Selvamicin, an atypical antifungal polyene from two alternative genomic contexts

Ethan B. Van Arnsm, Antonio C. Ruzzini, Clarissa S. Sit, Heidi Horn, Adrián A. Pinto-Tomás, Cameron R. Currie, and Jon Clardy

*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115; ‡Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706; §Centro de Investigación en Estructuras Microscópicas, Universidad de Costa Rica, San Pedro de Montes de Oca 2060, Costa Rica; ¶Centro de Investigación en Biología Celular y Molecular, Universidad de Costa Rica, San Pedro de Montes de Oca 2060, Costa Rica; and †Departamento de Bioquímica, Escuela de Medicina, Universidad de Costa Rica, San Pedro de Montes de Oca 2060, Costa Rica

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The bacteria harbored by fungus-growing ants produce a variety of small molecules that help maintain a complex multilateral symbiosis. In a survey of antifungal compounds from these bacteria, we discovered selvamicin, an unusual antifungal polyene macrolide, in bacterial isolates from two neighboring ant nests. Selvamicin resembles the clinically important antifungals nystatin A₁ and amphotericin B, but it has several distinct structural features: a noncautic 6-deoxymannose sugar at the canonical glycosylation site and a second sugar, an unusual 4-O-methylidigitoxose, at the opposite end of selvamicin’s shortened polyene macrolide. It also lacks some of the pharmacokinetic liabilities of the clinical agents and appears to have a different target. Whole genome sequencing revealed the putative type I polyketide gene cluster responsible for selvamicin’s biosynthesis, including a subcluster of genes consistent with selvamicin’s 4-O-methylidigitoxose sugar. Although the selvamicin biosynthetic cluster is virtually identical in both bacterial producers, in one it is on the chromosome, in the other it is on a plasmid. These alternative genomic contexts illustrate the biosynthetic gene cluster mobility that underlies the diversity and distribution of chemical defenses by the specialized bacteria in this multilateral symbiosis.

Significance

Bacteria use small molecules to mediate their relationships with nearby microbes, and these molecules represent both a promising source of therapeutic agents and a model system for the evolution and dissemination of molecular diversity. This study deals with one such molecule, selvamicin, which is produced by ant-associated bacteria. These bacteria protect the ants’ nests against fungal pathogens. Selvamicin is an atypical member of a clinically important class of antifungal agents, and it appears to have both better therapeutic properties and a different mechanism of action. Further, the genes for producing it are found on the bacteria’s chromosome in one ant nest but on a plasmid in another, illustrating the likely path by which it has spread.


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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. CP013854, CP013855, and CP013856); and National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession no. SRP075179).

To whom correspondence should be addressed. Email: jon_clardy@hms.harvard.edu.

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The compound’s UV-vis spectrum is characteristic of a polyene, with three prominent peaks (319, 334, 352 nm) consistent with a chromophore of five conjugated double bonds (SI Appendix, Fig. S1). Subsequent NMR analysis using a variety of 2D methods (COSY, TOCSY, HMBC, H2BC, and ROESY) revealed this compound to be an unreported polyene macrolide, which we have named selvamicin after the site of our original collection.

Selvamicin production can be greatly up-regulated by adding high concentrations of sodium butyrate (150 mM) to the culture medium (SI Appendix, Fig. S7), consistent with reports that butyrate can regulate secondary metabolism (13). We observed $^{13}$C labeling of selvamicin when [1-$^{13}$C] sodium butyrate was used, indicating that butyrate can also act as a metabolic precursor, and similar results were obtained with [1-$^{13}$C] propionate (SI Appendix, Fig. S8).

COSY and TOCSY correlations allowed us to construct two major fragments of the selvamicin macrolide: one from C2–C8 and another from C13 across the pentaene to the molecule’s terminus at C31 (overlap of the polyene resonances prevented definitive assignments of C19–C24, Fig. 2). HMBC couplings link the C2–C8 fragment to quaternary carbons at either end: an ester carbonyl at C1 (172.7 ppm) and a hemiketal at C9 (97.3 ppm). The hemiketal forms a six-membered ring established by a series of HMBC couplings from the hemiketal OH at C9, a tertiary alcohol and methyl substituent at C12, and the other bridgehead carbon at C13. H2BC correlations support the placement of substituents along the macrolide core of selvamicin (SI Appendix, Fig. S2). A series of ROESY correlations establishes an extended geometry for the C2–C8 aliphatic chain and a chair conformation for the hemiketal ring (SI Appendix, Fig. S2). These correlations, corroborated by available scalar coupling constants, allowed the assignment of relative stereochemistry from C4 to C13.

Our NMR analysis also revealed two sugars in the structure of selvamicin. COSY and HMBC couplings revealed their planar structures as 6-deoxy and 2,6-dideoxy hexoses, as shown in Fig. 2. To better resolve the crowded sugar CH signals and reveal additional peak fine structure, we reacted selvamicin with acetic anhydride to modify its free hydroxyl groups. In the acetylation product, the hemiketal at C9 was instead observed as a ketone, and with the exception of the tertiary alcohol at C12, all OH groups were acetylated (SI Appendix, Fig. S3). Scalar couplings and ROESY correlations allowed the acetylated sugars in this product to be assigned as (Ac)$_2$-β-6-deoxymannose and Ac-α-4-O-methylidigitoxose (SI Appendix, Fig. S4). The absolute configurations of the sugars were not determined.

A clear HMBC coupling from the anomeric proton of the β-6-deoxymannose places this sugar at C15 of selvamicin (Fig. 2). Whereas no HMBC couplings were observed for the anomeric proton of 4-O-methylidigitoxose, a series of ROESY correlations (1′′-H/27-H, 1′-H/33-H, 5′-H/34′-H) locates this sugar on the opposite side of the macrolide at C27. The $^1$H and $^{13}$C chemical shifts of the CH at position 27 support an oxygen substituent linking this sugar. From C25–C31, we observed broadened $^1$H and $^{13}$C resonances, which obscured the couplings needed to establish relative stereochemistry in this region. This peak broadening could reflect conformational flexibility near the 4-O-methylidigitoxose attachment.

Selvamicin’s structure diverges from the antifungal polyenes amphotericin B, nystatin A$_1$, and natamycin in several key respects. Its 30-membered polyene macrolide core is intermediate between that of the smaller antifungal natamycin and those of amphotericin B and nystatin A$_1$. Selvamicin’s unusual glycosylation is also noteworthy. The 6-deoxymannose replaces the mycosamine sugar common to most antifungal polyenes, and a second glycosylated nystatin analog named NPP was isolated from Pseudonocardia autotrophica. A diglycosylated nystatin analog named NPP was isolated from Pseudonocardia autotrophica, although the additional sugar, an N-acetylgalactosamine, is appended to the 4’ position of the mycosamine (14). A yet-unidentified nystatin analog from the ant-associated Pseudonocardia strain P1 also appears to have an additional sugar appendage at this same 4’ position (15).

![Key NMR correlations establishing the planar structure of selvamicin.](image_url)
A second glycosylation located instead on the opposite end of the macrolide, as in selvamicin, has been observed among the minor fermentation products of the nystatin A₂ producer Streptomyces noursei (nystatin A₃, Fig. 1, and NYST1070), and the candidin producer Streptomyces viridoflavus (candidin), with the second sugar located at C3ⱼ, the position corresponding to selvamicin’s 4-O-methylglitoxose attachment (16–18). Whereas structurally distinct from 4-O-methylglitoxose, these are also 2,6-dideoxy sugars (digitoxose, mycarose, and 2,6-dideoxy-L-erythro-hexopyranoses-3-ulose, respectively). Notably, in contrast to fermentations of S. noursei and S. viridoflavus, we observe the diglycosylated polypeptide selvamicin as the major polyene species, and neither monoglycosylated analog is detectable by LC-MS in extracts of LS1 or LS2.

The presence of 4-deoxymannose in place of mycosamine represents the only example to our knowledge of a noncationic sugar at that position in a glycosylated polypeptide natural product. Correspondingly, the usual paired carbamate substituent (C16 in nystatin and amphotericin B and C12 in natamycin) is absent in selvamicin. Instead, C12 bears a methyl group and a tertiary alcohol.

**Antifungal Activity and Solubility.** Liquid broth-based activity testing confirmed selvamicin’s antifungal activity against C. albicans (minimum inhibitory concentration, MIC = 23 μM), with similar activity observed across a panel of fungi (Saccharomyces cerevisiae, Aspergillus fumigatus, and Trichoderma harzianum, Fig. 3 and SI Appendix, Table S3). No activity was detected against either Gram-negative (Escherichia coli) or Gram-positive (Bacillus subtilis, Micrococcus luteus) bacteria. We note that selvamicin has more modest antifungal activity than clinically used antifungals such as nystatin A₁ (MIC = 1.0 μM against C. albicans). However, its improved aqueous solubility (2.3 mM compared with 0.3 mM for nystatin A₁) addresses a major limitation of clinically available antifungal polypeptides. Selvamicin’s improved solubility, despite its lack of charged carbohydrate and ammonium groups, is probably contributed by its second sugar moiety. Indeed, glycosylation has been reported to improve solubility dramatically in analogs of nystatin; NPP, a diglycosylated analog bearing N-acetylglucosamine, has more than 300-fold greater aqueous solubility than nystatin A₁ (14).

The activity of known antifungal polypeptides derives from interactions with ergosterol, the primary sterol of fungal plasma membranes. Such interactions can compromise membrane integrity and inhibit the function of membrane proteins (19, 20). Recent studies suggest that ergosterol sequestration into extracellular aggregates may be the dominant mechanism of action (21, 22), although several polypeptides, including nystatin and amphotericin B, have also long been known to permeabilize membranes by the formation of ergosterol-dependent transmembrane channels (23). The presumed geometry of these channels situates the charged end of the molecule at the lipid-water interface, with the polye and polyol interacting with ergosterol within the plasma membrane. The dramatically different electrostatic nature of selvamicin would likely preclude channel formation, with a hydrophilic yet uncharged sugar at each end of the molecule. We probed for an interaction with ergosterol using an established isothermal calorimetry assay for binding to liposome-embedded ergosterol (21, 24). These experiments showed no evidence for binding, in stark contrast to control experiments using nystatin A₁, suggesting that this interaction is much attenuated if present at all (SI Appendix, Fig. S9).

Further investigation of selvamicin’s mechanism of action is currently underway.

**Biochemical Activity Cluster.** To understand the genetic origins of selvamicin biosynthesis, we turned to the genomes of Pseudonocardia isolates LS1 (7) and LS2, which were sequenced using PacBio technology (25, 26). We readily identified a large type I PKS gene cluster in both genomes that matches the biosynthetic requirements for selvamicin (Fig. 4). The 109-kbp selvamicin biosynthetic gene clusters (BGC) from each isolate share perfect synteny and 98.4% nucleotide identity over their length. In contrast, the whole genomes differ more substantially. The average nucleotide identity (27) calculated across conserved replications on both chromosomes is only 83% and a comparison of housekeeping gene sequences places LS1 and LS2 into distinct clades previously established for ant-associated Pseudonocardia (28, 29). Overall, the two BGCs are much more similar to one another than are their bacterial hosts.

Surprisingly, the selvamicin BGC is situated in completely different genomic contexts in the two selvamicin producers; in LS1 it resides on the 6.1 Mbp circular chromosome, whereas in LS2 it is on a 376-kbp plasmid. The presence of an identical BGC in two divergent Pseudonocardia isolates, and in different genomic contexts, points to horizontal transfer. In keeping with BGC transfer, numerous mobile genetic elements including transposases and integrases flank it in both genomes (Fig. 4B). Mobile genetic elements are prominent features of both genomes. On the pLS2-1 plasmid containing the selvamicin BGC, an impressive 24% of all RAST-annotated genes are mobile genetic elements.

Selvamicin provides the most striking example yet for the emerging theme that plasmids drive the genetic, chemical, and functional diversity found in Pseudonocardia symbionts. Plasmid-derived BGCs for an antibacterial rebeccamycin analog and for the gerumycin depsipeptides feature in other ant-associated Pseudonocardia (7, 8). A rearranged variant of the gerumycin BGC also appears on the LS1 chromosome, suggesting that plasmid-mediated exchange also links the two gerumycin BGCs (7), but at greater evolutionary distance than for the virtually identical selvamicin BGCs.

**Biosynthesis.** The bioinformatically identified selvamicin cluster resembles known type I PKS-derived polypeptide BGCs (30–35), and a side-by-side comparison with the well-characterized nystatin BGC (36, 37) readily reveals the origins of selvamicin’s unusual structural features (Fig. 5). Both natural products derive from type I iterative PKSs with polyketide elongation modules spread across five genes (selnysB, -C, -I, -J, and -K). Relative to the corresponding genes for nystatin, selC and selF each lack two PKS modules, corresponding to the observed four-carbon truncations of selvamicin’s polype and polyol moieties opposite one another.

![Fig. 3.](image-url) Growth inhibition of C. albicans, S. cerevisiae, T. harzianum, and A. fumigatus by selvamicin.
The polyketide backbone of selvamicin can be traced through 14 PKS modules with ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains dictating the oxidation state of each malonyl or methylmalonyl unit (SI Appendix, Figs. S10 and S11). As often observed in type I PKS modules, there are several presumably inactive vestigial domains with mutations and/or truncations at their active sites: a DH and ER in module 13 and a KR in module 11. SelA, the putative PKS loading module for selvamicin’s pro-pionicated starter unit, shares several unusual features with known polyene loading modules (36). Unlike most type I PKS loading modules, SelA is a separate protein distinct from the first elongation module and a serine is found in place of the canonical KS.

The most significant divergence from nystatin’s BGC is a sub-cluster of seven sugar biosynthesis genes, selSI through selSV, found in the middle of the selvamicin BGC. These include a glycosyltransferase gene, selSP, and six genes consistent with 4-O-methyl digitoxose biosynthesis as a TDP-sugar from glucose-1-phosphate (SI Appendix, Fig. S12) (39). The putative 4-O-methyl digitoxose biosynthesis proteins are homologous to a similar suite of proteins responsible for digitoxose biosynthesis in the BGC for jadomycin B in Streptomyces venezuelae ISP5230 (40). However, the selvamicin sugar subcluster contains an additional O-methyltransferase gene (selSI) and lacks an NDP-sugar 4-ketoreductase, which would normally be required for digitoxose formation. Recently, 4-ketoreductase activity has been reported for a bifunctional S-adenosylmethionine-dependent methyltransferase involved in the biosynthesis of milramycin’s sugars (41–43). Similar bifunctional activity could be operative for the SelSI methyltransferase or alternatively this activity could require a separate 4-ketoreductase outside the selvamicin BGC in both the LS1 and LS2 genomes.

This sugar subcluster’s insertion within a cluster of familiar polyene biosynthetic genes fits well with the paradigm of modular subclusters recombining over the course of natural product evolution to generate new products (44). Presumably, a similar suite of genes synthesizes and attaches the digitoxose sugar to nystatin A3, although no such subcluster occurs in the nystatin BGC from Streptomyces noursei. Whole genome sequencing of this Streptomyces strain may eventually reveal the location of these genes. We note that nystatin A1 is a minor product of the nystatin BGC whereas selvamicin is the principal product of the selvamicin cluster. The 4-O-methyl digitoxose subcluster’s incorporation into the selvamicin BGC likely reflects selection for diglycosylation in the principal product. If this subcluster is truly modular, it presents an opportunity for appending 4-O-methyl digitoxose to other polyene scaffolds to create diglycosylated nystatin analogs, which are currently available only as minor products from S. noursei fermentation but have comparable anti-Candida potency to nystatin A1 (18). A solubility boost from an additional sugar would address a major pharmacological limitation of antifungals such as nystatin A1 and amphotericin B.
elution at 50% acetonitrile were most active in inhibition of C. albicans, representing a significant structural departure from the known antifungal polyenes. Preliminary studies also indicate that selvamicin’s atypical structure might be reflected in an unusual mechanism of action. In other ant-associated Pseudonocardia we have reported plasmid-encoded chemical defenses, and circumstantial evidence for the integration of plasmid-encoded biosynthetic pathways into chromosomes (7, 8). However, the variable genomic contexts for selvamicin’s biosynthetic gene cluster—on a plasmid or on a chromosome—provide the most convincing evidence to date for horizontal gene transfer, illustrating an environment in which frequently exchanged plasmids are the platforms for the formation and transmission of a dynamic suite of chemical defenses.

Materials and Methods

Selvamicin Production and Purification. Spores of Pseudonocardia LS1 were diluted into sterile double-distilled water and spread onto plates of ISP2 agar (BD Difco ISP; 60 mL agar per 150 × 15 mm Petri dish) supplemented with sodium butyrate (Aldrich, 150 mM final concentration, added after autoclaving), which were incubated at 30 °C for 14 d. Agar was then cut into squares and soaked in ethyl acetate overnight to extract organic components from the solid media. This extract was decanted and the agar was soaked in an additional volume of ethyl acetate for 3 h. The combined ethyl acetate extracts were concentrated in vacuo and adsorbed onto celite for drying packing onto a 10-g C18 SepPak column (Waters) that had been conditioned with acetonitrile and equilibrated with 30% acetonitrile in water. Fractions were eluted with a step gradient of 30%, 50%, 70%, and 100% acetonitrile in water and concentrated to dryness. Consecutive fractions from elution at 50% acetonitrile were most active in inhibition of C. albicans.

Conclusion

The systematic study of bacterially produced small molecules from well-defined ecological niches continues to uncover potentially useful molecules and to provide examples of the evolution of molecular diversity. Selvamicin, a previously unknown antifungal agent with activity against the human pathogen C. albicans, represents a significant structural departure from the known antifungal polyenes. Preliminary studies also indicate that selvamicin’s typical structure might be reflected in an unusual mechanism of action. In other ant-associated Pseudonocardia we have reported plasmid-encoded chemical defenses, and circumstantial evidence for the integration of plasmid-encoded biosynthetic pathways into chromosomes (7, 8). However, the variable genomic contexts for selvamicin’s biosynthetic gene cluster—one on a plasmid or on a chromosome—provide the most convincing evidence to date for horizontal gene transfer, illustrating an environment in which frequently exchanged plasmids are the platforms for the formation and transmission of a dynamic suite of chemical defenses.

Selvamicin eluted at 12.5 min. The overall yield of pure selvamicin isolated as an amorphous pale-yellow solid was 50 mg/L of agar. Selvamicin; [α]D 25° +128° (MeOH); UV (MeOH) λmax 305 (4.4), 319 (4.7), 334 (4.9), 352 (4.9) nm; NMR spectral data, see SI Appendix, Table S1; HR-ESI-MS m/z 951.4928 [M+Na]+ (calcd for C36H32NaO16; 951.4924).

Determination of Minimum Inhibitory Concentration. Fresh DMSO solutions of mezafungin and nystatin were prepared as serial dilutions and dispensed into clear flat-bottom 96-well plates in 4 replicates. A starting inoculum of the appropriate test strain in media was added to each well to yield a final concentration of 1% DMSO by volume. The plates were incubated at 30 °C with shaking at 200 rpm. Growth was assayed by OD600 readings taken on an MS plate reader (Molecular Devices). For E. coli, B. subtilis, and M. luteus, the starting inoculum consisted of an overnight culture in LB diluted into LB media at 10 μL/mL and final OD readings were taken at 22 h. For C. albicans and S. cerevisiae, the starting inoculum consisted of an overnight culture in yeast extract peptone dextrose (YPD) broth diluted to an OD600 of 0.05 in YPD broth and final OD readings were taken at 14 h. For T. harzianum and A. fumigatus, the starting inoculum consisted of a stock of concentrated conidia diluted into potato dextrose broth at 2 μL/mL and final OD readings were taken at 22 h. Using Prism (GraphPad), the OD data were normalized and fit to a Gompertz function, from which MIC values were extracted as described by Lambert and Pearson (45).

Genome Sequencing and Data Deposition. DNA isolation and genome sequencing was performed as described previously (7). The complete genome for Pseudonocardia LS2 (HH130630-07) has been deposited in the GenBank database (accession nos. CP013854, CP013855, and CP013856) and raw sequence data have been deposited in the Sequence Read Archive (accession no. SRR705179). The Pseudonocardia LS1 (HH130629-09) genome (7) can be accessed using GenBank accession nos. CP011868 and CP011869.

Additional experimental details are available in the SI Appendix, including preparation of Ac3-selvamicin, solubility determination, isothermal calorimetry...
sterol binding assay, induction with propionate and butyrate, and sequence comparisons and analysis.

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