Bionectriol A, a polyketide glycoside from the fungus *Bionectria* sp. associated with the fungus-growing ant, *Apterostigma dentigerum*

Elizaveta Freinkman [a], Dong-Chan Oh [b,†], Jarrod J. Scott [c], Cameron R. Currie [c], Jon Clardy [a,b,*]

[a] *Chemical Biology Graduate Program, Harvard Medical School, Boston, MA 02115, USA*
[b] *Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA*
[c] *Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706, USA*

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ABSTRACT

A new polyketide glycoside, bionectriol A (**1**), was produced by a fungal culture of *Bionectria* sp., which was isolated from a fungus garden of the fungus-growing ant *Apterostigma dentigerum*, in Costa Rica. The structure of bionectriol A was determined mainly through NMR and mass spectroscopic data, as well as UV and IR spectra. The relative configurations of the main chain, the pyranohexose, and the pentitol moiety were elucidated by ^{1}H–^{1}H coupling constants and ROESY NMR spectral analysis.

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Microbes that mediate complex ecological interactions among 'higher' organisms have recently emerged as rich sources of novel bioactive metabolites.[1–3] In one closely studied system, fungus-growing ants (Attini:Formicidae) cultivate a food fungus in specialized gardens (Agaricales:Basidiomycota:Lepiotaceae or Pterulaceae). These ants were recently shown to harbor mutualistic bacteria in the genus *Pseudonocardia* (Actinobacteria), which produce antibiotics that target *Escovopsis* sp. (Ascomycota), a specialized fungal pathogen of the ants' fungal gardens.[4,5] The ant-fungus mutualism originated approximately 50 million years ago in the Amazon Basin and evolved into more than 230 species of ants, each associated with lineages of mutualistic fungi, antagonistic fungi (*Escovopsis*), and mutualist bacteria (*Pseudonocardia*).[5,6] The diversity of the known microbial symbionts associated with fungus-growing ants, coupled with evidence that they produce diverse secondary metabolites (especially *Pseudonocardia*),[5,7] makes this ancient mutualism a potentially rich but unexplored source of natural products.

Even though the fungus-growing ant system has been studied for more than 130 years,[8] secondary metabolites from the microorganisms in this niche have received only limited attention.[9] However, our recent study on one of the fungus-growing ant symbiotic systems, *Apterostigma dentigerum* in Panama, revealed an anti-

fungal compound produced by the symbiotic actinomycete, *Pseudonocardia* sp. This new antifungal agent, a cyclic depsipeptide bearing unusual amino acids, selectively inhibits the antagonistic fungus over the mutualistic fungus, indicating that these symbioses are promising sources for bioactive natural products.[10]

Apterostigma occur throughout Central and South America and often nest in, or under decaying logs or in root banks near streams. Unlike most Attine ants that cultivate litter-decomposing fungi (Basidiomycota: Agaricales), *Apterostigma* grow a distantly related wood-gardening Basidiomycete (Pterulaceae).[11,12] The ants use insect feces and dead woody material to cultivate their food fungal crop.[13] In addition to the described fungal and actinomycete symbionts, it is possible that other microorganisms as yet unknown from the system could serve as additional sources for compound discovery. Several studies have shown that gardens from different fungus-growing ant species host a variety of microorganisms with unknown roles.[14–16] Here, we examine a fungus with an unknown role that was isolated from the garden of the fungus-growing ant *Apterostigma dentigerum*.

We have confirmed earlier findings[14–16] that fungus-growing ant gardens harbor additional fungal strains besides the cultivar and parasitic fungi. We isolated a strain, CC061026-06, from a nest of the attine ant *Apterostigma dentigerum* in Costa Rica. 28S rRNA sequencing and phylogenetic analyses were conducted to identify this strain. High bootstrap support indicates that its closest relative is *Bionectria ochroleuca*. Additionally, phylogenetic analysis shows that the strain is closely related to other strains of *Nectria*/*Bionectria*, and that this group is sister to *Hydropisphaera*. *Bionectria*, which is neither a known crop cultivated by *A. dentigerum* nor a known fungal pathogen; the exact role of CC061026-06 within the ant-fungal mutualism is

* Corresponding author. Tel.: +1 617 432 2845; fax: +1 617 432 6424.
   E-mail address: jon_clardy@hms.harvard.edu (J. Clardy).
† Present address: Natural Products Research Institute, College of Pharmacy, Seoul National University, San 56-1, Sillim, Seoul 151-742, Republic of Korea.

Table 1
NMR Data for bionectriol A (**1**) in CD_3OD

C/H	$\delta_H{}^a$	Mult (J in Hz)	$\delta_C{}^b$	#H
1			168.5	C
2			127.5	C
3	6.78	dd (10.0, 1.5)	146.5	CH
4	2.73	m	37.0	CH
5	3.82	m	82.3	CH
6			136.0	C
7	5.32	d (9.5)	133.0	CH
8	2.63	m	35.7	CH
9	3.68	d (9.5)	83.0	CH
10			134.7	C
11	5.37	d (9.0)	133.5	CH
12	2.72	m	34.2	CH
13	3.95	d (9.5)	87.3	CH
14			139.6	C
15	5.25	d (9.5)	129.9	CH
16	2.60	m	30.0	CH
17	1.28	m	44.5	CH_2
18	1.30	m	33.0	CH
19	1.15	m	26.0	CH_2
20	0.86	t (6.5)	10.8	CH_3
21	1.88	d (1.5)	11.8	CH_3
22	0.85	m	15.4	CH_3
23	1.67	s	10.0	CH_3
24	0.77	d (7.0)	16.6	CH_3
25	1.66	s	10.0	CH_3
26	0.80	d (7.0)	16.2	CH_3
27	1.60	d (1.0)	10.0	CH_3
28	0.98	d (6.5)	21.3	CH_3
29	0.84	m	18.4	CH_3
1'a	4.18	dd (11.0, 5.5)	66.6	CH_2
1'b	4.26	dd (11.0, 7.5)		
2'	4.15	ddd (7.5, 5.5, 1.5)	68.0	CH
3'	3.53	m	71.2	CH
4'	3.71	ddd (7.5, 6.0, 3.5)	71.7	CH
5'a	3.81	dd (11.0, 3.5)	63.9	CH_2
5'b	3.64	dd (11.0, 6.0)		
1''	4.35	bs	96.3	CH
2''	3.74	m	73.8	CH
3''	3.35	dd (9.5, 3.0)	74.5	CH
4''	3.53	m	67.6	CH
5''	3.04	ddd (9.5, 5.5, 2.5)	77.0	CH
6''a	3.72	m	62.0	CH_2
6''b	3.86	dd (12.0, 2.5)		

^{a} 600 MHz.
^{b} 150 MHz.

unknown. Species of *Bionectria* have, however, been identified from other insect systems including several wood-feeding bark beetle species[17,18] and some strains were isolated from oral secretions.[18] As part of our effort to probe the metabolic repertoire of the microorganisms in fungus-growing ant systems, we investigated the secondary metabolite profile of this fungal strain, *Bionectria* sp. Here, we report bionectriol A, a previously unreported polyketide glycoside, produced by strain CC061026-06.

The fungal strain was cultured in potato dextrose broth, with agitation at 250 rpm, at 30 °C for 12 days. An ethyl acetate extract of cultured cells and supernatant (total volume 600 mL) was subjected to reversed-phase flash column chromatography and final purification by HPLC to yield 3.7 mg bionectriol A (**1**). Bionectriol A was obtained as a viscous oil with the molecular formula $C_{40}H_{70}O_{14}$, as confirmed by ESIHRMS (obsd [M+Na]$^+$ at m/z 797.4670, calcd [M+Na]$^+$ 797.4663). This molecular formula was also supported by ^{1}H and ^{13}C NMR data (Table 1).
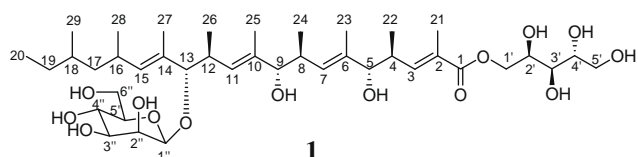

1

The planar structure of bionectriol A was elucidated mainly by two-dimensional NMR experiments, including ^{1}H-^{1}H COSY, TOCSY, HSQC, and HMBC spectra. The ^{13}C NMR and the HSQC spectra suggested the presence of one carbonyl carbon, four methine and four quaternary sp^2 resonances, eleven oxygenated methines and three oxygenated methylenes, two aliphatic methylene carbons, five aliphatic methine carbons, and 10 methyl groups. A network of COSY correlations between H-9 and H-8, between H-8 and H$_3$-24, between H-8 and H-7, and between H-7 and H$_3$-25 (long-range COSY correlation) constructed the C_6-substructure shown in Figure 1. In a similar manner, two more repeating C_6-substructures were assigned.

The HMBCs from H$_3$-23 to C-5, from H$_3$-25 to C-9, and from H$_3$-27 to C-13 connected these three consecutive and repeating units (Fig. 2). The termini of the repeating chain were assigned by the consecutive COSY and TOCSY correlations through H-15 to H-20 and the HMBCs from H-3 and H$_3$-21 to C-1 (Fig. 2).

The pentitol, the five-carbon polyhydroxy ester fragment, was identified on the basis of the COSY, TOCSY, and HMBC spectral analyses. This pentitol was connected to the C-1 ester carbonyl carbon (Fig. 2). The hexopyranose moiety was also elucidated by the COSY and HMBCs. Attachment of the sugar at C-13 was deduced from a HMBC from the acetal proton (H-1'', δ_H 4.35, δ_C 96.2). This sugar was identified as mannose by the ^{1}H–^{1}H coupling constant and ROESY spectral interpretation. The coupling constant between H-4'' and H-5'' (J = 9.0 Hz) established their axial–axial relationship. The ROESY correlations from H-1'' to H-3'' and H-5'' indicated that these protons were axial. An additional ROESY correlation between H-1'' and H-2'' showed that H-2'' is equatorial. The axial configuration of the anomeric proton assigned the glycosidic linkage as β-configuration, which was further supported by the one-bond C-H coupling constant of 157 Hz between the anomeric carbon and proton.[19] The geometry of all the double bonds in **1** was assigned as *E* based on the ROESY correlations as shown in Figure 2. The relative configuration between the hydroxy group at C-9 and the methyl group (C-24) at C-8 was established by ^{1}H–^{1}H coupling constant and ROESY correlations at these stereocenters. The large coupling constant (9.5 Hz) between H-8 and H-9 defined their *anti* relationship. Since H-8 and H-9 were fixed as *anti*, the ROESY couplings shown in Figure 3 also defined an *anti* relationship between the methyl group (C-24) and the hydroxy group at C-9. By a similar logic, the other two repeating units also have an *anti* relationship between their aliphatic methyl groups (C-22 and C-26) and the hydroxy group and the ester group, respectively.
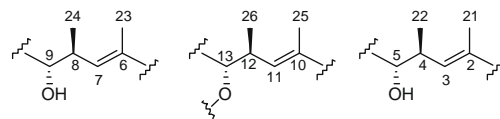
Figure 1. The repeating C_6-substructures in the structure elucidation of bionectriol A (**1**).
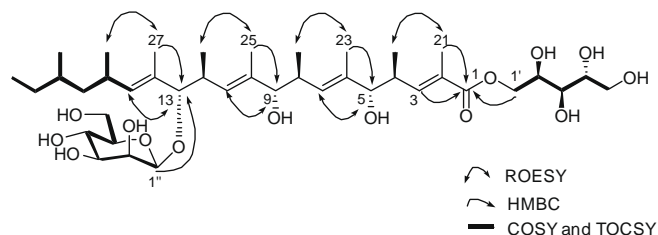
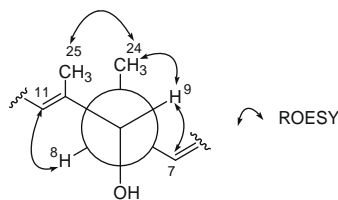**Figure 2.** Selected 2D NMR correlations for bionectriol A (**1**).

Figure 3. Rotamer of C-8 and C-9 stereogenic centers with ROESY correlations.

To determine the relative configuration of the pentitol moiety, we acquired ^1H–^1H coupling constants by utilizing selective 1D TOCSY spectra. The coupling constants and the carbon chemical shifts of the three consecutive stereogenic centers ($2''$, $3''$, and $4''$) indicated that the pentitol moiety has $2''R^*$, $3''R^*$, and $4''R^*$ relative configuration based on the published values of the identical pentitol moiety in roselipin 1B.[20]

The structure of bionectriol A suggests that it is most likely produced by a polyketide synthase (PKS) from one molecule of acetyl-coenzyme A (acetyl-CoA) and nine molecules of malonyl-CoA. The malonyl-CoAs are presumably methylated by C-methylation (CMeT) domains utilizing an S-adenosyl methionine (SAM) cofactor after each ketosynthase step, as has been documented in other fungal PKS systems.[21] The structure of the core of bionectriol A is consistent with an iterative PKS assembly model, in which a single pair of modules is responsible for incorporation of the third and fourth, fifth and sixth, and seventh and eighth two-carbon building blocks. We speculate that, after the bionectriol A core has been generated by the iterative PKS machinery, it undergoes mannosylation on the C-13 hydroxyl and esterification to a pentitol to yield **1**.

The closest known structural relatives of bionectriol A (**1**) are roselipins 1A and 1B, which were previously isolated from a marine fungal isolate of *Gliocladium roseum* KF-1040, the anamorph of *B. ochroleuca*.[20,22,23] The roselipins, which are diastereomers differing in configuration at C2′ and C4′, differ from bionectriol A by a double bond between C-14 and C-15. Bionectriol A is mannosylated, as are the roselipins.

The biological activity of **1** is currently under evaluation. Co-plating strain CC061026-06 with a variety of fungal isolates (*Escovopsis* spp.) revealed no significant antifungal activity. Also, an antifungal assay with pure bionectriol A against *Candida albicans* ATCC # 10231 showed no significant inhibition.

Bionectriol A represents a class of glycosylated, polyunsaturated polyols whose biological and ecological functions are unknown. While the other members of this class, the roselipins, were found in *Gliocladium* isolates associated with seaweed, bionectriol A was derived from a different context, a fungus-growing ant colony. These findings suggest that this class of compounds may play a role in interspecies interactions, although their activity remains unclear. Further investigation of the community of 'bystander' microbes associated with fungus-growing ants as well as the small molecules they produce, will be needed to understand the chemical ecology of this symbiosis.

Experimental section

General experimental procedures

Optical rotation was measured on a Jasco P-2000 polarimeter with a 5 cm cell. A UV–visible spectrum was acquired in methanol on an Amersham Ultrospec 5300 Pro UV/vis spectrophotometer with a path length of 1 cm. An IR spectrum was obtained on a Perkin Elmer 1600 FT-IR instrument. All NMR spectra were collected on a Varian Oxford AS600 instrument, operating at 600 MHz. Reversed-phase liquid chromatography-mass spectroscopy was carried out on an Agilent 1200 series LC instrument coupled to an Agilent 6130 Quadrupole mass spectrometer. High-resolution mass spectra were obtained on an Ultima Q-TOF instrument at the Mass Spectrometry Center, University of Illinois. HPLC was carried out using an Agilent 1100 series apparatus.

Collection and identification of fungal isolate

Filamentous fungi were isolated from a colony of *Apterostigma dentigerum* ants collected at the La Selva Biological Station in Costa Rica (10° 25′ 53″ N, 84° 00′ 13″ W) in October 2006. The isolation was conducted by plating garden substrate on potato dextrose agar plates containing antibacterial antibiotics, following previously published methods.[4] Fungal genomic DNA was isolated by use of the Epicenter MasterPure DNA purification kit protocol for plant tissue, without modification. A 900-basepair fragment of the gene encoding the 28S ribosomal RNA was amplified by polymerase chain reaction using primers LR5 (5′-TCC TGA GGG AAA CTT CG-3′) and LR0R (5′-ACC CGC TGA ACT TAA GC-3′), as described.[24] The PCR product was sequenced at the Dana Farber / Harvard Cancer Center DNA Sequencing Facility using the same primers. The phylogenetic placement for strain CC061026-06 was assessed using maximum likelihood (ML) analysis of ∼548 base pairs from 28S rDNA sequences. For this analysis we used 22 representative members of Hypocreomycetidae (Ascomycota), 19 of which were from the order Hypocreales.[25] Samples were first aligned in ClustalW using default parameters,[26] and ML analysis was performed using GARLI (Genetic Algorithm for Rapid Likelihood Inference).[27] Default settings were used to estimate the substitution model (general time reversible model), base pair frequencies, and among-site rate variation (gamma distribution).[28] Characters were all treated as unordered, and gaps as missing data. Bootstrap support for ML was calculated for internal branches after 100 pseudoreplicates. Output files from GARLI were imported into TREEVIEW to order taxa, display bootstrap values, and root the tree.[23]

Extraction, isolation, and characterization

The fungus was cultured in potato dextrose broth, with agitation, for 12 days at 30 °C. Fungal tissue and culture supernatant (total volume of 600 mL) were extracted with ethyl acetate (2 L). The extract was dried with sodium sulfate and filtered, and the solvent was removed in vacuo. The crude extract was subjected to reversed-phase flash column chromatography on C_{18} resin using a stepped gradient from 20% MeOH in H_2O to 100% MeOH. Fractions from this column containing bionectriol A (**1**) on the basis of LC–MS analyses were further purified by HPLC using an Altima C_{18} semipreparative column at a flow rate of 5 mL/min under isocratic conditions (35% acetonitrile in H_2O) to yield 3.7 mg bionectriol A.

Bionectriol A (1)

Amorphous solid; $[\alpha]_D$ +19 (c 0.31; MeOH); UV (MeOH) λ_{max} (log ε) 216 (sh, 4.0) nm; IR (NaCl disk) ν_{max} 3330, 2963, 1702, 1457, 1080, 1029 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 1; HRMS m/z found 797.4670 [M+Na]$^+$ (calcd for $C_{40}H_{70}O_{14}Na$, 797.4663).

Supplementary data

Supplementary data (experimental methods, ^{1}H, ^{13}C, 2D NMR spectra of **1**, and phylogenetic analysis of the strain CC061026-06) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.09.120.

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