Bacterial Associates of a Desert Specialist Fungus-Growing Ant Antagonize Competitors with a Nocamycin Analog

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ABSTRACT

Fungus-growing ants are defended by antibiotic-producing bacterial symbionts in the genus *Pseudonocardia*. Nutrients provisioned by the ants support these symbionts but also invite colonization and competition from other bacteria. As an arena for chemically-mediated bacterial competition, this niche offers a window into ecological antibiotic function with well-defined competing organisms. From multiple colonies of the desert specialist ant *Trachymyrmex smithi*, we isolated *Amycolatopsis* bacteria that inhibit the growth of *Pseudonocardia* symbionts. Using bioassay-guided fractionation, we discovered a novel analog of the antibiotic nocamycin that is responsible for this antagonism. We identified the biosynthetic gene cluster for this antibiotic, which has a suite of oxidative enzymes consistent with this molecule's more extensive oxidative tailoring relative to similar tetramic acid antibiotics. High genetic similarity to globally distributed soil *Amycolatopsis* isolates suggest that this ant-derived *Amycolatopsis* strain may be an opportunistic soil strain whose antibiotic production allows for competition in this specialized niche. This nocamycin analog adds to the catalog of novel bioactive molecules isolated from bacterial associates of fungus-growing ants and its activity against ant symbionts represents, to our knowledge, the first documented ecological function for the widely distributed enoyl tetramic acid family of antibiotics.

INTRODUCTION

Fungus-growing ants have well-defined associations with multiple microbial partners, forming a network of interactions that is a model system for the study of symbiosis.[1] Interspecies interactions throughout nature are mediated by chemical exchange, and molecular dissection of the fungus-growing ant system has offered a window into such interactions as well as a productive source of novel bioactive compounds such as the antifungals dentigerumycin, selvamicin, and cyphomycin.[2–4] Among their microbial associations, fungus-growing ants cultivate a basidiomycete fungus as their primary food source, which they must defend against a specialist ascomycete fungal pathogen *Escovopsis*. Symbiotic Actinobacteria in the genus *Pseudonocardia* grow on the cuticle of these ants and produce antifungal molecules that suppress the growth of *Escovopsis*.[2,5] These *Pseudonocardia* are transmitted vertically and are nourished by secretions from specialized crypts on the cuticle of these ants.[6–8]

Pseudonocardia isolates from fungus-growing ants are often observed to antagonize other strains of ant-associated *Pseudonocardia*, which are likely competitors in this niche.[9] The molecular basis for this antagonism is known for *Pseudonocardia* from a population of the Central American ant *Apterostigma dentigerum*, in which a plasmid-encoded rebeccamycin antibiotic antagonizes *Pseudonocardia* from neighboring ant colonies.[10] In a population of the North American ant *Trachymyrmex septentrionalis*, *Pseudonocardia* antagonize competitors using the thiopeptide antibiotic GE37468.[11] Actinobacteria other than *Pseudonocardia* have been detected on the cuticle of fungus growing ants in both culture-dependent and sequencing-dependent studies, and it remains debated whether these additional bacteria are transient or instead are maintained by the ants as part of a more complex cuticular microbiome.[12] Competition is expected when multiple bacterial strains co-occur in an ant population and conceptual frameworks have been developed for such bacteria-bacteria competition in the context of fungus-growing ants.[9,13,14]

Chemical defenses from bacterial symbionts of fungus-growing ants, both antibacterial and antifungal, have recently been a productive source for chemical discovery. We have turned our attention to the chemical ecology of desert-dwelling fungus-growing ants as their microbial partnerships are largely unexplored and their adaptation to a unique environment may include unique defensive compounds from their microbial associates. Over 200 species of fungus-growing ants are found in the Neotropics and while they are best known as the ubiquitous leaf cutter ants of Central and South American rain forests, they also include several desert specialist species found in southwestern North America.[15,16] *Trachymyrmex smithi* is one such desert-adapted fungus-growing ant, found in the Chihuahuan desert from New Mexico into Northeastern Mexico. Colonies of *T. smithi* can exceed 1000 individuals and the ants feed their fungal cultivar with mesquite leaflets and other collected plant matter. The ants appear to have adapted to fungal cultivation in dry desert soils by placing their fungus chambers deep underground.[16,17] Prior studies have noted the gray coating on the cuticle of these ants, presumed to be Actinobacteria,[16] but these microbes have not previously been characterized. In this study we have focused on the chemical basis for antibacterial niche defense by bacterial associates of the ant *T. smithi*. We find that these ants host not just *Pseudonocardia* but also Actinobacteria in the genus *Amycolatopsis* that produce a novel analog of the antibiotic nocamycin.

RESULTS AND DISCUSSION

Collection and Microbial Isolation. We began by collecting *T. smithi* ants from multiple sites in Las Cruces, New Mexico in July 2017 and July 2018 and isolated Actinobacteria from the cuticle of these ants. We used established methods for culturing symbiotic *Pseudonocardia* from live fungus-growing ants[18] and targeted our isolations to the propleural plates of these ants, a site known to house symbiotic *Pseudonocardia* in other *Trachymyrmex* ant species.[8,12] This targeted bacterial isolation approach aimed to avoid culturing transient bacteria that might be present on

the ant cuticle. Our isolations yielded not only *Pseudonocardia*, from 9 ant colonies, but also Actinobacteria from the closely related genus *Amycolatopsis*, from 5 ant colonies (Table S1).

Competition Assays. We wondered whether competition among these *T. smithi*-associated bacteria was mediated by antibacterial production, so we assessed competitive dynamics of a subset of our isolates, two *Pseudonocardia* and two *Amycolatopsis*, in an intruder assay (Figure 1). In this assay, an established bacterial colony on an agar plate is surrounded by an "intruder" strain of bacteria. A zone of inhibition surrounding the central colony indicates that it produces a diffusible antibacterial agent active against the intruder.[9,10] Our assay indicated robust inhibition of the *Pseudonocardia* isolates by both *Amycolatopsis* isolates, and indicated that the *Amycolatopsis* are resistant to their own antibacterials.

A

Resident Strain \ Intruder Strain	*Amycolatopsis* 17SM-2A	*Amycolatopsis* 17SM-8A	*Pseudonocardia* 17SM-1	*Pseudonocardia* 17SM-6
Amycolatopsis 17SM-2A	0	0	3.0	2.4
Amycolatopsis 17SM-8A	0	0	1.6	1.6
Pseudonocardia 17SM-1	0	0	0	0
Pseudonocardia 17SM-6	0	0	0	0

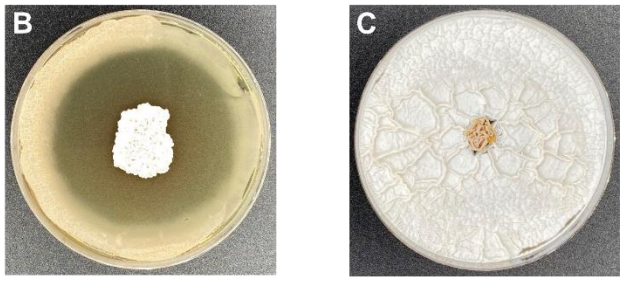

Figure 1. Intruder assay data. **A.** Zone of inhibition (cm) for resident-intruder pairings. **B.** Pairing showing strong inhibition of *Pseudonocardia* 17SM-1 intruder by *Amycolatopsis* 17SM-2A resident. **C.** Pairing showing no inhibition of *Amycolatopsis* 17SM-2A intruder by *Pseudonocardia* 17SM-1 resident.

Activity-Guided Fractionation and Structure Elucidation. To establish the molecular basis for this inhibition by the *Amycolatopsis* isolate with the greatest inhibitory activity, 17SM-2A, we cultured it on YMEA agar and carried out activity-guided fractionation of the ethyl acetate extract of these cultures. During fractionation we monitored antibacterial activity against the *Pseudonocardia* isolate 17SM-1 that was strongly inhibited in our intruder assay. Two rounds of fractionation by reversed-phase chromatography, using a C18 cartridge followed by preparative HPLC, revealed the active antibacterial molecule.

Detailed structural characterization by MS and by NMR established that the antibacterial molecule is a novel enoyl tetramic acid antibiotic, nocamycin V (Figure 2). High-resolution ESI-MS (*m*/*z* 520.2180 [M + H^+]; calcd for $C_{26}H_{34}NO_{10}$, 520.2183, Δ 0.6 ppm) supported a molecular formula with one additional oxygen relative to the known antibacterial nocamycin I. Correlations from 2D NMR experiments (COSY, HMBC, ROESY) allowed us to assign all NMR signals and establish that nocamycin V matches the structure of nocamycin I with the addition of a hydroxyl group at carbon 20 (Figure S2). ROESY correlations support the same stereochemistry of the bicyclic ketal ring system as for nocamycin I and our optical rotation measurement for nocamycin V is similar to that of nocamycin I, supporting the same absolute stereochemistry.

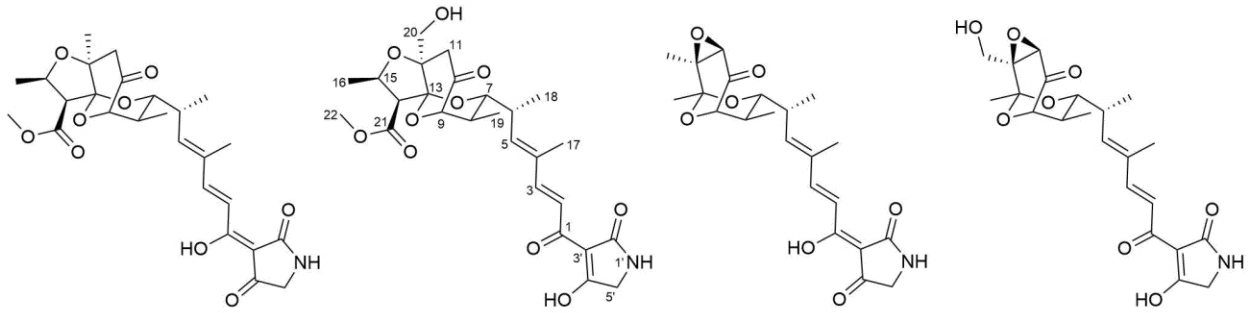

Figure 2. Select enoyl tetramic acid antibiotics.

Our NMR data, both in d_4-methanol and in d_6-DMSO, indicate that nocamycin V adopts the 1-keto-4′-enol tautomer in contrast to the 1-enol-4′-keto tautomer reported for nocamycin I. Notable spectral differences for this region include the C1 δ^{13}C value for nocamycin V appearing 8.16 PPM downfield relative to nocamycin I,[19] indicating a ketone group rather than an enol. The δ^{13}C values for the conjugated region of nocamycin V more closely match those for tirandamycin B, a related molecule that adopts the 1-keto-4′-enol tautomer, than nocamycin I (Figure 2).[20] Our data also are consistent with the empirical rule for keto-enol tautomers of this conjugated system proposed by Zhen *et al.*: a ^{1}H chemical shift value at C5 of less than 6.00 PPM indicates the 1-keto-4′-enol tautomer.[20] The apparent influence of a distal hydroxyl group on the keto-enol equilibrium, while unexpected, appears conserved for both the tirandamycins and nocamycins.

Structurally related antibiotics include nocamycins I and II, the engineered nocamycin analogs nocamycins III and IV, streptolydigin, and the tirandamycin family of compounds, all of which contain both a cyclic ketal moiety and the dienoyl tetramic acid moiety. These compounds have activity against Gram-positive bacteria, via inhibition of bacterial RNA polymerase.[21,22] Several tirandamycin compounds have demonstrated activity against vancomycin-resistant *Enterococcus faecalis* (VRE). For anti-VRE activity, tirandamycin B, which has the same hydroxyl placement as nocamycin V, is approximately 40-fold less potent than tirandamycin A.[23] The tirandamycins also have anti-filarial activity against the nematode parasite *Brugia malayi* via inhibition of asparaginyl-tRNA synthetase (AsnRS), and for this activity tirandamycin B is more potent than tirandamycin A.[24]

Activity Testing. To assess the ecologically-relevant antibiotic activity of nocamycin V, we performed agar diffusion assays using the pure compound against a panel of bacteria and fungi (Table 1). Consistent with the intruder assay data, the nocamycin V-producing isolate *Amycolatopsis* 17SM-2A is resistant to the compound, indicating self-resistance. Large zones of inhibition were observed for symbiotic *Pseudonocardia* isolates collected from *T. smithi*, from

other *Trachymyrmex* from the American Southwest, and from other fungus-growing ants. This finding is consistent with *Amycolatopsis* 17SM-2A effectively antagonizing bacterial competitors in the fungus-growing ant niche using nocamycin V. At the concentration tested, no inhibition was observed for the ant-associated *Amycolatopsis* isolates, suggesting that the resistance mechanism from 17SM-2A is shared among other *Amycolatopsis* isolates. Consistent with the known activity for nocamycin I, nocamycin V inhibited Gram-positive bacteria but not Gram-negative bacteria. We also observed no antifungal activity against *Escovopsis*, the ecologically-relevant fungal pathogen of fungus-growing ant nests.

Table 1. Inhibitory activity of nocamycin V. Zones of inhibition are reported for 10 µg nocamycin V in agar diffusion assays.

Organism	Zone of inhibition (cm)
Amycolatopsis 17SM-2A	0
Amycolatopsis 17SM-8A	0
Amycolatopsis 18SM-2A	0
Pseudonocardia 17SM-1	1.1
Pseudonocardia 17SM-3	1.6
Pseudonocardia 17SM-6	1.0
Pseudonocardia 18PO-3	2.2
Pseudonocardia 18AZ-4	1.6
Pseudonocardia 17SE-9	1.6
Pseudonocardia PLR1	2.2
Bacillus subtilis 3610	0
Escherichia coli	0
Escovopsis sp.	0
Saccharomyces cerevisiae	0

Nocamycin V Production Screen. As our intruder assay and agar diffusion assay data suggest that nocamycin V production by *Amycolatopsis* 17SM-2A could make it an effective competitor against other ant-associated bacteria in this niche, we wondered whether this molecule is broadly distributed among bacterial associates of *T. smithi* ants. We conducted an LCMS screen of all *Amycolatopsis* isolates that we recovered from *T. smithi* ants during our collections

in both 2017 and 2018, and also of our *Pseudonocardia* isolates from 2018 (Table 2). We detected nocamycin V in five out of 12 *Amycolatopsis* isolates; these nocamycin-containing isolates derive from three different *T. smithi* colonies and represent sampling across two years. We did not detect the compound in any of the *Pseudonocardia* isolates. These findings, based on our limited sampling, suggest that nocamycin-producing *Amycolatopsis* can effectively colonize *T. smithi* ants. We note that *Amycolatopsis* 17SM-8A, which also showed robust inhibition of *Pseudonocardia* in our intruder assay, does not produce nocamycin V. We are currently investigating other antibiotics from bacterial isolates from this niche.

Table 2. Nocamycin V production from Actinobacteria isolates, as detected by LCMS.

Bacterial Isolate[a]	Las Cruces, New Mexico Collection Site	Ant Colony	Collection date	Nocamycin V production
Amycolatopsis 17SM-2A	Tellbrook Park	17SM-2	07-2017	+
Amycolatopsis 17SM-2B	Tellbrook Park	17SM-2	07-2017	–
Amycolatopsis 17SM-8A	Tellbrook Park	17SM-8	07-2017	–
Amycolatopsis 17SM-8B	Tellbrook Park	17SM-8	07-2017	–
Amycolatopsis 18SM-1	Desert Trails Park	18SM-1	07-2018	–
Amycolatopsis 18SM-2A	Tellbrook Park	17SM-8[b]	07-2018	+
Amycolatopsis 18SM-2B	Tellbrook Park	17SM-8[b]	07-2018	–
Amycolatopsis 18SM-2C	Tellbrook Park	17SM-8[b]	07-2018	–
Amycolatopsis 18SM-3A	Tellbrook Park	18SM-3	07-2018	+
Amycolatopsis 18SM-3B	Tellbrook Park	18SM-3	07-2018	+
Amycolatopsis 18SM-3C	Tellbrook Park	18SM-3	07-2018	+
Amycolatopsis 18SM-4	Desert Trails Park	18SM-4	07-2018	–
Pseudonocardia 18SM-5A	Tellbrook Park	18SM-5	07-2018	–
Pseudonocardia 18SM-5B	Tellbrook Park	18SM-5	07-2018	–
Pseudonocardia 18SM-6	Desert Trails Park	18SM-6	07-2018	–
Pseudonocardia 18SM-7	Desert Trails Park	18SM-1	07-2018	–

[a]For multiple bacterial strains from the same ant colony, each isolate comes from a different ant
[b]Re-sampling of 17SM-8 colony in 2018

Genome and Biosynthetic Gene Cluster Analysis. We were interested in identifying the nocamycin biosynthetic gene cluster (BGC) to understand the genetic basis for the compound's unique oxidation pattern and to understand how it arrived in this unique niche. We used PacBio sequencing to obtain a complete genome for *Amycolatopsis* isolate 17SM-2A and identified the nocamycin V BGC using AntiSMASH v6.0.[25] The nocamycins, tirandamycins, and streptolydigin consist of a hybrid PKS-NRPS scaffold elaborated through oxidative tailoring. The BGC for nocamycin I was recently characterized in the strain *Saccharothrix syringae* NRRL B-16468.[26] As expected, the nocamycin V BGC is broadly similar with homologs present for all genes in the nocamycin I BGC (Figure 3, Table S3). The nocamycin V BGC has a distinct gene order and bears three additional genes absent in the nocamycin I BGC: these code for the putative transcriptional regulator NmvT and two putative cytochromes P450, NmvR and NmvS. In total, the nocamycin V BGC has four putative cytochromes P450, including homologs for the two P450s in the nocamycin I cluster, NmvG and NmvO.

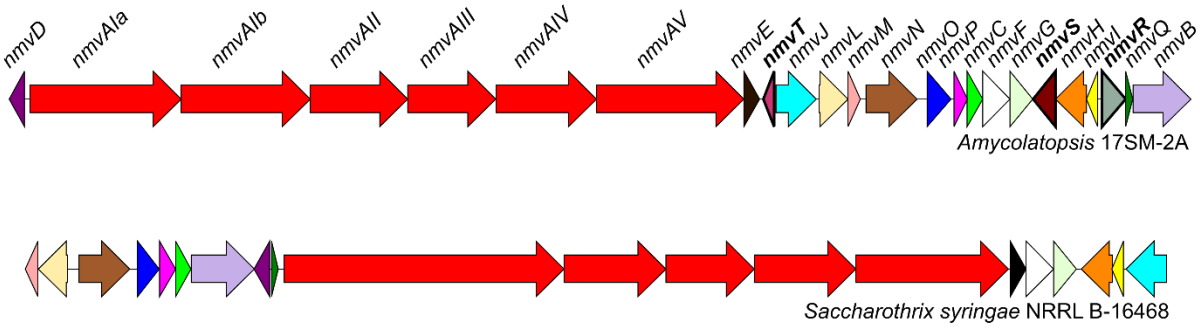

Figure 3. Biosynthetic gene clusters for nocamycin V (top) and nocamycin I (bottom). Color coding denotes homologous genes. Genes in bold are unique to the nocamycin V BGC.

Because nocamycin V bears only a single additional oxidation relative to nocamycin I—the C20 hydroxyl group—the presence of not one but two additional oxidative enzymes was unexpected. The complex oxidation patterns observed for the polycyclic ketal headgroups of the nocamycins and tirandamycins have inspired mechanistic studies to untangle the sequence of

oxidative tailoring steps.[19,26–30] Remarkably, a single cytochrome P450 enzyme, TamI, accomplishes three distinct oxidative steps as part of a cascade of oxidative reactions on the tirandamycin scaffold, resulting in installation of a hydroxyl group at C18, analogous to the C20 hydroxyl of nocamycin V.[28,29] From a phylogeny of cytochromes P450 from tirandamycin and nocamycin BGCs (Figure S5), we find that NmvR is homologous to TamI, which could indicate a role in installation of the C20 hydroxyl group. A complex interplay of oxidations is likely and future investigation of the biosynthesis of nocamycin V would contribute to our understanding of multifunctional enzymes and co-dependent oxidations.

We further examined the genome of *Amycolatopsis* 17-SM2A to understand the origin of this strain and the distribution of this molecule beyond this specialized niche. By searching the NCBI nucleotide databases we identified four additional strains, all *Amycolatopsis* with BGCs highly homologous to the nocamycin V cluster, that we predict are also producers of nocamycin V (Table S4, Figure S6). Three of these strains are from soil while one is a plant endophyte, and all share greater than 97% 16S sequence identity with *Amycolatopsis* 17-SM2A. The most similar strain, *Amycolatopsis keratiniphila* strain FH 1893 (isolated from a soil sample from India), shares 94.3% average nucleotide identity with *Amycolatopsis* 17-SM2A across the entire genome. This extremely high similarity to soil isolates is consistent with *Amycolatopsis* 17-SM2A being acquired by the ants from soil with nocamycin V aiding its competition with symbiotic *Pseudonocardia* on the ants. *Pseudonocardia* are vertically transmitted by fungus-growing ants while non-*Pseudonocardia* isolates from these ants are assumed to be acquired from the environment.[12]

Dienoyl tetramic acid antibiotics, particularly tirandamycins, are encountered with some frequency in studies of bacteria from diverse environments. Production of tirandamycins has been reported from at least 10 strains of *Streptomyces* of both marine and terrestrial origin.[20,22,31,32] Nocamycin I has been isolated from multiple Actinobacteria: *Saccharothrix syringae* NRRL B-16468 and *Microtetraspora caesia* ATCC 31295.[33,34] To our knowledge, however, a niche

competition role for nocamycin V is the first ecological function ascribed to an antibiotic from the dienoyl tetramic acid family.

CONCLUSIONS

Our study of an unexplored ecological niche, the microbial community of the desert-adapted fungus-growing ant *T. smithi*, has revealed a novel antibacterial compound that mediates bacterial competition. Nocamycin V belongs to a family of compounds of clinical interest with activity against antibiotic-resistant bacteria. It is, to our knowledge, the first enoyl tetramic acid antibiotic ascribed an ecological function and it adds to the diverse catalog of novel bioactive molecules from fungus-growing ant microbiomes. Bacterial competitors, such as this nocamycin V-producing *Amycolatopsis* strain that likely came from soil, further expand the roster of organisms associated with fungus-growing ants that interact using small molecules. Insect microbiomes, especially those from underexplored environments, are rich and largely untapped reservoirs of bioactive chemistry.

MATERIALS AND METHODS

General chemical analysis procedures. UV-visible absorbance spectra were collected on an Agilent 8453 spectrophotometer. High resolution mass spectrometry and MS/MS analysis was performed on a Waters Xevo G2-XS qTof mass spectrometer. LCMS analysis was performed on an Agilent 1200 series HPLC coupled to an Agilent 6120 mass spectrometer. NMR experiments were performed on a Bruker AVANCE III 500 MHz NMR spectrometer fitted with either a broadband probe or with an inverse TXI probe. Chemical shifts were referenced to the residual solvent peak. Optical rotation was measured on a Jasco P-2000 polarimeter.

Bacterial collection and isolation. Actinobacteria were isolated from the propleural (laterocervical) plates of *Trachymyrmex smithi* worker ants by scraping with a sterile pick and

plating onto chitin agar as described previously.[18] Ants were collected in Las Cruces, New Mexico under permits from the city of Las Cruces. Isolate details including specific collection location, collection date, and ant species are described in Table S1.

Intruder assays. A concentrated suspension of spores (5 uL) was spotted in the center of agar plates (YMEA 1% agar; 25 mL agar per 100 x 15 mm plate). The plates were incubated at 30°C for 7 days. A suspension of the "intruder" spores (15 uL concentrated spores in 315 uL sterile water) was spread using a dampened sterile cotton swab over the rest of the surface area on the plate. After 7 days, the radius of the inhibitory zone was measured. Two replicates were performed for each pairing.

Activity-guided fractionation and isolation of nocamycin V. A dilute spore suspension of *Amycolatopsis* 17SM-2A was spread onto 19 YMEA agar plates (60 mL agar per 150 x 15 mm plate, 200 µL spore suspension per plate), which were incubated at 30 °C for 14 days. The agar was then cut into 1 cm x 1 cm squares and soaked in EtOAc for approximately 18 h. This extract was decanted, the agar was soaked in additional EtOAc for 4 h, and the combined EtOAc portions were concentrated *in vacuo* and redissolved in 10 mL MeOH. The MeOH-soluble extract was absorbed onto celite. The celite-adsorbed extract was dry-packed onto a 5 g C_{18} SepPak column (Waters) that had been conditioned and pre-equilibrated with 20% MeCN in H_2O containing 0.1% formic acid. Fractions were collected during elution with a step gradient of 20%, 40%, 60%, 80%, and 100% MeCN, all containing 0.1% formic acid. Two consecutive fractions from elution at 60% MeCN showed the most inhibition of *Pseudonocardia* 17SM-1. These active fractions were combined and subjected to semi-preparative reversed-phase HPLC (Phenomenex Kinetex 5 µm Biphenyl 250 mm x 10 mm column, 2.5 mL/min) at 35% MeCN in H_2O with 0.1% formic acid for 1 min followed by a gradient of 35% to 100% MeCN in H_2O containing 0.1% formic acid over 20 min. Fractions were collected and the HPLC fraction with greatest activity corresponded to

nocamycin V, which eluted at 13.1 minutes. Nocamycin V was isolated as an amorphous yellow solid in a yield of 8.9 mg.

Nocamycin V: $[\alpha]_D^{23}$ -54° (EtOH); UV (EtOH) λ_{max} 253, 288, and 333 nm; NMR spectral data, see Supporting Information, Table S2; HR-ESI-MS *m/z* 520.2180 [M+H$^+$] (calcd for $C_{26}H_{34}NO_{10}$, 520.2183, Δ 0.6 ppm).

Genome sequencing. Using the Qiagen Gentra Puregene Yeast/Bact. kit, genomic DNA was isolated from pelleted cell mass from a culture of *Amycolatopsis* 17SM-2A grown in YMEA liquid media. Library preparation and sequencing by PacBio RS II (2 SMRT cells) was performed by the UC Riverside genomics core facility. De novo genome assembly was performed using HGAP 4 via the de novo genome assembly tool within the SMRT Analysis (v6) software package, which yielded a single contig. Additional polishing was carried out using the Resequencing tool within SMRT Analysis. The genome has been deposited in the GenBank database (accession number CP092497), and raw sequence data have been deposited in the Sequence Read Archive (accession number PRJNA808064).

Biosynthetic gene cluster annotation and analysis. Gene detection and annotation was performed using RAST v2.0.[35] Biosynthetic gene cluster detection was conducted using antiSMASH v6.0.[25] Open reading frames of less than 150 nucleotides were omitted from further analysis. The nocamycin V biosynthetic gene cluster was identified in additional genomes in GenBank through both the Cluster Blast feature within antiSMASH and a BlastP search of the Dieckmann cyclase NmvC from *Amycolatopsis* 17SM-2A against the nonredundant protein sequences database. Protein sequences for cytochromes P450 were aligned using MUSCLE and a maximum likelihood phylogeny was generated using PhyML v3.0 within Phylogeny.fr.[36–38]

LCMS Screen for nocamycin V. Bacterial isolates were grown on YMEA agar at 30°C for 14 days, after which the agar was cut into 1 cm × 1 cm squares and soaked in EtOAc. These

extracts were concentrated *in vacuo* and redissolved in MeOH. The resulting extract solutions were subjected to LCMS analysis (Eclipse XDB-C18 5 µm 150 mm x 4.6 mm column, 1.0 mL/min) with a gradient of 10% acetonitrile in water with 0.1% TFA to 100% acetonitrile with 0.1% TFA over 8 minutes. Nocamycin V was observed at a retention time of 6.7 minutes.

Agar diffusion assays. Agar lawns were prepared by mixing an inoculum of test strain with soft YMEA agar (4 mL; 55°C; 0.75% agar). The mixture was poured on top of a plate of YMEA agar (25 mL per 100 x 15 mm dish) and left to solidify. Three evenly spaced holes were punched through both layers of agar using a sterilized metal punch (5 mm diameter). Two wells were loaded with a 10 ug solution of nocamycin V in 25 µL of 1:1 DMSO and water. The third well was loaded with a 25 µL of a control solution of 1:1 DMSO and water. The diameter of the zone of inhibition was monitored over several days and measured once growth stopped. The zones of inhibition for *Amycolatopsis* 17SM-2A and 17SM-8A were reported after 2 days. The zones of inhibition for *Pseudonocardia* 17SM-1 and *Amycolatopsis* 18SM-2A were reported after 5 days. The zones for *Pseudonocardia* 17SM-6 and PLR1 were reported after 6 days. The zones for *Pseudonocardia* 17SM-3, 17SE-9, 18PO-3, and 18AZ-4, and for *E. coli, B. subtilis, S. cerevisiae* and *Escovopsis sp.* were reported after 7 days. The average diameter taken from the two zones on the final day of zone growth was reported.

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Supporting Information: Summary of bacterial strains used; mass spectra, NMR spectra, and NMR spectral data for nocamycin V; summary of nocamycin V BGC genes; phylogeny of cytochromes P450 from tirandamycin and nocamycin BGCs; summary of all putative nocamycin V BGCs.

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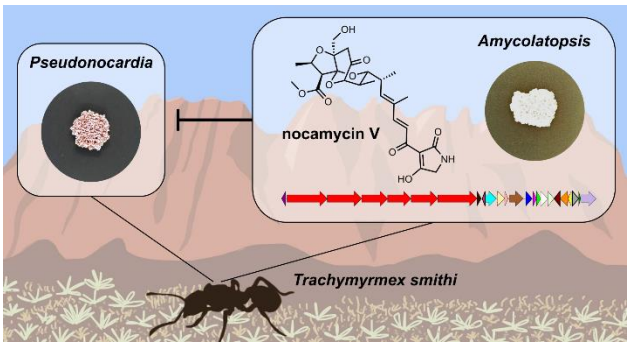
TOC graphic:


Pseudonocardia
Amycolatopsis
nocamycin V
Trachymyrmex smithi