cm), indicating a high concentration of antibiotic in these fractions (Figure 5). These fractions eluted at 60-80% acetonitrile + 0.1% formic acid.



Fraction	*Acetonitrile Content of Mobile Phase*	*Zone of Inhibition (cm)*
F6	60%	1.75
F7	80%	2.00
F8	80%	1.80
F9	100%	1.35
F10	100%	1.00
Crude Extract	--	1.45

FIG 5. Spot-on-lawn assay results of fractions collected from ethyl acetate extract of 17SE-10. Ten fractions were collected through reverse-phase column chromatography with a C-18 column, using acetonitrile with 0.1% formic acid in varying concentrations as the mobile phase. Fractions were evaporated and rehydrated in methanol. Fractions were tested for activity against PLR-2, a strain of *Pseudonocardia.* Activity of fractions was compared against the crude, unfractionated 17SE-10 ethyl acetate extract as well as pure methanol (neg. control). The diameter of each circular zone was measured and recorded.

Because there was high activity in the sixth fraction to elute from the column, fraction 6 was further fractionated through reverse-phase HPLC. Twenty fractions were collected

using acetonitrile + 0.1% formic acid in varying concentrations as the mobile phase. Fractions were analyzed for absorbance at 300 nm using UV/Vis spectroscopy. Activity of these fractions was also tested against PLR-2 in a spot-on-lawn assay. Notable peaks of high-intensity absorbance eluted in fractions 2 and 3, fraction 5, and fractions 14 and 15 (Figure 6a). Only fractions 14 and 15 had strong antibiotic activity against PLR-2 (Figure 6b). This data indicates that the target antibiotic is likely concentrated in fractions 14 and 15, and that the compound absorbs UV light at 300 nm. The lack of other UV-absorbance peaks in fractions 14 and 15 suggest that these fractions are relatively purified.

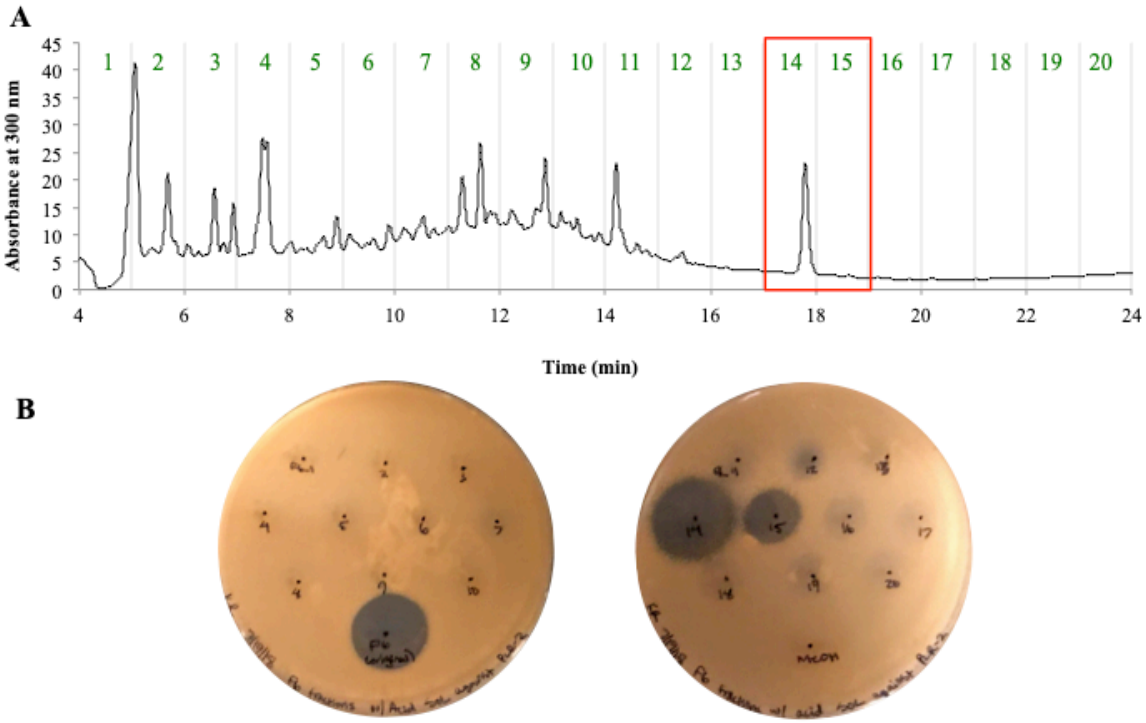


FIG 6A. UV chromatogram (λ = 300 nm) for the fractionation of F6 from SepPak chromatography using reverse-phase HPLC. F6 was separated into 20 different fractions using acetonitrile with 0.1% formic acid in varying concentrations (30% to 100% over 20 minutes, flow rate = 2.500 mL/min) as the mobile phase. Bars represent collected fractions. Active fractions 14 and 15 are boxed in red.
FIG 6B. Spot-on-lawn activity assay of fractions from HPLC fractionation. Fractions were evaporated and rehydrated in methanol. Activity of fractions was compared against the unfractionated F6 fraction (pos. control) and pure methanol (neg. control).

High-Resolution Mass Spectrometry and Preliminary Compound Identification

Fraction 14 from the HPLC fractionation was analyzed through HR-LCMS coupled with UV/Vis spectroscopy. Compounds with high-intensity absorbance at 325 nm eluted at 4.69 minutes and high total ion counts were present in both positive and negative modes at a retention time of 4.9 minutes.

In negative-ion mode, strong-intensity peaks were present at m/z values of 1307.2408 and 653.1171 (Figure 7a). Of these, m/z = 1307.2408 likely represents the $[M\text{-}H^+]^-$ peak. In positive-ion mode, strong peaks were present at m/z values of 1309.2550, 1291.2444, 653.3744, and 646.1260 (Figure 7a). Of these, m/z = 1309.2550 likely represents the $[M+H^+]^+$ peak. Based on the observed masses of positive and negative ions, the exact mass of the compound was calculated as 1308.2479 ± 0.0002 Da.

This exact mass was searched for in the Dictionary of Natural Products, and matched only one compound, antibiotic GE37468A.[35] This antibiotic was discovered as a natural product made by *Streptomyces* sp. from soil in Italy.[36] The exact mass of GE37468A is 1308.2493 Da, which closely matches the exact mass determined for the antibiotic from 17SE-10. Based on previous literature characterizing GE37468A, this antibiotic shares a similar mass spectrometry pattern to the active compound of 17SE-10, with the peaks of strongest intensity occurring around the m/z value of 1291. For these reasons, the active compound from 17SE-10 was characterized as antibiotic GE37468A based on initial assessment.

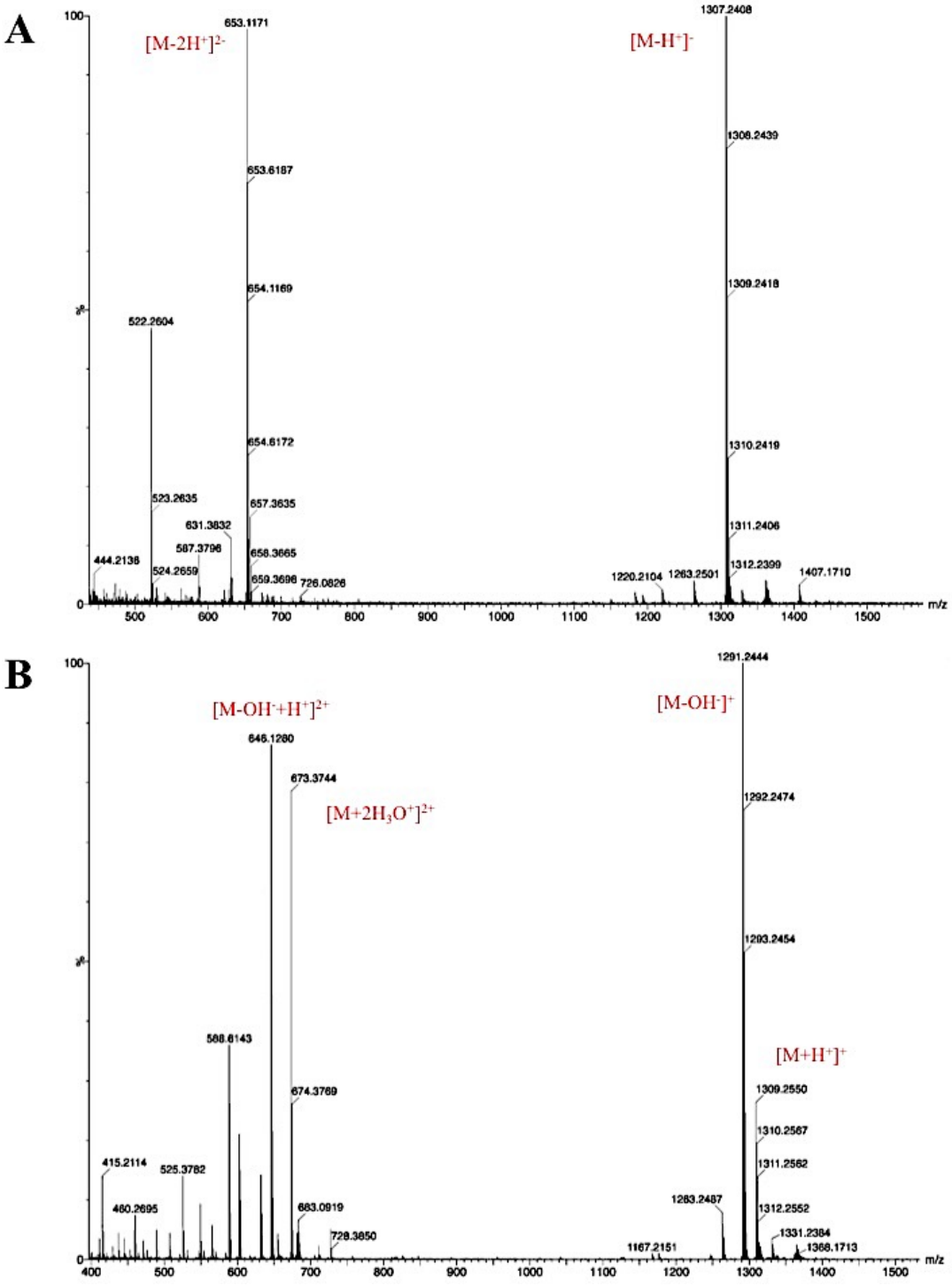


FIG 7. High-resolution LCMS spectra of F6-14 in each negative ion (A) and positive ion (B) modes. Spectra were captured at a retention time of approximately 4.9 minutes. From this data, the exact mass of the active compound was calculated as 1308.2479 Da.

Comparative Metabolomics of *Streptomyces* ATCC 55365 and *Pseudonocardia* 17SE-10

Antibiotic GE37468A was first characterized and studied as a natural product obtained from *Streptomyces* sp. ATCC 55365. To confirm the characterization of the target antibiotic as GE37468A, purified active antibiotics from each 17SE-10 and *Streptomyces* sp. ATCC 55365 were analyzed through high-resolution liquid chromatography and mass spectrometry (HR-LCMS/MS). UV/Vis chromatograms at 305 nm and the fragmented and unfragmented ions from mass spectrometric analysis were compared between the two compounds. For both active fractions, a compound eluted off of the column with high UV absorbance at a retention time of 4.6 minutes (Figure S1). Both mass spectra shared identical ions at very similar relative ion counts (Figure 8). The m/z = 1309.26 ion for each strain was fragmented (collision energy = 28-33 eV), and the ion fragments of each strain all matched in m/z values with similar relative ion counts. Resulting ions are all likely from the fragmentation of antibiotic GE37468A, as labeled on the figure (Figure 9). Collectively, this data strongly suggests that the active compounds of *Streptomyces* sp. and 17SE-10 are the same molecule, which is antibiotic GE37468A.

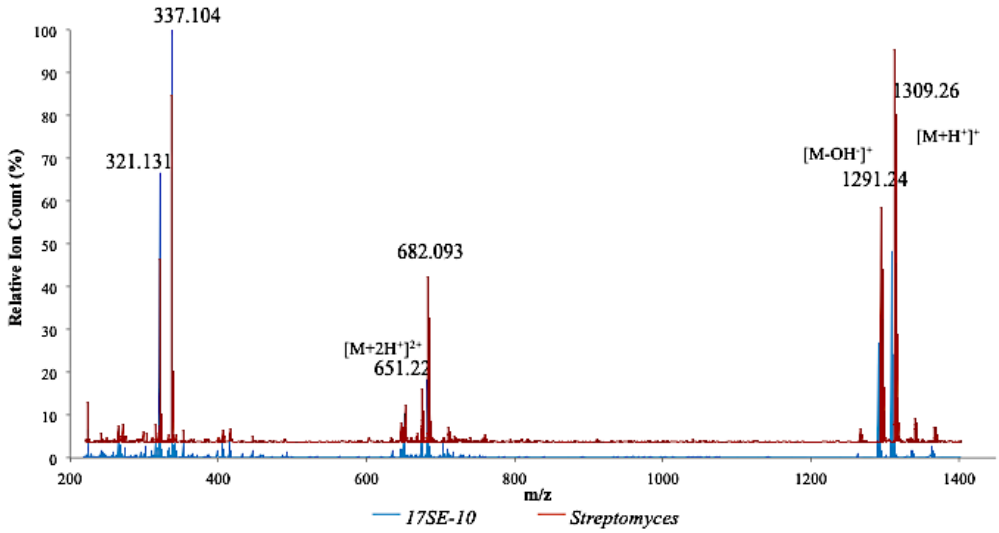
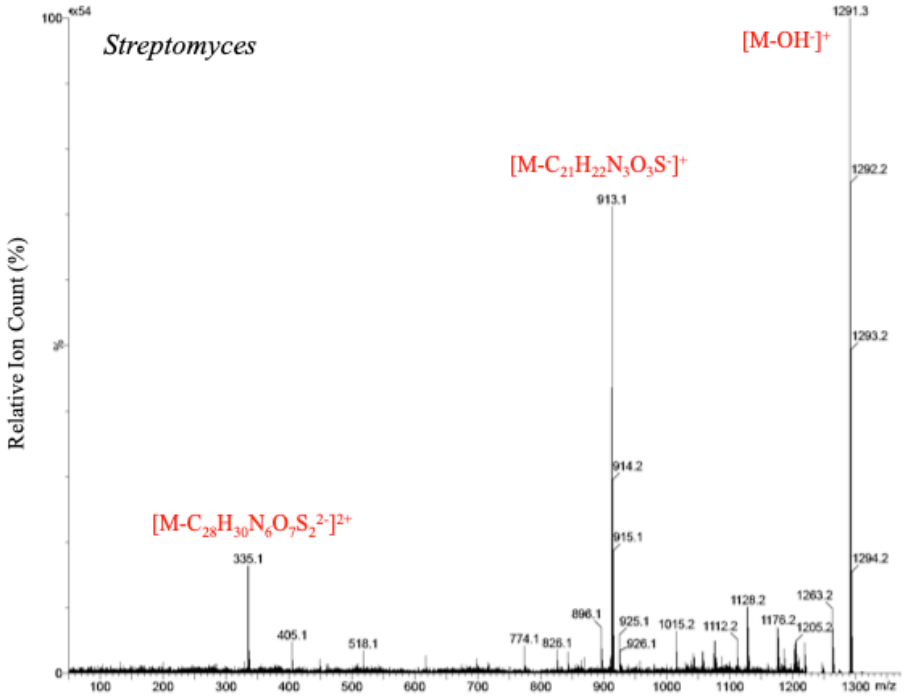


FIG 8. Overlain mass spectrums of 17SE-10 and *Streptomyces* sp. for compounds that eluted at a retention time of 4.6 minutes.

A



B

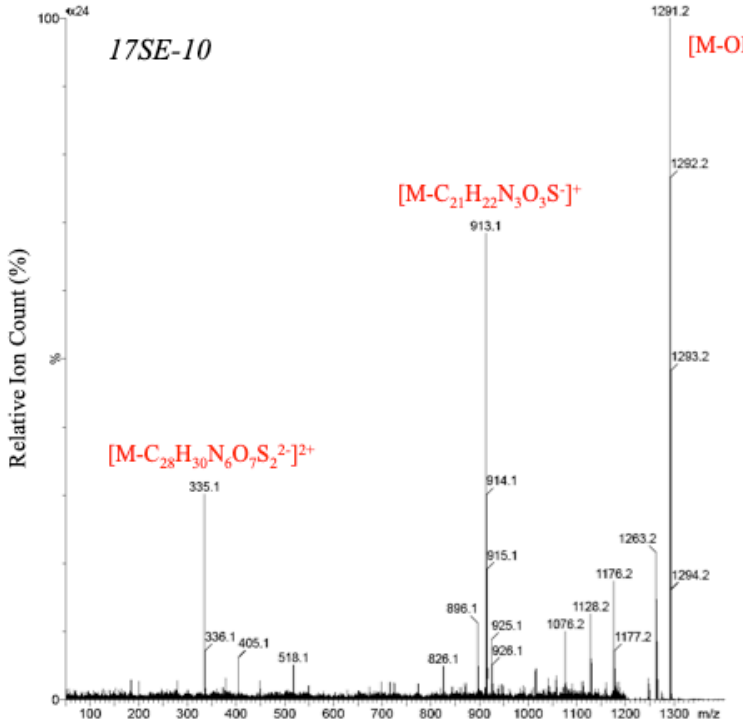


FIG 9A. MS/MS spectrum of fragmented *Streptomyces* sp. ion at m/z = 1309.26. The positive ion was eluted after 4.639 minutes and was fragmented at the collision energy of 28-33 eV.
FIG 9B. MS/MS spectrum of fragmented *Streptomyces* sp. ion at m/z = 1309.26. The positive ion was eluted after 4.647 minutes and was fragmented at the collision energy of 28-33 eV.

DISCUSSION

Data from this study indicates that the tested *Pseudonocardia* strain associated with *Trachymyrmex septentrionalis* produces antibiotic GE37468A. No prior studies have demonstrated the production of this compound by actinobacteria associated with *Trachymyrmex* ants. In fact, this compound has only previously been studied from *Streptomyces* sp. ATCC 55365 in Gerenzano, Italy.[36] 17SE-10 *Pseudonocardia* thus serves as a novel source for a well-studied compound. This compound has never before been identified as part of a defensive symbiosis, and has never been found in the western hemisphere. This evidence suggests that the biosynthetic gene cluster for antibiotic GE37468A may have somehow migrated from Italy to the United States, and may have been incorporated by *Pseudonocardia* specifically for use in this defensive symbiosis. This novel source for the antibiotic therefore poses new questions related to chemical ecology and evolution, and further studies will be required to determine how the *Trachymyrmex*-associated *Pseudonocardia* came to acquire and produce this chemical.

Since its discovery in 1996, many researchers have studied antibiotic GE37468A to clarify its structure and mechanism of activity. The antibiotic belongs to the family of ribosomally encoded post-translationally modified peptides (RiPP) and prohibits bacterial growth by inhibiting the transcriptional elongation factor (EF-Tu), thus preventing protein synthesis.[37] The molecule consists of a large macrocycle with ten modified amino acids, as well as a four-residue tail of dehydrated amino acids (Figure 10).[36]

Antibiotic GE37468A is considered a thiazolyl peptide, or a thiopeptide. Many thiopeptides have been extensively studied due to their potent inhibitory effects on the protein synthesis of a broad range of gram-positive bacteria and malarial parasites.[38] In

particular, thiopeptides have demonstrated antibiotic activity against common human pathogens that are prone to antibiotic resistance, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Corynebacterium aurimucosum*, *Streptococcus mutans*, and *Streptococcus sobrinus*.[37, 38, 39] Nevertheless, due to poor pharmacokinetics and low water solubility, pharmacological uses of thiopeptides have been limited.[37]

Recent studies suggest, however, that bacteria in the human microbiome naturally produce thiopeptides. For example, prior studied have demonstrated the presence of the biosynthetic gene cluster for lactocillin, a thiopeptide antibiotic, in the genomes of oral and vaginal human commensal bacteria. Researchers hypothesized that in this context, lactocillin is involved in a defensive symbiosis that selectively protects human hosts from pathogenic infections. This conclusion was supported by experiments that demonstrated lactocillin activity at low inhibitory concentrations selectively against vaginal and oral pathogens and not commensal bacteria.[39]

Given that thiopeptides are likely already produced and present in humans, it is promising that these antibiotics can be modified for pharmacological usage to treat common antibiotic-resistant bacterial infections. To account for the low blood solubility of thiopeptides, directed drug delivery mechanisms may be necessary to target the antibiotic selectively to pathogens in the body. Some studies have tried to incorporate the biosynthetic gene clusters for thiopeptides, including GE37468A, into the genome of existing human symbionts as a way to localize production of antibiotics to specific areas in the human body.[37]

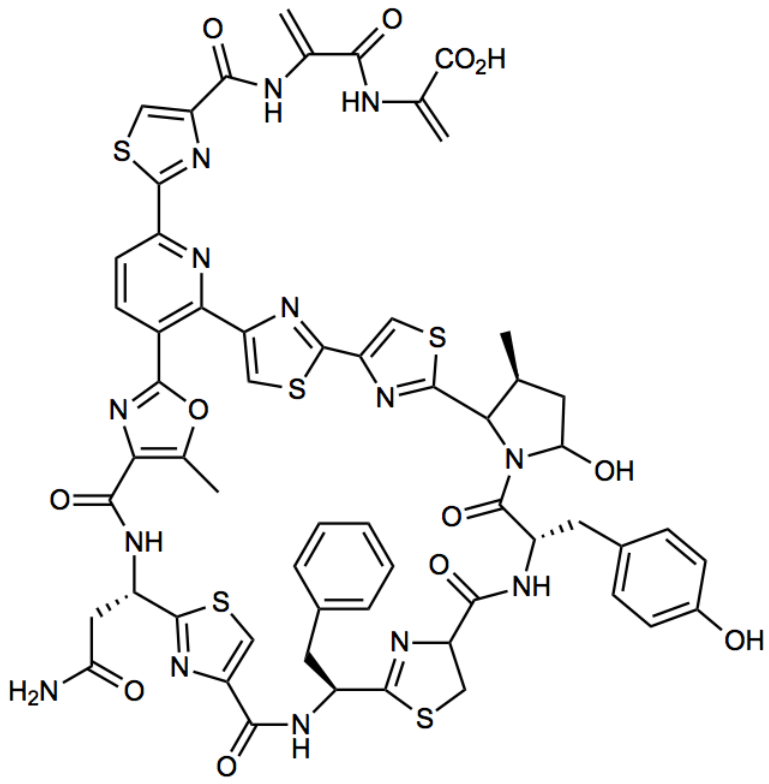


FIG 10. Structure of antibiotic GE37468A.

Other approaches may include directed delivery of the antibiotic using nanoparticle technology[39] or liposomes.[41] By exploring alternative drug-delivery mechanisms, thiopeptides such as antibiotic GE37468A could prove effective for treating patients with otherwise fatal antibiotic-resistant infections.

Apart from its potential applications to pharmacology, the discovery of antibiotic GE37468A production by ant symbionts poses additional questions to chemical ecology. Since this antibiotic has never before been characterized as part of a defensive symbiosis, this novel source for the molecule provides the new insight that the chemical may be involved in the interspecies interactions of organisms in complex relationships. It is possible that other

ant-symbiotic *Pseudonocardia* could produce this antibiotic as well – especially other bacteria associated with *Trachymyrmex septentrionalis* ants from different ant colonies. Further studies are thus required to assess the activity of other *Pseudonocardia* symbionts of *Trachymyrmex septentrionalis* ants to determine the scope of antiobiotic GE37468A production within this system.

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REFERENCES

1. Spellberg, B. & Gilbert, D. N. The Future of Antibiotics and Resistance: A Tribute to a Career of Leadership by John Bartlett. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **59,** S71–S75 (2014).
2. Ventola, C. L. The Antibiotic Resistance Crisis. *Pharm. Ther.* **40,** 277–283 (2015).
3. Tommasi, R., Brown, D. G., Walkup, G. K., Manchester, J. I. & Miller, A. A. ESKAPEing the labyrinth of antibacterial discovery. *Nat. Rev. Drug Discov.* **14,** 529–542 (2015).
4. Moloney, M. G. Natural Products as a Source for Novel Antibiotics. *Trends Pharmacol. Sci.* **37,** 689–701 (2016).
5. Xie, J. L., Polvi, E. J., Shekhar-Guturja, T. & Cowen, L. E. Elucidating drug resistance in human fungal pathogens. *Future Microbiol.* **9,** 523–542 (2014).
6. Center for Disease Control. Antibiotic Resistance Threats in the United States, 2013 | Antibiotic/Antimicrobial Resistance | CDC. (2013). Available at: https://www.cdc.gov/drugresistance/threat-report-2013/index.html. (Accessed: 22nd August 2018)
7. Sanglard, D. Emerging Threats in Antifungal-Resistant Fungal Pathogens. *Front. Med.* **3,** (2016).
8. Perlin, D. S., Shor, E. & Zhao, Y. Update on Antifungal Drug Resistance. *Curr. Clin. Microbiol. Rep.* **2,** 84–95 (2015).
9. Brown, D. G., Lister, T. & May-Dracka, T. L. New natural products as new leads for antibacterial drug discovery. *Bioorg. Med. Chem. Lett.* **24,** 413–418 (2014).
10. Singh, S. B. Confronting the challenges of discovery of novel antibacterial agents. *Bioorg. Med. Chem. Lett.* **24,** 3683–3689 (2014).

11. Bush, K. The coming of age of antibiotics: discovery and therapeutic value. *Ann. N. Y. Acad. Sci.* **1213,** 1–4 (2010).
12. Foster, J. W. & Woodruff, H. B. Antibiotic Substances Produced by Bacteria. *Ann. N. Y. Acad. Sci.* **1213,** 125–136 (2010).
13. Genilloud, O. Actinomycetes: still a source of novel antibiotics. *Nat. Prod. Rep.* **34,** 1203–1232 (2017).
14. Nett, M., Ikeda, H. & Moore, B. S. Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat. Prod. Rep.* **26,** 1362–1384 (2009).
15. Dixon, R. A. Natural products and plant disease resistance. *Nature* **411**, 843–847 (2001).
16. Clay, K. Defensive symbiosis: a microbial perspective. *Functional Ecology* **28**, 293–298 (2014).
17. Currie, C. R., Scott, J. A., Summerbell, R. C. & Malloch, D. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398,** 701–704 (1999).
18. Currie, C. R. A Community of Ants, Fungi, and Bacteria: A Multilateral Approach to Studying Symbiosis. *Annu. Rev. Microbiol.* **55,** 357–380 (2001).
19. Poulsen, M., Erhardt, D. P., Molinaro, D. J., Lin, T.-L. & Currie, C. R. Antagonistic Bacterial Interactions Help Shape Host-Symbiont Dynamics within the Fungus-Growing Ant-Microbe Mutualism. *PLoS ONE* **2,** (2007).
20. Sosa-Calvo, J. *et al.* Cyatta abscondita: Taxonomy, Evolution, and Natural History of a New Fungus-Farming Ant Genus from Brazil. *PLoS ONE* **8,** (2013).
21. Reynolds, H. T. & Currie, C. R. Pathogenicity of Escovopsis weberi: The Parasite of the Attine Ant-Microbe Symbiosis Directly Consumes the Ant-Cultivated Fungus. *Mycologia* **96,** 955–959 (2004).

22. Currie, C. R., Bot, A. N. M. & Boomsma, J. J. Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites - Currie - 2003 - Oikos - Wiley Online Library. (2003). Available at: https://onlinelibrary.wiley.com/doi/abs/10.1034/j.1600-0706.2003.12036.x. (Accessed: 21st August 2018)
23. Cafaro, M. J. *et al.* Specificity in the symbiotic association between fungus-growing ants and protective Pseudonocardia bacteria. *Proc. Biol. Sci.* **278,** 1814–1822 (2011).
24. Barke, J. *et al.* A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant Acromyrmex octospinosus. *BMC Biol.* **8,** 109 (2010).
25. Haeder, S., Wirth, R., Herz, H. & Spiteller, D. Candicidin-producing Streptomyces support leaf-cutting ants to protect their fungus garden against the pathogenic fungus Escovopsis. *Proc. Natl. Acad. Sci.* **106,** 4742–4746 (2009).
26. Boya P, C. A. *et al.* Imaging mass spectrometry and MS/MS molecular networking reveals chemical interactions among cuticular bacteria and pathogenic fungi associated with fungus-growing ants. *Sci. Rep.* **7,** 5604 (2017).
27. Oh, D.-C., Poulsen, M., Currie, C. R. & Clardy, J. Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. *Nat. Chem. Biol.* **5,** 391–393 (2009).
28. Van Arnam, E. B. *et al.* Selvamicin, an atypical antifungal polyene from two alternative genomic contexts. *Proc. Natl. Acad. Sci. U. S. A.* **113,** 12940–12945 (2016).
29. Van Arnam, E. B., Ruzzini, A. C., Sit, C. S., Currie, C. R. & Clardy, J. A Rebeccamycin Analog Provides Plasmid-Encoded Niche Defense. *J. Am. Chem. Soc.* **137,** 14272–14274 (2015).
30. Rabeling, C., Cover, S. P., Johnson, R. A. & Mueller, U. G. PDF | A Review Of The North

American Species Of The Fungus-Gardening Ant Genus Trachymyrmex (Hymenoptera: Formicidae). (2007). Available at: https://www.researchgate.net/publication/228628938_A_Review_Of_The_North_American_Species_Of_The_Fungus-Gardening_Ant_Genus_Trachymyrmex_Hymenoptera_Formicidae. (Accessed: 27th June 2018)

31. Sosa-Calvo, J., Jesovnik, A., Okonski, E. & Schultz, T. Locating, collecting, and maintaining colonies of fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). *Sociobiology* **62,** 300–320 (2015).
32. Rodrigues, A., Mueller, U. G., Ishak, H. D., Bacci, M. & Pagnocca, F. C. Ecology of microfungal communities in gardens of fungus-growing ants (Hymenoptera: Formicidae): a year-long survey of three species of attine ants in Central Texas. *FEMS Microbiol. Ecol.* **78,** 244–255 (2011).
33. Ishak, H. D. *et al.* Microbiomes of ant castes implicate new microbial roles in the fungus-growing ant Trachymyrmex septentrionalis. *Sci. Rep.* **1,** (2011).
34. Mendes, T. D. *et al.* Anti-Candida Properties of Urauchimycins from Actinobacteria Associated with Trachymyrmex Ants. *BioMed Res. Int.* **2013,** (2013).
35. Dictionary of Natural Products 27.1. Available at: http://dnp.chemnetbase.com/faces/chemical/ChemicalSearch.xhtml. (Accessed: 5th December 2018)
36. Ferrari, P., Colombo, L., Stella, S., Selva, E. & Zerilli, L. F. Antibiotic GE37468 A: A Novel Inhibitor of Bacterial Protein Synthesis. *J. Antibiot.* **48**, 1304–1311 (1995).

37. Young, T. S. & Walsh, C. T. Identification of the thiazolyl peptide GE37468 gene cluster from Streptomyces ATCC 55365 and heterologous expression in Streptomyces lividans. *PNAS* **108**, 13053–13058 (2011).

38. Bagley, M. C., Dale, J. W., Merritt, E. A. & Xiong, X. Thiopeptide Antibiotics. *Chem. Rev.* **105**, 685–714 (2005).

39. Donia, M. S. *et al.* A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. *Cell* **158**, 1402–1414 (2014).

40. Singh, R. & Lillard, J. W. Nanoparticle-based targeted drug delivery. *Exp. Mol. Pathol.* **86**, 215–223 (2009).

41. Allen, T. M. & Cullis, P. R. Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Deliv. Rev.* **65**, 36–48 (2013).

APPENDIX

TAB S1. All strains of *Pseudonocardia* and *Escovopsis* used.

Strain Name	*ID*	*Ant Species*	*Origin*
17SE-3	EV170527-03	*T. septentrionalis*	Long Island, NY
17SE-9	EV170527-09	*T. septentrionalis*	Long Island, NY
17SE-10	EV170527-10	*T. septentrionalis*	Long Island, NY
17SE-11	EV170527-11	*T. septentrionalis*	Long Island, NY
17SM-1	EV170708-01	*T. smithi*	Las Cruces, NM
17SM-6	EV170708-06	*T. smithi*	Las Cruces, NM
17SM-14	EV170708-14	*T. smithi*	Las Cruces, NM
17SM-15	EV170708-15	*T. smithi*	Las Cruces, NM
PLR-2	EC080529-05	*A. dentigerum*	Panama
GA6-1	EV151025-09-1	*A. dentigerum*	Panama
Escovopsis sp.	EV151027-02-2	*A. dentigerum*	Panama

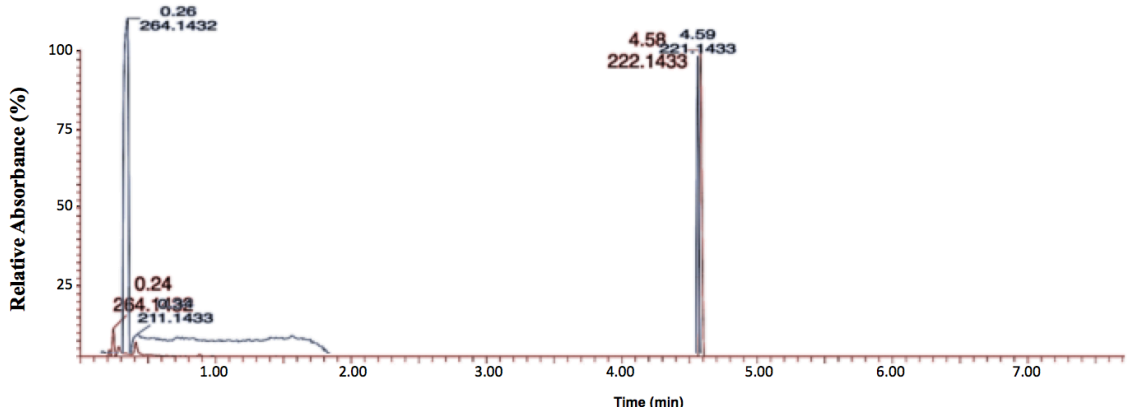

FIG S1. UV/Vis chromatogram of purified fractions from *Streptomyces* ATCC 55365 (red) and 17SE-10 (blue) obtained during ESI-TOF HR-LCMS/MS analysis of the two compounds. Compounds from each strain with a retention time around 4.6 minutes were used for further analysis.