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Article

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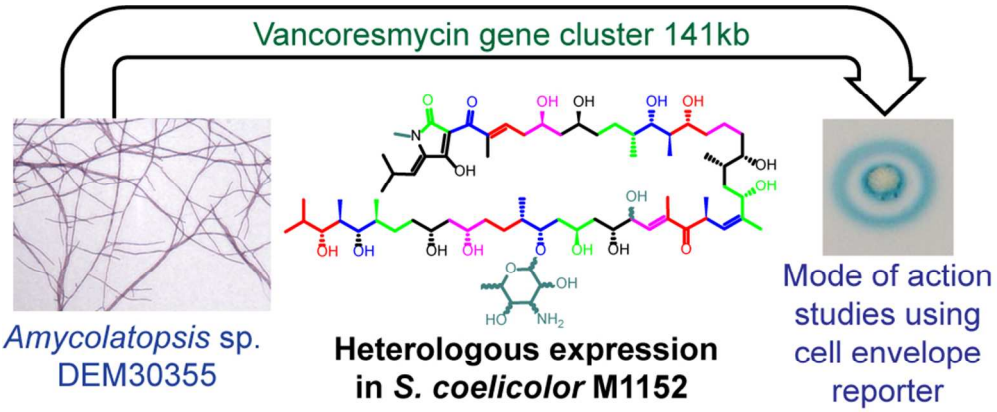

TOC graphic

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Mode of action and heterologous expression of the natural product antibiotic vancoresmycin

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Abstract

Antibiotics that interfere with the bacterial cytoplasmic membrane have long term potential for the treatment of infectious diseases, as this mode of action is anticipated to result in low resistance frequency. Vancoresmycin is an understudied natural product antibiotic consisting of a terminal tetramic acid moiety fused to a linear, highly oxygenated, stereochemically complex polyketide chain. Vancoresmycin shows minimum inhibitory concentrations (MICs) from 0.125 to 2 μg/mL against a range of clinically relevant, antibiotic-resistant Gram-positive bacteria. Through a comprehensive mode-of-action study, utilising *Bacillus subtilis* reporter strains, $DiSC_3(5)$ depolarization assays and fluorescence microscopy, we have shown that vancoresmycin selectively targets the cytoplasmic membrane of Gram-positive bacteria via a non-pore forming, concentration-dependent depolarization mechanism. Whole genome sequencing of the producing strain allowed identification of the 141 kbp gene cluster encoding for vancoresmycin biosynthesis and a preliminary model for its biosynthesis. The size and complex structure of vancoresmycin could confound attempts to generate synthetic analogues. To overcome this problem and facilitate future studies, we identified, cloned and expressed the 141 kbp biosynthetic gene cluster in *Streptomyces coelicolor* M1152. Elucidation of the mode-of-action of vancoresmycin, together with the heterologous expression system will greatly facilitate further studies of this and related molecules.

To combat the growing threat posed by antimicrobial resistance (AMR), there is an urgent need to discover new mode-of-action (MOA) antibiotics that can overcome pre-existing antibiotic resistance mechanisms.[1, 2] The bacterial cell membrane is an essential macromolecular structure that plays a critical role in cellular respiration and in the transport of nutrients and is therefore a promising target for the development of new antibiotics. In addition, it is anticipated that antibiotics that target the intrinsic structure of the bacterial cytoplasmic membrane, rather than having a proteinogenic-target, cannot be easily countered through simple point mutations and would thus exhibit low resistance frequencies. However, only a handful of clinically used antibiotics are known to employ this general MOA (e.g. telavancin and daptomycin).[3-7] The study of antibiotics that interfere with the bacterial cytoplasmic membrane in novel ways is therefore a key area for the development of new antimicrobials refractory to the emergence of resistance.

Vancoresmycin is a structurally unique, large tetramic acid containing polyketide natural product antibiotic, originally isolated from the fermentation broth of the actinomycete *Amycolatopsis* sp. ST 101170, which displays sub-microgram per millilitre minimum inhibitory concentrations (MICs) towards a wide range of Gram-positive pathogens, including vancomycin-resistant *Enterococcus* spp. (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Figure 1).[8]

Initial work on the MOA of vancoresmycin by Henrich *et al.* involved attempts to generate resistant mutants in *Streptococcus pneumoniae*. Exposure of cultures of *S. pneumoniae* to 0.5 μg/mL of vancoresmycin resulted in a low resistance frequency of 2×10^{-7} and only marginal increases in MIC (from 0.4 to 0.5-0.7 μg/mL).[9] Transcriptional profiling of the resultant mutants revealed that the differentially expressed genes mainly encode for proteins which associate or depend on the cytoplasmic membrane for activity.[10]

We have used a wide range of methods to study the mode of action of vancoresmycin and propose that it targets the cytoplasmic membrane of Gram-positive bacteria via a non-pore forming, concentration-dependent depolarization mechanism. To facilitate future development of such a highly complex polyketide natural product, we have also identified and heterologously expressed the

biosynthetic gene cluster in *Streptomyces coelicolor* M1152. Analysis of the gene sequence suggests a probable pathway for the biosynthesis of vancoresmycin.

RESULTS AND DISCUSSION

Isolation of vancoresmycin from *Amycolatopsis* sp. DEM30355. As part of a wider screen for novel and under-characterized antibiotics from the Demuris strain collection[11] we identified *Amycolatopsis* sp. DEM30355 as a producer of vancoresmycin. *Amycolatopsis* sp. DEM30355 was cultivated in ISP2 media in a stirred-tank bioreactor. After 103.5 h, Amberlite™ XAD-16 was added to the bioreactor. Subsequent elution with methanol (MeOH), extraction with ethyl acetate and purification by multi-step chromatography gave access to sufficient quantities of vancoresmycin to allow investigation of its mode of action. Structural confirmation was provided by ESI-MS analysis ($[M+H]^+$ = 1343.8921 m/z; $[M+H_{theo}]^+$= 1343.8926; Δm = 0.37 ppm) and NMR spectroscopy (Supporting Figure S1 and Supporting Table S2).

Vancoresmycin is a potent and selective antibiotic towards Gram-positive pathogenic bacteria. Vancoresmycin was tested against a panel of 25 clinically relevant Gram-positive pathogens, including 15 antibiotic-resistant isolates (Supporting Table S3). Low MICs were observed against 11 MRSA strains (0.25 – 0.5 μg/mL) and 10 VRE strains (0.5 – 1 μg/mL), demonstrating that vancoresmycin is a potent antibiotic against the Gram-positive ESKAPE pathogens. Interestingly no antimicrobial activity was observed against both Gram-negative organisms (*Escherichia coli* NCTC 10418) and Eukaryotic microorganisms (including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* 972 / ATCC 24843).

Vancoresmycin upregulates LiaRS, indicative of cell envelope stress. In an initial assay to identify the mode of action of vancoresmycin, we employed a panel of six *B. subtilis* reporter strains in which a *lacZ* gene is fused to promoters responsive to inhibition of DNA gyrase, cell wall damage, interference with fatty acid synthesis, DNA-damage, RNA polymerase inhibition or cell envelope

stress (Supporting Figure S2).[12-14] Upregulation was only observed with the cell envelope stress (LiaRS) reporter strain. Other antibiotics known to upregulate LiaRS include vancomycin (binding to D-Ala-D-Ala dipeptide of non-cross-linked peptidoglycan)[15], nisin (membrane depolarization via lipid II-mediated pore formation)[16], bacitracin (lipid II biosynthesis or recycling)[17] and tunicamycin (MraY in the lipid II biosynthesis pathway) [18]. Therefore, this result was consistent with a MOA resulting in an increase in cell envelope stress (Figure 2).[14]

Vancoresmycin does not act on the bacterial cell wall. The lack of a cell wall makes L-form strains an interesting model to probe the mode of action of an antibiotic, as compounds that specifically act on cell wall peptidoglycan synthesis or its assembly should not inhibit the proliferation of L-forms.[19] We therefore compared the sensitivity of walled and L-form *B. subtilis* strains to vancoresmycin, to control compounds carbenicillin (cell wall) and nalidixic acid (NAL, gyrase inhibitor), and to carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP, protonophore) (Table 1). As expected, carbenicillin was active only on walled cells because it inhibits cell wall assembly. In contrast, vancoresmycin showed similar activity against both *B. subtillis* strains, as did CCCP and NAL, thus ruling out the cell wall synthetic machinery as the principal cellular target of vancoresmycin.

Vancoresmycin targets the cytoplasmic membrane. Next, we examined the ability of vancoresmycin to interfere with the integrity of the cytoplasmic membrane, through use of the voltage sensitive dye $DiSC_3(5)$. $DiSC_3(5)$ accumulates in well-energized cells, resulting in a quenching of overall fluorescence in the cell suspension. Antibiotics that lead to an increased membrane ion-permeability and, consequently, to a dissipation of membrane potential, trigger a release of the dye into the surrounding medium. This release can be measured as fluorescence dequenching.[20] The ability of vancoresmycin to cause membrane depolarization was tested both at sub-lethal and lethal concentrations (Supporting Figure S3). Even at the highest concentration, no complete membrane depolarization was observed. Instead, the depolarization was gradual, concentration-dependent, and incomplete (Figure 3). These results indicate that vancoresmycin indeed depolarizes the cytoplasmic membrane to a degree that is sufficient to explain the growth inhibition. However, the

observed gradual and partial depolarization is seemingly inconsistent with pore formation, ruling it out as the molecular mechanism by which vancoresmycin kills.

Single cell analysis of membrane depolarization and pore formation. The use of depolarization kinetics to rule out pore-formation *in vivo* has a commonly overlooked caveat: a significant cellular heterogeneity giving full depolarization of a subset of cells, could cause partial depolarization erroneously interpreted as non-pore forming activity. To test this, we developed a microscopic single-cell assay which combines both the voltage-sensitive dye $DiSC_3(5)$, and Sytox Green, a membrane impermeable DNA stain commonly used as a reporter for pore formation (Figure 4).[21] Upon addition of nisin, which assembles into large non-specific pores in the cytoplasmic membrane, a bright Sytox Green staining and a simultaneous homogeneous loss of $DiSC_3(5)$-fluorescence was observed, consistent with membrane depolarization caused by pore formation.[16] In comparison, addition of gramicidin, which forms small cation-specific channels, triggered full and homogeneous depolarization without Sytox Green staining.[22] In contrast to both of these control antimicrobials, vancoresmycin triggered a strikingly heterogeneous dissipation of membrane potential. Crucially, individual cells exhibited partial depolarization levels, thus ruling out pore formation as a mode of action for vancoresmycin. Moreover, no significant uptake of Sytox Green was observed in vancoresmycin-treated cells, again inconsistent with pore formation. Based on these data, we conclude that vancoresmycin kills bacteria by membrane depolarization but does not utilize the common mechanisms of pore or cation-selective channel-formation. Rather, we propose that vancoresmycin causes depolarization by perturbing the membrane bilayer integrity and thereby its tight barrier function. Formally, however, we cannot yet rule out a mode of action based on vancoresmycin acting as an ion carrier. Rather than depolarising the membrane through bilayer perturbations, the observed gradual reduction of membrane potential could also be due to specific inhibition of the respiratory chain. Indeed, a concentration-dependent reduction of *B. subtilis* respiration is observed upon incubation with vancoresmycin (Supporting Figure S5). However, the respiratory chain of *B. subtilis* requires membrane potential for proper activity[23, 24] and the observed

inhibition could, thus, be a consequence rather a cause for the depolarization. Since vancoresmycin retains strong antibacterial activity against both streptococci and enterococci that do not encode a TCA-cycle or a classical respiratory chain, we strongly favour the former option.

Heterologous expression of vancoresmycin. Future development of vancoresmycin would likely involve deriving a structure activity relationship via a range of molecular analogues. However, the large and complex structure of vancoresmycin presents an extreme challenge for chemical synthesis. Therefore, the production of analogues would be reliant on either semi-synthetic modification of the natural product, requiring access to large quantities of material from a reliable heterologous production host, or through genetic manipulation of the biosynthetic cluster. Thus, to facilitate these future studies, we developed a heterologous expression system for vancoresmycin.

The biosynthetic gene cluster responsible for the production of vancoresmycin was identified via whole genome sequencing of *Amycolatopsis* sp. DEM30355, using a combination of PacBio and Illumina sequencing technologies. Assembly of the sequence data produced a draft 9.7 Mb genome in 13 contigs. Bioinformatic analysis (AntiSMASH 4.0) of the draft genome identified 30 potential secondary metabolite gene clusters. Initial analysis of the clusters revealed 10 encoding type 1 polyketide synthase products (PKS). However, only cluster 13 on contig 5 contained sufficient polyketide modules (22) to account for the formation of vancoresmycin.

To test whether this cluster was responsible for the production of vancoresmycin, a high-molecular-weight P1 artificial chromosome (PAC) library was obtained consisting of 2,688 strains with an average insert size of 138 kb, by BioS&T Inc. (detailed information in SI). The entire library was screened by PCR using primers designed to amplify genes located at the start, middle and end of the proposed cluster (var1, var14, var+1). PCR screening identified a single PAC (3-3B9) which had the correct PCR profile. This PAC was cloned into *S. coelicolor* M1152 by intergeneric conjugation and production of antibiotic activity was analysed by plug assay. Of the 15 exconjugants obtained, 9 produced an inhibition zone and the expected blue ($LacZ^+$) reporter gene halo using the *B. subtilis*

LiaRS reporter strain. Culture supernatant of a typical ex-conjugant was semi-purified by SPE and HPLC and subsequently analysed via LC-MS, giving a peak at 9.5 min identical to the mass of vancoresmycin (Figure 5).

This peak was present in extracts from the *Amycolatospis* parent strain DEM30355 but absent from those of the M1152 host strain. The fragmentation pattern of this peak in positive and negative MS mode was consistent with previously reported vancoresmycin data, confirming that PAC 3-3B9 encodes all the genes necessary for production (Supporting Figure S7 and Supporting Figure S8). Additional peaks present in M1152::PAC3-3B9 were, due to their molecular weight, fragmentation pattern and absence in the native host strain, proposed to be intermediates in biosynthesis or shunt metabolites (Supporting Figure S9).

To confirm the sequence of the gene cluster producing vancoresmycin, the heterologous host M1152::PAC3-3B9 was sequenced using Illumina MiSeq and found to contain a 184 kb insert which contained Cluster 13. At 141 kb, the vancoresmycin biosynthetic gene cluster is, to our knowledge, the largest antibiotic gene cluster heterologously expressed to date.[25]

Gene cluster assignment and proposed biosynthetic pathway. The, 141 kb pks1/nrps, vancoresmycin biosynthetic gene cluster contains thirty eight genes. Including eight type1 pks genes that span the majority (108 kb) of the cluster (Figure 6a). BlastP analysis identified the remaining genes as being involved in peptide biosynthesis, late stage tailoring reactions and mycosamine biosynthesis (Supporting Table S4). Sequence domain analysis of the eight type1 pks genes revealed a total of 101 enzymatic domains organised into 23 modules (Figure 6b)[26], with a single non-functional ketoreductase (KR) in module 11. We propose the biosynthesis of the polyketide component of vancoresmycin starts at *var13* through the loading of an isobutyrate unit, followed by condensation of 10 malonyl-CoA and 13 methylmalonyl-CoA units.

Based on analogy to previous studies examining the biosynthesis of tetramic acid containing natural products in both bacteria[27, 28] and fungi[29, 30], we suggest that the tetramic acid moiety is derived from leucine[31] which is *N*-methylated by methyltransferase (*var17*), condensed with the polyketide

backbone (mediated by either *var16* or *var31*), and finally cyclised (potentially mediated by *var18*).[32] Subsequent tailoring steps and putative enzymes include: (1) hydroxylation of *C*-26 (*var19*); (2) attachment of mycosamine (*var24* or *var32*); (3) a non-enzymatically controlled ketoenol tautomerisation; (4) reduction of the tetramic acid side chain (*var14*). The absolute stereochemistry of vancoresmycin is unknown, but we can propose a stereochemical assignment based on domain analysis of the KR domains (Figure 6C).[33]

CONCLUSIONS

In summary, we propose that the primary MOA of vancoresmycin involves a non-pore-forming depolarization of the bacterial membrane and we speculate that vancoresmycin causes this depolarization by perturbing the membrane bilayer integrity. To aid future development of this antibiotic, a heterologous expression system for biosynthetic cluster of vancoresmycin was constructed in *S. coelicolor* M1152. At 141 kb, it may be the largest gene cluster to be expressed in a surrogate host and is to our knowledge the first heterologously produced antibiotic from the genus *Amycolatopsis.* This study will hopefully pave the way for future development of vancoresmycin derivatives and other antibiotics from the genus *Amycolatopsis.*

MATERIALS AND METHODS

Fermentation of DEM30355 and purification of vancoresmycin. *Amycolatopsis* sp. DEM30355 was cultivated for 103.5 h at 30 °C in ISP2 media supplemented with glycerol (0.4% yeast extract, 1% malt extract, 0.4% glucose and 1% glycerol) at a 500 L scale. The cell mass was removed by centrifugation (disc stack centrifuge, Satorious) and subsequent filtration (Satorious depth filters). 10 kg of Amberlite™ XAD-16 resin was washed with deionised water and then applied to the filtered broth in batch absorption. The material was eluted off the beads with 40 L of 100% MeOH and concentrated under reduced pressure to an aqueous residue. The concentrate was adjusted to pH 4 using aqueous sulphuric acid and extracted twice with ethyl acetate (EtOAc) at equal volume. The organic extracts were combined and evaporated to dryness under reduced pressure to yield 231.11 g of crude extract.

A sample of DEM30355 crude extract (5.335 g) was triturated in water (100 mL, acidified to pH 4 with 2 M HCl aqueous solution) and EtOAc (100 mL). The insoluble material (1.338 g) was isolated by filtration then subjected to silica gel chromatography (eluent = linear gradient from 100% dichloromethane (DCM) to 100% MeOH, Biotage® SNAP 50 g cartridge). Vancoresmycin containing fractions were combined and the solvent was removed under reduced pressure to yield 0.555 g of material. This material was subjected to silica gel chromatography (eluent = linear gradient from 90% DCM / 10% MeOH, to 100% MeOH, Biotage® SNAP 50 g cartridge). Vancoresmycin containing fractions were combined and the solvent was removed under reduced pressure to yield 0.181 g of material. This material was subjected to size exclusion chromatography (eluent = MeOH, Sephadex LH-20). The vancoresmycin containing fractions were combined and the solvent was removed under reduced pressure to yield 70 mg of material. A portion (48 mg) of this material was dissolved in MeOH (0.3 mL) and subjected to reversed-phase chromatography (eluent = linear gradient from 60% water/ 40% acetonitrile (MeCN) to 100 % MeCN, Biotage® SNAP Ultra C18 12 g cartridge). The organic solvent was removed under reduced pressure and dried by lyophilisation, to yield vancoresmycin (18 mg) as a white amorphous solid resulting in an approximate titre of 2.3 mg/L.

MIC determination against a panel of bacterial isolates. Broth microdilution was performed in accordance with ISO 20776-1:2006 (ISO, 2006), against a collection of 30 bacterial isolates. The collection included 10 isolates acquired from the National Collection of Type Cultures (NCTC, Colindale, UK), 10 MRSA strains frequently encountered in Europe and 10 clinically isolated VRE.

Vancoresmycin was prepared at a stock concentration of 10 mg/mL in 100% DMSO, which was tested in Mueller-Hinton broth at a concentration range of 0.004 to 4 µg/mL, and inoculated with a final concentration of 5 x 10^5 CFU/mL of each isolate. An inhibitor-free control was also included. Incubation occurred at 37 ± 0.5 °C for 18 h.

The MIC was defined as the lowest concentration of vancoresmycin inhibiting growth after overnight incubation and determined spectrophotometrically at 640 nm. A spectrophotometric reading of ≤0.01, after deduction of the background absorbance, was the cut-off for inhibition. All suspensions showing inhibition were subcultured onto blood agar and incubated for 18 h at 37 ± 0.5 °C. Colony counts were compared to a cut-off for bactericidal activity, determined using the calculation: initial inoculum x aliquot plated x allowable viable percent. The minimum bactericidal concentration (MBC) was defined as the lowest vancoresmycin concentration eliciting bactericidal activity after subculture onto an antibiotic-free medium.

Reporter strain panel. A Kirby-Bauer disc diffusion assay was performed with the *B. subtilis* reporter strain panel, gyrA, ypuA, fabHA, ϕ105, helD and liaI [12-14]. The nutrient agar was supplemented with X-Gal (100 µg/mL).

Inhibition assay against cell wall deficient bacteria (L-form). Rod shaped cells were maintained in NB supplemented with 1 mM ITPG while the L-form strain was maintained in NB/MSM as previously reported.[19]

The antibiotics nalidixic acid, CCCP, vancoresmycin and carbenicillin were diluted in NB/MSM. The walled cell culture was diluted to a final OD_{600nm} of 0.005 and grown under shaking in NB/MSM supplemented with 1 mM ITPG overnight at 37 °C, before visual inspection of the growth. A dense L-form culture (2 days growth) was diluted 1:100 and incubated without shaking in the presence of compounds at 30 °C for 72 h before visual inspection of growth.

Membrane depolarization assay. Vancoresmycin-triggered changes in cell membrane potential levels were measured using the voltage-sensitive dye $DiSC_3(5)$ (AnaSpec) as described earlier [20]. In brief, *B. subtilis* 168CA was grown to an OD_{600} of 0.2 in LB medium at 37 °C while shaking. The cells were pelleted and re-suspended in pre-warmed LB containing 10 µg/mL chloramphenicol and 0.5 mg/mL bovine serum albumin (BSA) (Sigma-Aldridge), and 150 µL aliquots of the cell suspension were transferred in triplicates to a black flat bottomed 96-well plate (Greiner Bio-One). The cell suspensions were incubated for 6 min in the presence of the $DiSC_3(5)$ (final concentration of 0.5 µM and 1% DMSO) to obtain a baseline, followed by addition of vancoresmycin in final concentrations of 200 ng/mL, 100 ng/mL, 50 ng/mL, and 25 ng/mL. Fluorescence measurements were taken every minute with a Fluostar Optima fluorimeter (BMG) using 610/10 nm excitation, and 660/10 emission filters.

$DiSC_3(5)$ and Sytox Green combined microscopy. *B. subtilis* 168CA was grown to an OD_{600} of 0.2 in LB medium at 37 °C while shaking, followed by simultaneous staining with 2 µM $DiSC_3(5)$ and 50 nM Sytox Green for 5 min at 37 °C. The ability vancoresmycin to depolarize individual cells and to trigger influx of Sytox Green as an indication for pore formation, were assayed by addition of vancoresmycin to a final concentration of 100 ng/mL for 1 min, followed by transfer to microscopy slides covered with 1.2% agarose in water and fluorescence imaging. As a positive control for channel-mediated depolarization without pore formation, cells were incubated for 1 min with gramicidin (mix of gramicidin A, B, C, and D, final concentration of 5 µg/mL).[22] As a positive control for pore formation, cells were incubated for 1 min with 10 µM of the pore-forming lantibiotic, nisin.[16] Microscopy was carried out with Nikon Eclipse Ti (Nikon Plan Apo 1.40 Oil Ph3 objective) and the images acquired with Prime 4.2 sCMOS camera (Photometrics) and Metamorph 7 (Molecular Devices). Quantification of the fluorescent intensity of $DiSC_3(5)$ and Sytox Green for individual cells was carried out in ImageJ v.1.48 (National Institutes of Health). ROI's were determined manually for each cell in an image field (n=156-202) by drawing a line with an appropriate pixel width along the longitudinal axis of the cells, and by measuring the average pixel intensity.

Resazurin assay. The active respiration of *B. subtilis* was assessed by following redox-dependent conversion of cell-permeable resazurin into fluorescent resorufin using commercially available alamarBlue™ Cell Viability Reagent following manufacturers' instructions (Thermo Fisher Scientific). In brief, *B. subtilis* 168CA was grown to an OD_{600} of 0.2 in LB medium at 37 °C while shaking, followed by transfer to a microtiter plate as 100 µl aliquots, and incubation with 5 µl of the alamarBlue™ reagent for 60 min upon shaking at 37 °C. The formation of resorufin was detected with BMG Clariostar fluorimeter as an increase in fluorescence at 595±5 nm upon excitation with 550±5 nm light. As positive controls, cells heat-inactivated at 85 °C for 15 min, and cells depleted for O_2 by flushing with argon for 45 min were measured in parallel to cells treated with vancoresmycin. To prevent cell growth affecting the measurement, all samples were growth-inhibited by addition of 50 µg/ml chloramphenicol.

DNA sequencing and bioinformatic analysis. High molecular chromosomal DNA of *Amycolatopsis* DEM30355 was extracted according to standard procedures.[34] DNA sequencing was performed using Pacific Biosciences (PacBio) and Illumina (GATC) technology. Contigs were assembled using the SMRT portal of PacBio. The annotation of open reading frames (ORFs) and assignment of putative gene functions were done using a combination of RAST and blastP.[35, 36] Putative NP-encoding gene clusters were identified using antiSMASH3.[37]

The nucleotide sequence of the vancoresmycin cluster was deposited in the NCBI database with the accession number (*will be submitted upon acceptance*).

PAC library construction and PCR screening for the vancoresmycin gene cluster. A genomic library of *Amycolatopsis* strain DEM30355 DNA was constructed in pESAC13 (by BioS&T Inc.). High molecular weight DNA was partially digested with Sau3AI and ligated with *Bam*HI-digested pESAC13, resulting in loss of the pUC19 component present in the original vector. The supplied genomic library was screened for clones containing the predicted vancoresmycin biosynthesis gene cluster, by PCR against several genes across the full length of the cluster (see Supporting Table S6 for targets and primer sequences). PCR amplification was performed using Herculase II Fusion according to manufacturer's protocols. This resulted in one PAC clone (3-3B9) being identified which was transferred together with pR9604 into *E.coli* ET1256. We subsequently conjugated the plasmid pEASAC13_3-3B9 into *S. coelicolor* M1152 as described previously.[38]

Analysis of heterologous expression. The parent strain M1152 and the vancoresmycin cluster containing heterologous host M1152::3-3B9 were grown for 5 days at 30 °C on ISP2 plates (5 plates in total) (0.4% yeast extract, 1% malt extract, 0.4% glucose and 1% agar). The plates were homogenized using a syringe, freeze-thawed and subsequently extracted with 100 mL of acetone. The solid particles were removed via filtration and the resulting liquid was concentrated under reduced pressure. The extracts were loaded onto a Thermo Scientific™ HyperSep™ C18 Cartridge and the vancoresmycin containing fraction at 75-100% MeOH (M1152::3-3B9) and corresponding fraction from M1152 were collected. Subsequently these extracts were purified using an Agilent 1260 HPLC system equipped with a Phenomenex Synergy 4 µm 150 x 4.6mm column with a Hichrom C18 guard column. Mobile solvent systems were water buffered with 0.1% (vol/vol) formic acid (FA) (solvent A) and MeCN buffered with 0.1 % FA (solvent B). The area between 22 min to 30 min was collected. These fractions were injected into a LC-MS (Bruker micrOTOF). The LC consists of an Agilent 1260 HPLC system equipped with a Zorbax Eclipse Plus column (3.5 µm 100 x 4.6 mm).

Comparative analysis of vancoresmycin production in *Amycolatopsis* isolate DEM30355 vs. *S. coelicolor* M1152::3-3B9. The *Amycolatopsis* isolate DEM30355 and the heterologous host *S. coelicolor* M1152::3-3B9 were grown on eight ISP2 plates at 30 °C on ISP2 plates until the onset of vancoresmycin production. The plates were homogenized using a syringe and subsequently freeze-thawed. 50 mL of methanol was added, solid particles were removed via filtration and the resulting liquid was concentrated under reduced pressure to give 150 mL of plate extract. Each extract was loaded onto a Thermo Scientific™ HyperSep™ C18 Cartridge and the vancoresmycin containing fractions were collected and analysed on a LC-MS (Bruker micrOTOF, Agilent 1260 HPLC system, Zorbax Eclipse Plus column (3.5 µm 100 x 4.6 mm)), calibrated against vancoresmycin standards. The *Amycolatopsis* isolate DEM30355 produced 2.7 mg/L vancoresmycin while the heterologous host *S. coelicolor* M1152::3-3B9 had a vancoresmycin titre of 2.2 mg/L of agar.

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ASSOCIATATED CONTENT

The supporting Information is available free of charge on the ACS Publications website at DOI: *to be assigned.*

Microorganisms used; Mass spectrum and NMR data of vancoresmycin; Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of vancoresmycin; Reporter panel analysis of vancoresmycin; Concentration-dependent growth inhibition of *B. subtilis* by vancoresmycin; Calibration of $DiSC_3(5)$ assay; Concentration-dependent inhibition of *B. subtilis* respiratory chain activity by vancoresmycin; Postulated protein function in the vancoresmycin gene cluster based on BlastP homology search; Oligonucleotides used in the screening for the vancoresmycin cluster; HPLC fractionation of heterologous host and wild-type; Fragmentation pattern of the heterologously produced vancoresmycin in positive and negative mode; LC-MS extracted ion chromatogram of vancoresmycin; Mass and fragments of additional peaks in M1152::3-3B9 fraction.

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Table 1. Comparison of antibiotic activities on walled and L-form strains of *B. subtilis*.

Antibiotic	MIC (µg/ml)*		Ratio L-form
	L -form	Walled	/ walled
Vancoresmycin	0.05	0.025	2
CCCP	2.6	2.6	1
NAL	25	12.5	2
Carbenicillin	>100	2.5	>40

* Osmoprotective conditions to enable L-form growth.

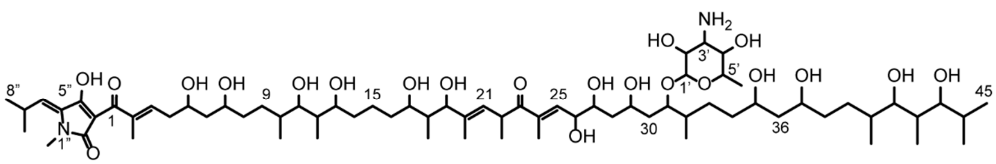


Figure 1. Structure of Vancoresmycin

Figure 2. Analysis of the mode of action of vancoresmycin using the cell envelope reporter *liaI-lacZ* in a disc diffusion assay. This *B. subtilis* reporter strain has the promoter of *lia* fused to *lacZ*, so that expression of *liaI* can be visualised by a blue colour in the presence of X-Gal. A blue halo surrounding a zone of inhibition indicates that the compound is inhibiting some aspect of cell envelope synthesis. Discs impregnated as follows (1) vancoresmycin 1 µg, (2) nisin 200 µg, (3) bacitracin 500 µg, (4) tunicamycin 10 µg, (5) vancomycin 1.5 µg, (6) tetracycline 10 µg.

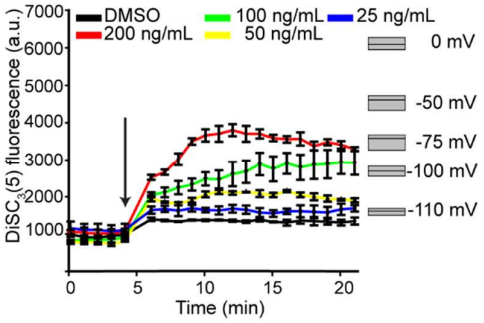


Figure 3. Dissipation of membrane potential triggered by vancoresmycin. *B. subtilis* membrane potential levels were quantified following addition of different levels of vancoresmycin using the fluorescent voltage-sensitive dye $DiSC_3(5)$. The time point of antibiotic addition is indicated with an arrow. The graph depicts the average and standard deviation of three technical replicates. The calibration depicted to the right represents the mean and the 95% confidence interval of $DiSC_3(5)$-fluorescence in cells with pre-defined membrane potential levels (Supporting Figure 5).

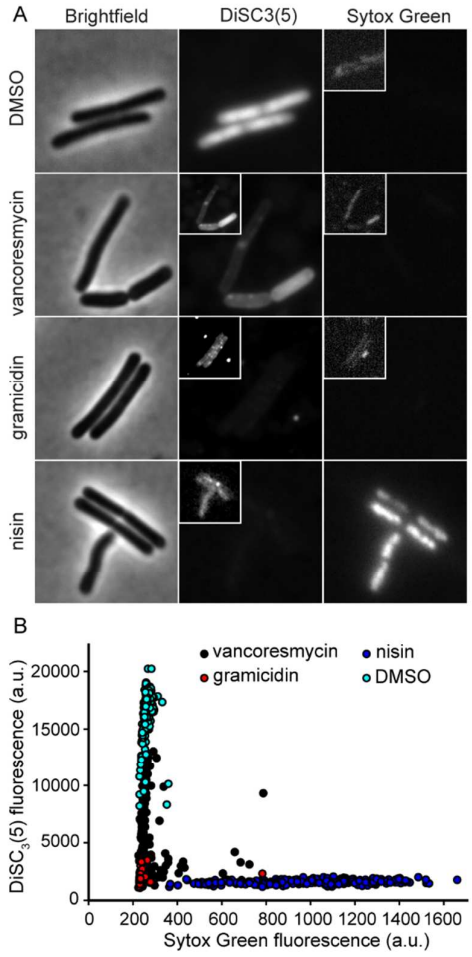


Figure 4. Single-cell measurement of membrane potential and permeability. (A) Phase contrast (left panels) and fluorescence microscopy of *B. subtilis* cells stained with the voltage-sensitive dye $DiSC_3(5)$ (middle panels), and the membrane permeability-indicator Sytox Green (right panels) in the presence and absence of 100 ng/mL vancoresmycin. As positive controls, the cells were treated with 5 µg/mL gramicidin (membrane depolarization without pore formation), and 10 µM nisin (membrane depolarization through pore formation). The large fluorescence images depict the cells with identical contrast settings. In the smaller inserts the contrast of the fluorescence image is increased to reveal weakly stained cells. (B) The cellular $DiSC_3(5)$ and Sytox Green fluorescence values were quantified for cells treated with vancoresmycin (100 ng/mL), gramicidin (5 µg/mL), and nisin (10 µM) The scatter plot depicts the fluorescence intensity values of individual cells (n = 112) for both dyes. Note that, unlike in the fluorimetric measurement based on fluorescence quenching, high cellular $DiSC_3(5)$-fluorescence indicates high membrane potential.

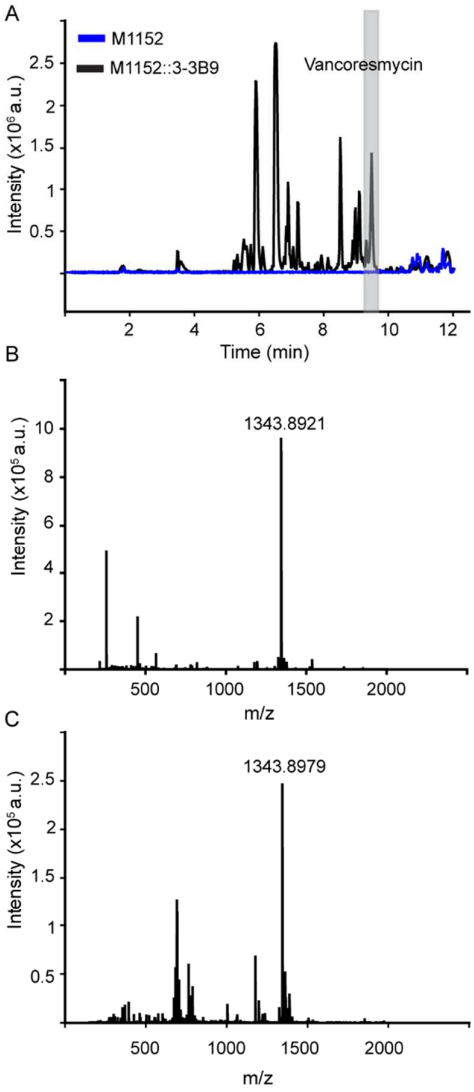


Figure 5. Detection of vancoresmycin in the heterologous host M1152. (A) Plate extracts of *S. coelicolor* M1152 and *S. coelicolor* M1152::3-3B9 were fractionated via SPE. The vancoresmycin containing fraction 75-100% MeOH and corresponding fraction from wild type M1152 were injected into a C18 reverse phase column (Supplementary Figure 6). The area of 22min-30min was collected and injected into a LC-MS system. The peak highlighted in grey corresponded to the mass expected for vancoresmycin. (B) MS spectrum of vancoresmycin purified from the native host *Amycolatopsis* DEM30355. (C) MS spectrum of vancoresmycin purified from the heterologous host *S. coelicolor* M1152::3-3B9

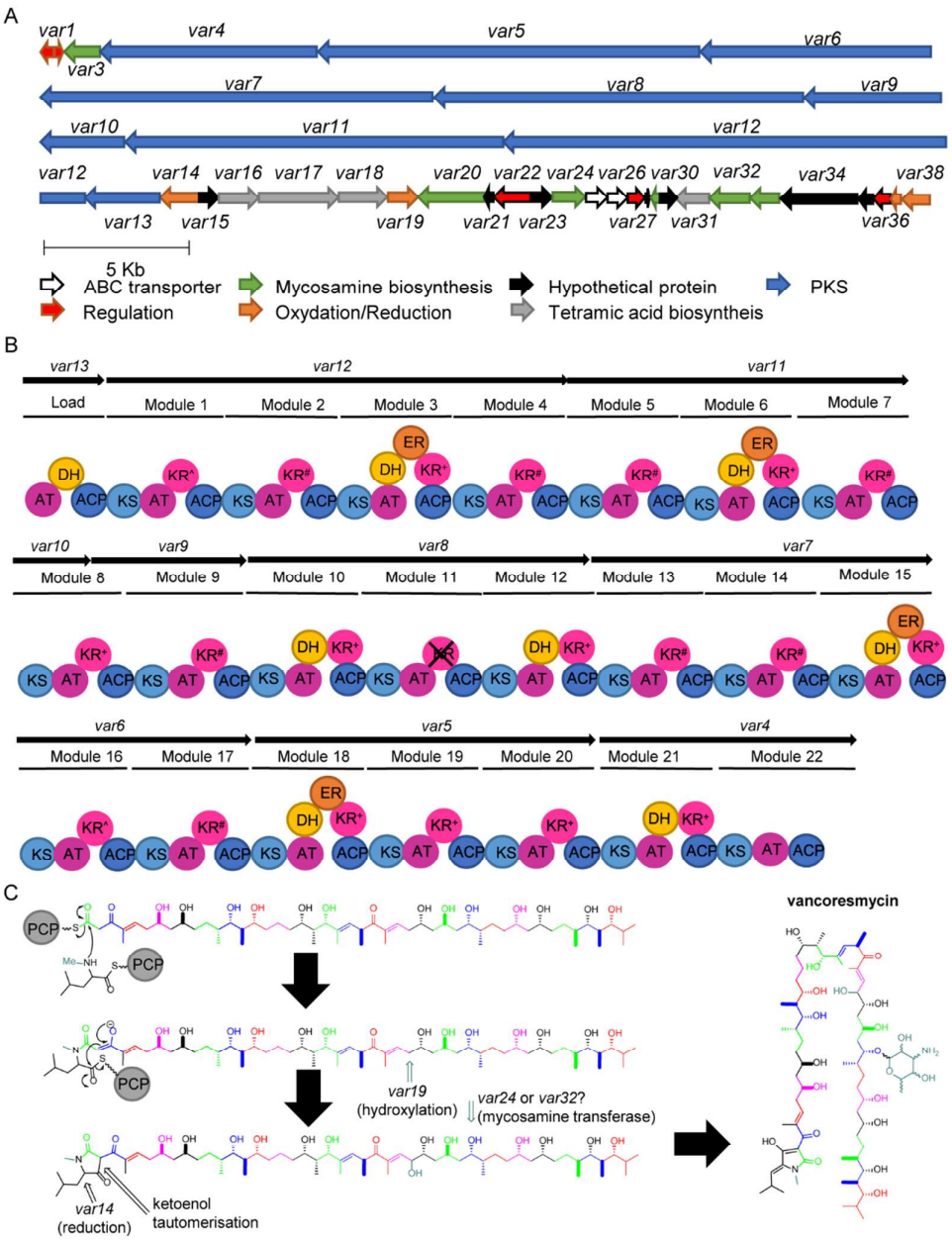
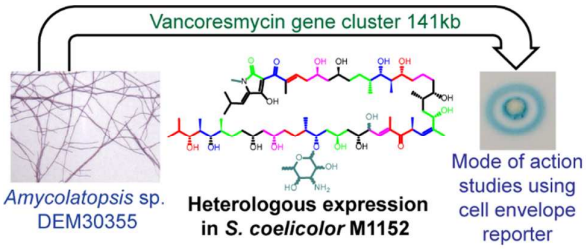


Figure 6. Vancoresmycin gene cluster and bioinformatic analysis. (A) Organization of the vancoresmycin biosynthetic gene cluster. (B) Module and domain organization of the PKS encoded by the cluster. The ketoreductase domain in module 11 is predicted to be non-functional due to modifications in the catalytic region. ACP, acyl carrier protein; DH, deydratase; ER, enoylreductase; KR*, ketoreductase predicted to generate a 2*R*, 3*R*-acyl thioester intermediate; KR^{+}, ketoreductase predicted to generate a 2*R*, 3*S*-acyl thioester intermediate; KR$^{\#}$, ketoreductase predicted to generate

a 2*S*, 3*S*-acyl thioester intermediate; KR^, ketoreductase predicted to generate a 2*S*, 3*R*-acyl thioester intermediate. (C) Predicted structure of the full assembled pks intermediate attached to a peptidyl carrier protein (PCP) and following tailoring steps to give the fully synthesized vancoresmycin.



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