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Coronamycins, peptide antibiotics produced by a verticillate *Streptomyces* sp. (MSU-2110) endophytic on *Monstera* sp.

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Coronamycin is a complex of novel peptide antibiotics with activity against pythiaceous fungi and the human fungal pathogen *Cryptococcus neoformans*. It is also active against the malarial parasite, *Plasmodium falciparum*, with an IC_{50} of 9·0 ng ml^{-1}. Coronamycin is produced by a verticillate *Streptomyces* sp. isolated as an endophyte from an epiphytic vine, *Monstera* sp., found in the Manu region of the upper Amazon of Peru. Bioassay-guided fractionation of the fermentation broths of this endophyte on silica gel and HPLC chromatography yielded two principal, inseparable, peptides with masses of 1217·9 and 1203·8 Da. Three other minor, but related components, are also present in the preparation. Amino acid analysis of coronamycin revealed residues of component 1, component 2, methionine, tyrosine and leucine at a ratio of 2 : 2 : 1 : 1 : 3. Other compounds with antifungal activities are also produced by this endophytic streptomycete.

INTRODUCTION

A widely accepted definition of endophytes is that they are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects (Bacon & White, 2000). While the symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens (Bacon & White, 2000). The most frequently isolated endophytes are fungi (Bacon & White, 2000), but bacteria also form endophytic relationships. The vast majority of plants have not been studied for the endophytes that may inhabit them. Endophytes are likely to be a rich and reliable source of genetic diversity and biological novelty (Bacon & White, 2000), and experience has shown that novel endophytic microbes usually produce novel natural products (Strobel, 2002, 2003).

One of the most promising endophytic micro-organisms to be isolated would be an actinomycete, or specifically a streptomycete, since these organisms often produce antibiotics. Recently, one of the first antibiotic-producing endophytic streptomyctes was isolated from a medicinal plant (snakevine, *Kennedia nigriscans*) used by Aboriginal Australians to treat and dress bleeding wounds (Castillo *et al.*, 2002). The snakevine consistently yielded a yellowish-orange culture of *Streptomyces* sp. which was subsequently demonstrated to produce a unique family of functionalized peptide antibiotics, the munumbicins (Castillo *et al.*, 2002). The rationale to direct a study of the snakevine plant was related to the traditional ethnobotanical knowledge of Aboriginal people. Other streptomycetes may be found as endophytes in other higher plants, and ethnobotanical or other approaches could be used to find them.

Plants growing in areas of great biodiversity have the prospect of harbouring endophytes with great biodiversity (Strobel, 2003). The Manu region of the upper Amazon in Peru is a worthy place to search for interesting and useful endophytic micro-organisms since it is one of the most biologically diverse places on earth. The search is driven

The GenBank accession number for the sequence reported in this paper is AY327845.

by the need to find and develop new products for medicine and agriculture (Strobel, 2003). Herein, we report recovery of what we believe to be the first verticillate endophytic *Streptomyces* sp. from any higher plant. This actinomycete was recovered from a small epiphytic vine commonly known locally in the Manu region as 'follow-me vine' (*Monstera* sp.). This endophytic micro-organism demonstrated antimicrobial activity and thus it was subjected to further study to determine the amount, nature and biological activity of the components in its fermentation broth. This report describes the isolation and activity spectrum of the novel biologically active compounds produced by this organism.

METHODS

Isolation of endophytes. Stems (about 0·2–0·4 cm in diameter) of *Monstera* sp. were obtained in the Lake Sandoval area of the Bahuaja Sonene Park Nacional in the upper Amazon region of Peru at 12° 36′ 25′′ S, 69° 01′ 54′′ W. The small stems of this epiphytic plant were thoroughly treated with 70 % ethanol, the outer bark was carefully removed with a sterilized sharp blade, and then endophytic micro-organisms were isolated on water agar according to methods described earlier (Castillo *et al.*, 2002). One endophytic micro-organism was of primary interest, because of its activity against the plant-pathogenic fungus *Pythium ultimum*. This organism, initially designated as isolate P-25-2-4, was deposited in the Montana State University Mycological (MONT) culture collection as *Streptomyces* MSU-2110. Small pieces of PDA (potato dextrose agar), mostly containing spores, were stored in 15 % (v/v) glycerol at −70 °C.

Microscopic techniques. Scanning electron microscopy was performed on MSU-2110 by placing agar pieces and γ-irradiated carnation pieces supporting bacterial growth into packets of Whatman no. 1 filter paper, made by folding the filter paper over a piece of cork (1·5 cm). The packets were tied with cotton string and two removable split shot sinkers (approx. 3·25 g each) were attached to the packets to hold them under the surface of the dehydrating solutions and the liquid CO_2 during critical-point drying. The preparation was then processed through buffer and alcohol solutions as previously described (Castillo *et al.*, 2002). The dehydration process was done slowly to prevent spore shrivelling, which may occur during rapid dehydration. Ultimately, the biological material was dried, sputter-coated with gold and examined with a JEOL 6100 scanning electron microscope.

Because the spores and sporophores of the bacterium appeared fragile and easily disrupted, the organism was subjected to the relatively new technique of environmental scanning microscopy, which requires no coating of the specimen with conducting material, and preserved the sporophore intact (spores attached). Fresh specimens were examined by and images were recorded with a Philips XL 30 ESEM FEG. A gaseous secondary electron detector was used with a spot size of 3, at 15 kV. The temperature was 4 °C with a chamber pressure which ranged from 5 to 6 torr providing humidity up to 100 % at the sample.

DNA isolation, amplification of 16S rDNA, cloning, and sequencing. Isolate P-25-2-4 was grown on PDA in a 9 cm Petri plate for 14 days at 23 °C. The colonies were scraped directly from the surface of the agar culture. Extraction of DNA was done with Qiagen's DNeasy mini kit according to the manufacturer's instructions.

A partial 16S rDNA fragment of about 920 bp was amplified from genomic DNA of isolate P-25-2-4 by PCR using the bacterial primers 16S-bact-27f (5′-AGA-GTT-TGA-TCM-TGG-CTC-AG-3′) and 16S-bact- 907r (5′-CCG-TCA-ATT-CMT-TTR-AGT-TT-3′) (Lane, 1991). The reaction was performed in a 25 µl final volume containing 0·1 µg genomic DNA, 15 mM of each primer, 10 mM of the four dNTPs and 1·25 units Nova*Taq* polymerase (Novagen) in *Taq* buffer (Novagen) containing 15 mM magnesium chloride. The following cycle parameters were maintained: 95 °C for 5 min followed by 34 cycles of 40 s at 95 °C, 40 s at 50 °C and 40 s at 72 °C, followed by 5 min at 72 °C. The PCR product was purified and desalted using the QIAquick PCR purification kit (Qiagen).

The PCR product was cloned into a pGem-T easy vector (Promega) according to the manufacturer's instructions. Transformation of the cloned PCR product into *Escherichia coli* DH5α was performed as previously described (Stinson *et al.*, 2003). The transformed cells were plated on LB agar supplemented with 30 µg ampicillin ml^{-1}, in the presence of IPTG and X-Gal for blue/white selection. White single colonies were grown in LB broth and DNA was extracted using a Perfectprep Plasmid Mini Kit (Eppendorf) according to the manufacturer's instructions. Presence of the insert was confirmed by DNA digestion with *Eco*RI (Promega).

The plasmid inserts were sequenced by the Plant-Microbe Genomics Facility at Ohio State University using an Applied Biosystems 3700 DNA Analyser and BigDye cycle sequencing terminator chemistry and the universal primers T7 and Sp6 designed for sequencing from pGem-T easy.

Coronamycin isolation procedures. *Streptomyces* sp. MSU-2110 was grown on PDA plates for at least 7 days at 23 °C. One quarter of the agar plate was used to inoculate 1 litre of Potato Sucrose Natural Broth (PSNB) medium in a 2 litre flask and the flask was left standing at 25 °C for 3–4 weeks. PSNB is a high-nutrient medium with sucrose and natural potato pellets as carbon source. It is prepared with 20 g sucrose and 15 g potato pellets (Basic American) in 1 l double-distilled H_2O. Thick pink-purple-brown layers of the organism developed on the surface of the liquid after 2–3 weeks.

To extract the secondary metabolites, the culture was filtered through two layers of cheesecloth and the filtrate was extracted three times, each with 0·5 vol. methylene chloride. The organic solvent was pooled and dried under flash evaporation at 40 °C. The yield of dried residue was about 150 mg l^{-1}.

The dried residue was dissolved in 5 ml chloroform and applied to a 3 × 15 cm column of Selecto silica gel (32–63 particle size; Selecto Scientific). The column was first rinsed with at least 200 ml chloroform followed by a series of 100 ml chloroform/methanol (v/v) mixtures in the following order: 200 : 1, 100 : 1, 50 : 1, 10 : 1, 9 : 1, 5 : 1, 4 : 1, 3 : 1, 2·5 : 1. Each fraction, after solvent evaporation, was tested for biological activity against *Pythium ultimum*. The last fraction (no. 9) was the only active fraction. Approximately 1 mg of material recovered from fraction 9 was subjected to reverse-phase HPLC on a Varian-Microsorb-mv 100 Å 250 × 10 mm RPC-18 column. Gradient elution was performed using a methanol/water/0·1 % formic acid solvent system at a flow rate of 0·5 ml min^{-1}, starting with 60 % (v/v) methanol and increasing the methanol concentration linearly over 60 min to a final value of 100 %. The elution process was monitored at 280 nm. The major bioactive fraction eluted at 40 min. The bioactive material was subjected to further purification by an additional HPLC step, with the same column and solvents, but with UV monitoring at 220 nm on an HPLC program in which the methanol concentration was adjusted from 60 % to 65 % over 30 min, held at 65 % for 60 min and then raised to 100 % to wash the column. A biologically active peak eluted at 28 min. This fraction was concentrated and then subjected to a final HPLC step utilizing a Waters Symmetry 4·6 × 150 mm, 3·5 µm RPC-18 column under the same conditions as in the previous stage. A biologically active fraction eluted at 36·43 min. Approximately 0·2 mg of this product was obtained per litre of starting bacterial culture fluid.

Bioassays in plate wells. Fractions from the silica gel and HPLC columns were assayed for antifungal activities by dissolving known amounts of the material from each fraction in methanol and spotting the fluid into depressions of a 24-well test plate. After evaporation of the solvent, 1 ml potato dextrose broth (PDB) was added to the wells and a $2 \times 3 \times 3$ mm block of PDA harbouring the fungus *Pythium ultimum* was placed into each well and then monitored for growth. Appropriate controls, without exposure to the column fractions, were performed during the course of each experiment.

Various plant-pathogenic fungi were subjected to coronamycin treatment in order to obtain minimal inhibitory concentration values (MICs at 48 h) as the first well in the plate showing no growth, with organisms and methods as previously outlined (Castillo *et al.*, 2002). Dr John Menge of the Department of Plant Pathology, University of California, Riverside, provided a culture of *Phytophthora cinnamomi*. All other plant pathogens were obtained from the MSU mycological collection.

Microbroth dilution assays of human-pathogenic yeasts were performed as described in the NCCLS M27A (NCCLS, 1997) with the addition of Alamar Blue (Trek Diagnostics Systems) as a colorimetric indicator of viability (Espinel-Ingroff *et al.*, 1999). The MIC was defined as that concentration of compound resulting in no visible growth of the test organism after 24–48 h incubation, with the exception of *Cryptococcus neoformans*, which was read at 72 h (Castillo *et al.*, 2002). Flucytosine was included as a control antifungal compound.

Bioassays on disks. Coronamycin was tested by disk diffusion at 20 μg per disk against a representative panel of human-pathogenic bacteria as described by Castillo *et al.* (2002). This panel included *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 10031), *Enterococcus faecium* (ATCC 49624), *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* VRE (ATCC 51299) and *Streptococcus pneumoniae* (ATCC 10031).

Human cell line assays. Coronamycin was tested against human cancer cell line BT20 (breast epithelial cancer, ATCC) and human primary mammary epithelial cells (HMECs) (Clonetics) using a CellTitre 96 Aqueous Non-Radioactive Cell Proliferation Assay kit from Promega. The BT20 cells were grown in the medium recommended by ATCC and were seeded into 96-well clear flat-bottom plates at 3000 cells per well in 100 μl. The primary mammary cells were cultured as recommended by the manufacturer in mammary epithelial cell growth medium (MEGM) and were seeded at 3200 cells per well in 100 μl. After seeding, the cells were incubated for 5 h at 37 °C before addition of coronamycin. Twofold serial dilutions of the coronamycin were made in the culture medium, and a volume equal to that of the seeded wells for each dilution was added to the cells. Each plate also contained wells with cells plus medium only, and wells with medium only, as controls. After addition of the compounds, the plates were incubated at 37 °C for 48 h or 96 h, for the HMEC and BT20 cell lines, respectively. The proliferation assay was performed using the manufacturer's protocols. The IC_{50} of coronamycin was defined as the concentration of compound which gave 50 % viability.

Antimalarial assay. Cultures of *P. falciparum* strain CSC-1 (Honduras) were maintained according to previously published methods except that human serum was replaced with Albumax I (Gibco BRL): 6 % (w/v) stock solution in RPMI 1640 medium containing 0·1 mg hypoxanthine ml^{-1}, stored at −20 °C (Trager & Jensen, 1976, 1978). Data are reported as IC_{50} values. Details of the assay, in particular the use of labelled phenylalanine and the Giemsa staining procedures, are described by Castillo *et al.* (2002).

Amino acid analyses. The HPLC-purified bioactive fraction (coronamycin) was dissolved in 50 % (v/v) methanol in water and subjected to hydrolysis by HCl according to previously described methods (Castillo *et al.*, 2002). Samples then were analysed by pre-column 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatization followed by reverse-phase HPLC (Cohen & Michaud, 1993). An Alliance/Millenium HPLC system (Waters) equipped with an AccQTag amino acid analysis column was used. The AQC chemistries were done according to the manufacturer's instructions. Moles of each amino acid were initially determined using molar absorption coefficients derived from amino acid standards. All analyses were done at least twice on samples prepared from at least two individual fermentations of the micro-organism.

Mass spectroscopic analyses. A mass spectrum was obtained of HPLC-purified material and spectral data were obtained on a Bruker Biflex-III MALDI/TOF mass spectrometer according to methods used previously (Castillo *et al.*, 2002). External calibration for the initial determination of the molecular masses was done with adrenocorticotropic hormone fragment 18-39 (mass 2465·2 Da). Fragmentation ion (MS/MS) data on the sample were obtained by application of LC/MS on a Bruker Esquire 3000 system with the sample in acetonitrile at a flow rate of 5 μl min^{-1}. The counter-current drying gas was at 250 °C with a flow rate of 4·0 l min^{-1}.

Materials. All solvents used for HPLC were HPLC grade. Those used for extraction were ACS grade. All other reagents were obtained from Sigma, including adrenocorticotropic hormone fragment 18-39, standard amino acids, and radiolabelled phenylalanine. Taxol was supplied by the National Cancer Institute.

RESULTS AND DISCUSSION

Isolation and identification of the endophyte

A small stem of *Monstera* sp. (Fig. 1), after being subjected to procedures for endophyte isolation, yielded at least one micro-organism that outwardly resembled a *Penicillium*

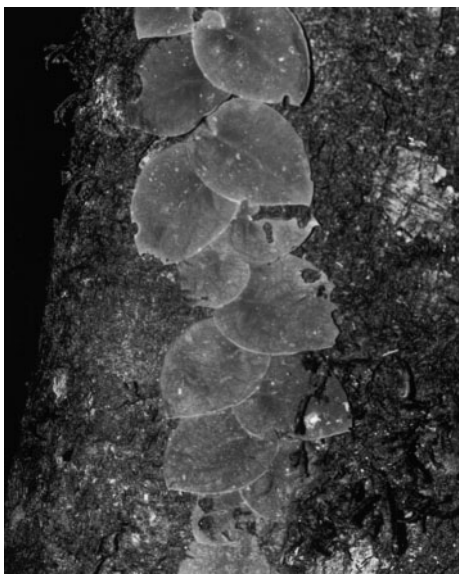

Fig. 1. The plant specimen of *Monstera* sp. growing on a tree trunk in the upper Amazon of Peru from which the endophytic *Streptomyces* sp. MSU-2110 was isolated.

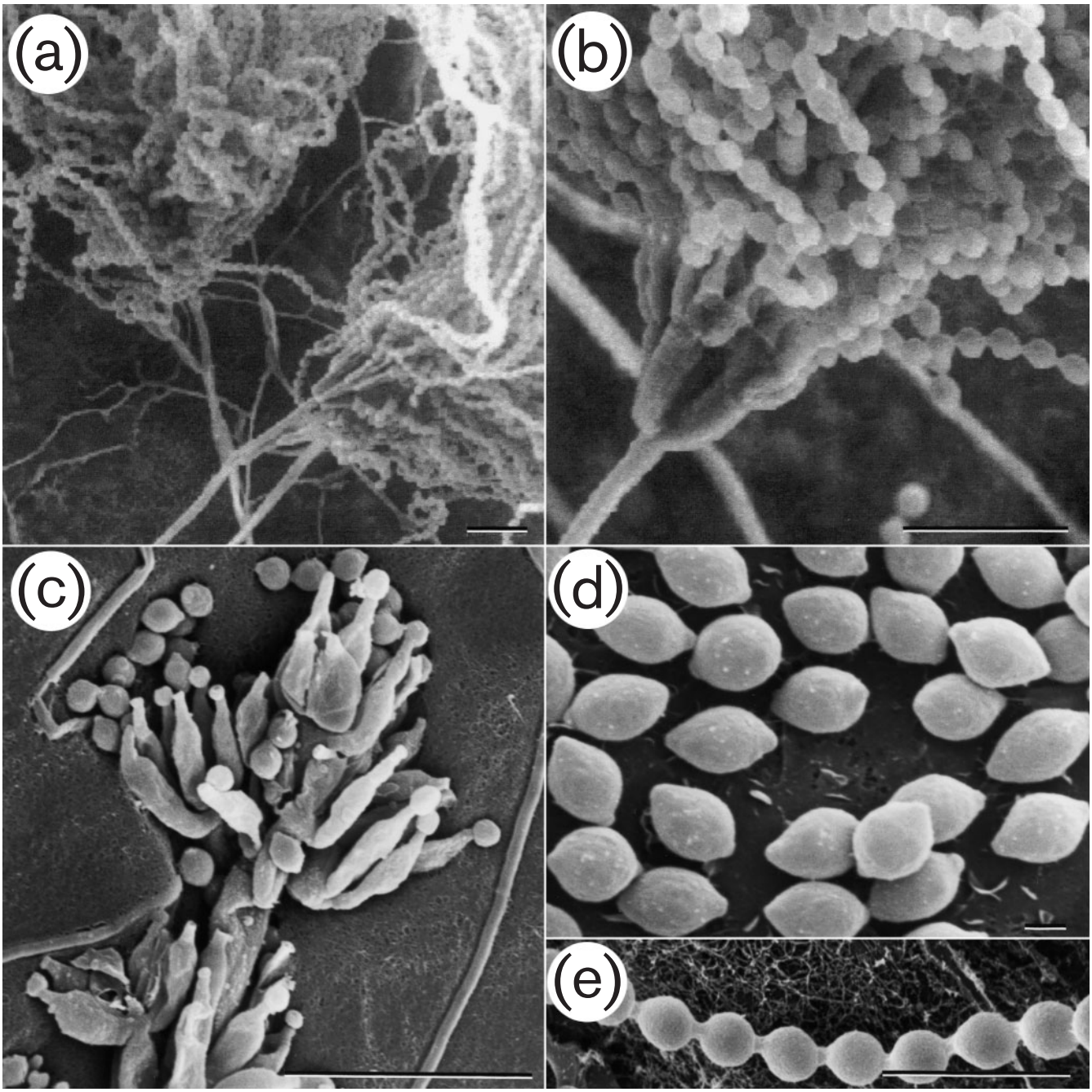

Fig. 2. Electron microscopy of *Streptomyces* sp. MSU-2110. (a, b) Environmental scanning electron micrographs of fruiting structures (a), and obpyriform verticils arranged in a whorl (b). (c–e) Regular scanning electron micrographs illustrating the verticils without spores (c), the characteristic lemon-shaped individual spores (d), and a chain of spores (e). Bars, 10 μm (a–c); 1 μm (d).

sp. Closer examination, however, revealed that the organism was not a *Penicillium* sp., an *Aspergillus* sp. or a *Verticillium* sp. because it had exceedingly small spores. This organism, initially labelled as isolate P-25-2-4, was of interest because of its antifungal activity. The organism was not isolated from any of a number of other trees, shrubs and vines growing within a few hundred metres of the source plant, including *Heliconia* sp., *Piper* sp., *Paullinia paullinoides*, *Philodendron* sp., *Ochroma pyramidale* and *Caryota urens*. When transferred and grown on PDA, isolate P-25-2-4 produced a whitish fluffy growth that after a few days gradually developed into a mycelium with a powdery felt-like surface and a pinkish-tan coloration that became more brownish with time (up to 2 weeks).

An examination of isolate P-25-2-4 by environmental scanning electron microscopy revealed that the spores are borne on verticillate structures: a series of obpyriform verticils arranged in a whorl-like fashion, mostly, but not exclusively, at a terminus of a hyphal strand (Fig. 2). The verticils are enteroblastic conidiogenesis cells approximately 5·2–5·7 μm in length and 1·6 μm in diameter at the widest point. The individual spores are lemon-shaped, ranging from 2·7 to 2·8 μm in length and from 1·5 to 1·6 μm in diameter (Fig. 2). Streptomycetes with a verticillate sporulation habit were placed in the genus *Streptoverticillum* (Locci & Schofield, 1989), although this genus was later transferred to the genus *Streptomyces* (Witt & Stackebrandt, 1990). Examination of the morphology of these organisms (Locci & Schofield, 1989; Miyadoh, 1997) revealed none with a sporulation pattern identical to P-25-2-4.

A partial 16S rDNA gene (912 bp in length) from P-25-2-4 was amplified by PCR and sequenced (GenBank accession no. AY327845). The sequence was searched against the GenBank database and found to possess close similarity to some members of the order Actinomycetales. In addition, there was 94 % homology (642/682) between its partial 16S rDNA and that of *Streptomyces caelestis* (previously described as a *Streptoverticillum*). There was no significant molecular relatedness to *Penicillium* spp. or *Aspergillus* spp. Other organisms showing a high degree homology were *Kocuria kristinae* (99 %) and *Rothia amarae* (94 %).

On the basis of the above morphological and molecular data, isolate P-25-2-4 fits into the streptoverticillium group of *Streptomyces*. It was placed on deposit in the Montana State University culture collection as *Streptomyces* sp. MSU-2110. To our knowledge, this is the first verticillate endophytic streptomycete to be reported in the literature.

Chemical characterization of coronamycin

The antimicrobial compounds (bioactive fraction) isolated from this endophtyic streptomycete have been designated collectively as coronamycin because the sporophore of this organism somewhat resembles a crown (Fig. 2); the source organism was isolated from a plant in the predominantly Spanish-speaking country of Peru, and *corona* is the Spanish for 'crown'.

Only final-step HPLC preparations of the coronamycin were used for chemical and biological characterization of the compound. The major components of the final HPLC separation step consisted of peaks with retention times of 11·14, 22·12 and 36·43 min (Fig. 3). Only the last peak at 36·43 min possessed biological activity. Analysis of the UV absorption spectrum of coronamycin (fraction at 36·43 min), in methanol, produced peaks at 208 nm and 214 nm, and a broad band at 270 nm with millimolar ε values of 2·86, 2·03 and 0·23, respectively. The absorption band at 208 nm suggested the presence of amido- chromophoric groups, which would be consistent with the presence of one or more peptide bonds in the molecule (Silverstein *et al.*, 1991). The broad band at 270 nm hinted at the presence of an aromatic moiety in the molecule (Silverstein *et al.*, 1991). The ^{1}H NMR spectrum (Fig. 4) suggested that the primary nature of the coronamycin is that of a functionalized peptide.

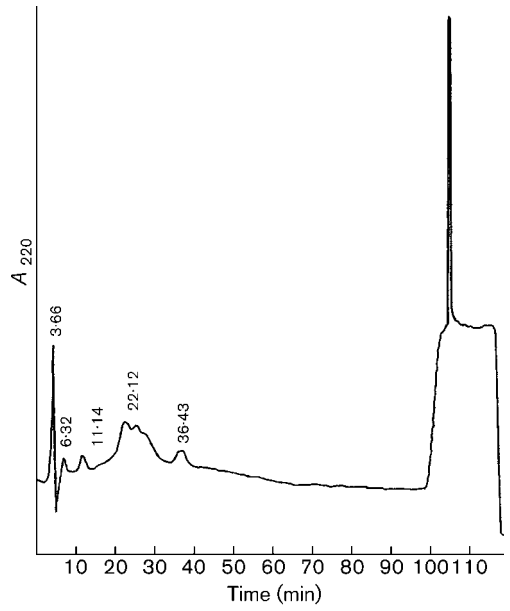

Fig. 3. HPLC elution pattern of coronamycin from a Waters Symmetry 4·6 × 150 mm, 3·5 μm C-18 column under the conditions described in Methods. The bioactive peak (coronamycin) eluted at 36·43 min.

Amino acid analysis of coronamycin revealed five major components (Table 1). These components chromatographed with retention times identical, within experimental error, to those of threonine, α-aminobutyric acid, tyrosine,

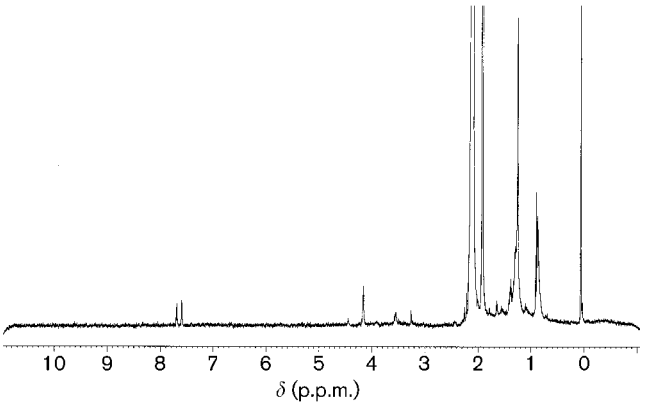

Fig. 4. ^{1}H NMR spectrum of coronamycin taken in 99·95 % deuterated acetonitrile in a Bruker 500 MHz NMR instrument (Castillo *et al.*, 2002). The peak at 0 p.p.m. is consistent with the tetramethylsilane (TMS) standard and the peak at 2·2 p.p.m. is the deuterated acetonitrile solvent peak.

Table 1. Amino acid composition of coronamycin

Results are presented as the mean ± SD of three individual amino acid analyses of the purified coronamycin. The number in parentheses following each amino acid residue indicates the tentative number of moles of that residue per mole of coronamycin (mass 1203) based on 1 mol% being 11.

Amino acid	Molar percentage
Component 1	20·4 ± 3·4 (2)
Component 2	25·2 ± 1·4 (2)
Tyrosine	9·3 ± 0·4 (1)
Methionine	9·1 ± 1·9 (1)
Leucine	33·7 ± 0·3 (3)

methionine and leucine. When hydrolysed coronamycin was combined with a mixture of amino acid standards prior to chromatography, the components identified as tyrosine, methionine and leucine co-migrated with their respective amino acid standard. However, the component tentatively identified as threonine produced a baseline-resolved peak migrating immediately after threonine and before alanine. We term this peak 'component 1'. The component tentatively identified as α-aminobutyric acid ran as a partially (approx. 20 %) resolved leading shoulder of α-aminobutyric acid. We term this peak 'component 2'. The molar ratios of component 1 : component 2 : tyrosine : methionine : leucine were 2 : 2 : 1 : 1 : 3, respectively (Table 1). The amino acid detection system employs a fluorescent amino-reactive agent; therefore the unidentified components are amino compounds. In addition, these compounds are released by acid hydrolysis of the parent compound and are at least partially resistant to this treatment, suggesting that they have chemical properties akin to those of amino acids. Unusual amino acids are commonly found in biologically active peptides produced by endophytes and on occasion these may have elution times close or identical to those of the standard amino acids (Ballio *et al.*, 1994; Strobel *et al.*, 1999; Miller *et al.*, 1998). In addition, the hydrolysis of peptides containing unusual amino acids can cause the elimination of labile substituent groups (e.g. dechlorination of chlorothreonine). This results in the production of amino acid products which are readily identified but not identical to those in the native peptide. These considerations, taken together with the results of extensive prior structural analysis of antibiotics produced by endophytes (Ballio *et al.*, 1994; Strobel *et al.*, 1999; Miller *et al.*, 1998), suggest that coronamycin is largely peptidyl in nature.

Mass spectrometry of coronamycin (36·43 min fraction), isolated independently from several different preparations, consistently revealed the presence of a mixture of compounds, with the major component having a mass of 1203·4 Da, and another predominant component with a mass of 1217·9 Da. On the basis of signal intensity it is estimated that these two major components make up about 80 % of the total weight of coronamycin. Three minor components had masses of 1185·8, 1199·8 and 1233·5 Da, respectively, and make up the difference in the total weight of the sample. None of these components appeared to be sodiated. The 14 Da difference in mass between the 1203 Da component and the 1217 Da component could represent a methylene ($-CH_2$) group. The minor 1199 Da component differed from the 1217 Da component by 18 Da, consistent with the loss of a water molecule. The 1185 Da component could be one methylene group different from 1199 Da component. The minor 1233 Da component differs in mass by 16 Da from the 1217 Da component, which may be accounted for by the loss of one oxygen atom. These relatively minor mass differences are consistent with the family of ions being derived from a common core structure. The structural relatedness of these compounds is also suggested by the concise and consistent amino acid analytical data (Table 1). The LC/MS/MS analysis provided evidence that the molecules of coronamycin were all related by virtue of having the same major fragments after MS/MS of the individual components. As an example, MS/MS of component ions 1217, 1203 and 1233 each yielded daughter ions at 435, 546, 631 and 960, suggesting the existence of a common core structure. Coronamycin evidently consists of a family of structurally related compounds; this is often the case for peptide antibiotics (Ballio *et al.*, 1994; Strobel *et al.*, 1999; Miller *et al.*, 1998). These highly similar compounds are quite difficult to resolve using currently available chromatographic methods.

The broad UV absorbance at 260–280 nm, of a known amount of coronamycin, is consistent with the presence of one tyrosine residue based on the molar absorption coefficient of tyrosine (Table 1). Furthermore, the signals at 7·6–7·7 p.p.m. in the ^{1}H NMR spectrum of coronamycin (Fig. 4) can be attributed to the resonances of the aromatic ring in tyrosine. The major signal at 1·3 p.p.m. is ascribed to the methylene proton resonances in fatty acids (Pouchert & Campbell, 1974). This conclusion is further supported by the presence of multiple upfield ^{13}C resonances (20–60 p.p.m.) in the ^{13}C spectrum (data not shown). The other signals in the ^{1}H spectrum are consistent with shifts commonly associated with peptides (Ballio *et al.*, 1994). The collective spectral and analytical data are consistent with coronamycin having a peptide core functionalized by one of a number of fatty acid moieties with varying degrees of oxidation, hydration and carbon chain length. This type of peptide antibiotic has been described by us and others, exemplified by the pseudomycins and cryptocandins (Ballio *et al.*, 1994; Strobel *et al.*, 1999; Miller *et al.*, 1998). It is to be noted that other highly functionalized peptides have been virtually inseparable, as with commercially available echinomycin, which contains a family of related compounds (Waring, 1979; Castillo *et al.*, 2003). It is important to note the strongly hydrophobic nature of coronamycin, imparted by the lipid component and the three leucine residues. This results in a strong tendency of

the compound to adhere to glass surfaces, creating potential errors in dilution bioassay experiments, and may contribute to its relatively poor yields from liquid culture.

A search of the *Chapman & Hall Dictionary of Natural Products on CD ROM*, 2002, did not reveal chemical identity of coronamycin with any previously described natural products. The peptidyl compound actinomycin F_1, produced by *Actinomyces* sp., has a mass of 1217 Da but does not have any amino acids in common with coronamycin. Similarily, antibiotic A_{41030} has a mass of 1233 Da and also does not share any amino acids with coronamycin. The closest chemical relative of coronamycin appears to be polymyxin B_1, produced by *Bacillus polymyxa*, which has a mass of 1203 Da, is a cyclic peptide that contains leucine, but not tryrosine or methionine. Actinomycin D_{11} has a mass of 1203 but has no amino acids in common with coronamycin. Interestingly, all other compounds listed in the *Dictionary* whose masses are similar to that of coronamycin each possess one or more sugar residues. Coronamycin thus appears to represent a novel group of bioactive substances.

Biological activities of coronamycin

Coronamycin had an MIC value of 2 μg ml^{-1} against the pythiaceous fungus *Pythium ultimum*, and relatively low MICs against related oomycetes such as *Aphanomyces cochlioides* and *Phytophthora cinnamomi*. Other plant-pathogenic fungi, representing the three major families of plant pathogens, were not nearly as sensitive to coronamycin as *Pythium* or *Aphanomyces* (Table 2).

Since coronamycin demonstrated activity against some micro-organisms, it was tested by disk diffusion at 20 μg per disk against a representative panel of human-pathogenic bacteria as described by Castillo *et al.* (2002). Inhibition was observed only against *Streptococcus pneumoniae*, with a 7 mm zone of inhibition. No further antibacterial assays of coronamycin were pursued.

In comparable disk diffusion assay tests, coronamycin displayed activity against a number of human fungal pathogens and thus was subjected to MIC tests. The pathogen most sensitive to coronamycin was *Cryptococcus neoformans*, having an MIC of 4 μg ml^{-1} at 72 h (Table 2). The MIC for *C. neoformans* at 48 h was 0·065 μg ml^{-1}. MICs for most of the other yeasts tested were greater than 16 μg ml^{-1} (Table 2). Flucytosine was used as a control and the MICs obtained were within the expected range.

The best bioactivity of coronamycin was against the malarial parasite *Plasmodium falciparum*, indicating that it possesses enough activity to make it pharmacologically interesting. Coronamycin consistently showed anti-*P. falciparum* activity with IC_{50} values of $9 \pm 7{\cdot}3$ ng ml^{-1}. It was during the antimalarial testing of coronamycin that the ability of this compound to adhere to glassware was noted. Care was taken to ensure that all of the compound in a given test container was in solution, and plasticware was used for all of the antimalarial assays. The low IC_{50} values of coronamycin are in the same range as chloroquine, the gold standard antimalarial compound, which has an IC_{50} of 7·0 ng ml^{-1}. These initial results on coronamycin strongly suggest that it may have potential as an

Table 2. MIC values against plant-pathogenic fungi and yeasts

The MIC was determined as the lowest concentration of coronamycin producing no growth of the test organism (see Methods for details). Flucytosine used as a comparative test substance for the yeasts. ND, Not determined.

Test organism	Coronamycin MIC (μg ml^{-1})	Flucytosine MIC (μg ml^{-1})
Pythium ultimum	2	ND*
Phytophthora cinnamomi	16	ND
Aphanomyces cochlioides	4	ND
Geotrichum candidum	>500	ND
Aspergillus fumigatus	>500	ND
Aspergillus ochraceus	>500	ND
Fusarium solani	>500	ND
Rhizoctonia solani	>500	ND
Cryptococcus neoformans (ATCC 32045)	4	8
Candida parapsilosis (ATCC 90018)	>32	0·12
Candida albicans (ATCC 90028)	16–32	0·5
Saccharomyces cerevisiae (ATCC 9763)	>32	⩽0·06
Candida parapsilosis (ATCC 22019)	>32	0·5
Candida albicans (ATCC 24433)	>32	1
Candida krusei (ATCC 6258)	>32	16
Candida tropicalis (ATCC 750)	>32	⩽0·06

antimalarial drug, especially if choroquine-resistant *P. falciparum* biotypes are sensitive to coronamycin.

Cytotoxicity testing of coronamycin against a primary mammary epithelial cell line (HMEC) gave an IC_{50} of 5–10 μg ml^{-1}, whereas taxol yielded a value of 30–40 μg ml^{-1}. In the case of the breast cancer cell line (BT20) coronamycin had an IC_{50} of 5–10 μg ml^{-1}, whereas the IC_{50} of taxol was 0·008 μg ml^{-1}. It would appear that coronamycin does not have potential as an anticancer drug; however, its cytotoxicity to primary mammary epithelial cells is within the range of taxol.

Other inhibitory compounds of *Streptomyces* sp. MSU-2110

When alternative methods were applied to the separation of the bioactive components of this organism, still other peptide antibiotics active against *Pythium ultimum* were detected. One peptide complex has compounds of masses 1053 and 1102 Da. These masses are identical to components of kakadumycin and echinomycin, which are DNA-intercalating antibiotics (Waring, 1979; Castillo *et al.*, 2003) found as products in various streptomycetes. However, these inhibitory compounds are different from coronamycin, kakadumycin and echinomycin; amino acid analyses indicate that they contain leucine, tyrosine, isoleucine and other amino acids and that they are novel.

Conclusions

Coronamycin is a novel peptide antibiotic active against a relatively small number of human- and plant-pathogenic fungi that were tested. Nevertheless, since it possesses activity against the pythiaceous fungi (Table 2), it may have potential agricultural applications. Moreover, its activity against the malarial parasite, *P. falciparum*, makes it an interesting candidate for further testing, especially since its toxicity against a primary human cell line is more than two orders of magnitude greater than its concentration for effectiveness against *P. falciparum*. It is possible that chemical modifications to its structure, in a manner similar to those made to the lipopeptide antimycotic pseudomycin (Zhang *et al.*, 2001), could reduce its mammalian cell toxicity.

Nearly 75 % of the world's antibiotics are produced by the *Streptomyces* spp. (Arai, 1976; Goodfellow *et al.*, 1988; Demain, 1981). These bacteria have, for the most part, been obtained from soil samples in various parts of the world (Waksman, 1967). The advent of new strains of drug-resistant infectious bacteria, new agents of disease, and old agents of disease whose menace has never been truly contained, e.g. malaria, all emphasize the need for a continued search for novel antibiotics (NIH, 2001). In the meantime, many large pharmaceutical companies have given up or reduced discovery work for control of infectious diseases. It is apparent that plants can serve as a reservoir of endophytic streptomycetes, and evidence thus far indicates that the antibiotics from these sources are novel, interesting and hold pharmaceutical and agricultural promise (Castillo *et al.*, 2002, 2003; Kunoh, 2002). This microbiological reserve residing in the rainforests of the world has never been tapped for its antibiotic potential (Strobel, 2002). The coronamycins provide another example of the potential that endophytes hold for antibiotic discovery.

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