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Ecomycins, unique antimycotics from *Pseudomonas viridiflava*

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C.M. MILLER, R.V. MILLER, D. GARTON-KENNY, B. REDGRAVE, J. SEARS, M.M. CONDRON, D.B. TEPLOW AND G.A. STROBEL. 1998. A novel family of peptide antimycotics, termed ecomycins, is described from *Pseudomonas viridiflava*, a plant-associated bacterium. Ecomycins B and C have molecular masses of 1153 and 1181. They contain equimolar amounts of a β hydroxyaspartic acid, homoserine, threonine, serine, alanine, glycine and one unknown amino acid. Fatty acids were detectable after hydrolysis, methylation and gas chromatography and mass spectroscopy. The ecomycins have significant bioactivities against a wide range of human and plant pathogenic fungi. The minimum inhibitory concentration values for ecomycin B were 4·0 μg ml^{-1} against *Cryptococcus neoformans* and 31 μg ml^{-1} against *Candida albicans*. *Pseudomonas viridiflava* also produces what appears to be syringotoxin, an antifungal lipopeptide previously described from *Ps. syringae*.

INTRODUCTION

Pseudomonas viridiflava is a member of a group of plant-associated fluorescent bacteria that is either weakly pathogenic or saprophytic (Lelliot *et al*. 1966). This group differs from the more commonly known organism, *Ps. syringae*, in a number of biochemical tests, including its inability to utilize sucrose (Lelliot *et al*. 1996). *Pseudomonas viridiflava* has a tendency to be associated with the leaves of lettuce (*Lactuca sativa*) and many grass species. Isolates of *Ps. syringae*, and related species, produce a number of lipopeptide antimycotics (Segre *et al*. 1989; Ballio *et al*. 1990, 1991, 1994; Isogai *et al*. 1990; Harrison *et al*. 1991). These compounds may help assure the survival of the pseudomonads on the leaf, given the strong competition with other saprophytic and parasitic microbes (Harrison *et al*. 1991). In addition, the specificity and potency of these compounds make them attractive as human antifungal therapeutic agents. Unfortunately, the medical uses of these antifungal agents have not been exploited. The increase in incidence of fungal infections in humans makes the need for potent antifungal agents particularly acute. This need has come with the advent of the international AIDS epidemic, chemotherapy of cancer patients and the profusion of organ transplant patients, all of which produce immunocompromised patients susceptible to fungal infection.

The first pseudomonad-derived antimycotics considered for application on human fungal infections were the pseudomycins derived from strain 16H, a genetically-modified isolate of *Ps. syringae* (Harrison *et al*. 1991; Ballio *et al*. 1994). However, the pseudomycins are highly sensitive to a pH above 6·5. In our quest for other antimycotics, we acquired various isolates of *Ps. viridiflava* that produce unique antifungal lipopeptides. These antimycotics were isolated and chemical and biological comparisons made with other known antimycotics. This report summarizes the observations on antifungal activities of certain isolates of *Ps. viridiflava*, and the isolation and partial characterization of these novel antimycotics, the ecomycins.

MATERIALS AND METHODS

Bacterial strains

Grasses from several parts of the world, including California (USA), Israel and Europe, were sampled for bacteria. Those

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plants selected for bacterial isolation generally possessed some chlorotic symptoms on the leaf blade. Within 48 h leaf tissue samples were cut into 5 mm square pieces and these tissues soaked overnight in 0·1 mol l^{-1} sodium phosphate (pH 6·8)-buffered saline (0·1 mol l^{-1} NaCl) at 4 °C. After 12 h, the saline solution was collected, left at room temperature for 1 h and then streaked onto a semi-selective medium (King's B (KB), containing 1^{-1}: 20 g protease peptone (Difco, Detroit, MI, USA), 1·5 g KH_2, PO_4, 1·5 g $MgSO_4 . 6H_2O$, 15 ml glycerol, 100 mg cycloheximide and 15 g agar (King *et al.* 1954)). In approximately 1–2 d, only one yellowish colony type appeared on the semi-selective plates. These yellowish colonies were transferred back to a KB plate. Each was tested for its antimycotic activity by spotting cells from a single colony onto a potato dextrose agar (PDA) plate and allowing growth for 5 d. The colonies were removed with a sterile swab and the plate exposed to chloroform vapours for 20 min followed by dissipation of the vapours for an additional 30 min to kill remaining bacterial cells. The plate was then oversprayed with a spore suspension of *Candida albicans*. Isolates possessing antifungal activity were further characterized via tests such as hypersensitivity on tobacco, arginine dehydrolyase and oxidase activities plus fluorescence on KB agar. In addition, extensive carbon source utilization tests, including Biolog (Biolog Inc., Hayward, CA, USA) were performed. On the basis of all available data, isolates EB-273 from California (USA), EB 274 from California (USA) and EB 227 from Israel were each identified as antimycotic-producing strains of *Ps. viridiflava*.

Fungal strains

All fungi used for test purposes for sensitivity to the ecomycins were obtained from the American Type Culture Collection and the mycological collection at Montana State University (Bozeman, MT, USA).

Ecomycin isolation procedures

Cells from a single colony of EB 273 were used to inoculate 15 ml of potato dextrose (PD) broth and the culture was grown overnight with shaking at room temperature (*ca* 23 °C). This culture was then used to inoculate 2800-ml flasks, each containing 1000 ml of PD broth, which were grown for 6 d at 27 °C as still cultures. The entire culture, including cells, was mixed with an equal volume of acetone and then trifluoroacetic acid (TFA) was added to a final concentration of 0·1% (v/v). The acetone/culture mixture was left overnight at 4 °C and then cells and debris were removed by centrifugation at 10 000 *g* for 20 min. The supernatant liquid was taken to dryness by flash evaporation at 50 °C, and the residue resuspended in 1000 ml of water containing 1% TFA. This solution was then applied to an Amberlite XAD-2 (20–60 mesh ; Merck, Darmstadt, Germany) column (1 × 30 cm) that had been previously equilibrated with an aqueous 0·1% TFA solution. The 1000-ml sample was loaded onto the column at the rate of 1 ml min^{-1} with the aid of a peristaltic pump. After loading, the column was washed successively with a column volume equivalent of H_2O : isopropanol (20 : 80 v/v), and two column volumes of H_2O : isopropanol (30 : 70 v/v) containing 0·1% TFA. The bioactive compounds were then eluted with a linear gradient of 40–100% isopropanol with 0·1% TFA. Fractions of 10 ml were collected and bioactivity determined by spotting 20 μl of each fraction onto a 50% yeast/mannitol (Difco) agar plate. After drying, the plates were overlaid with *C. albicans*. Fractions containing activity, as indicated by zones of inhibition of the *C. albicans*, were pooled and then dried by flash evaporation. The dried residue was washed three times with 15 ml of CH_3OH and the solution filtered through a 0·45 μm teflon filter and dried by flash evaporation. These semi-purified fractions were used in the preliminary biological assay and characterization tests and are referred to as the semi-purified ecomycin preparations. The semi-purified preparations were subjected to high-performance liquid chromatography (HPLC) analysis on an Altima C-18 column (7·8 × 250 mm ; Alltech, Deerfield, IL, USA) and eluted with a linear gradient starting with 100% CH_3OH : H_2O (1 : 1 v/v) containing 0·1% TFA and finishing with 100% acetonitrile : isopropanol (4 : 1 v/v) containing 0·1% TFA. The gradient was run over 35 min at a flow rate of 2 ml min^{-1}. u.v. absorbance was monitored at 254 nm and those fractions containing bioactivity were rerun on the same column under different solvent conditions. The starting solution was 100% acetonitrile : H_2O (1 : 3 v/v) containing 0·1% TFA and the final solution 100% acetonitrile : H_2O (1 : 1 v/v) containing 0·1% TFA. The gradient of the two solutions as run linearly at a flow rate of 3 ml min^{-1} over 25 min monitoring at 214 nm. Fractions were also assayed for sensitivity to *C. albicans*.

Amino acid analysis

Lyphilized HPLC-purified compounds were dissolved in methanol, placed in 6 × 50 mm glass tubes, dried *in vacuo* and then placed in a hydrolysis cylinder (Millipore, Marlborough, MA, USA; part no. 007603). Approximately 300 μl of 6 mol l^{-1} HCl (Pierce Chemical Co., Rockford, IL, USA) were added to the cylinder, which was then alternately purged with nitrogen and evacuated three times before being sealed under vacuum. Vapour phase hydrolysis was performed by heating at 110 °C for 22 h. After cooling, the cylinders were dried *in vacuo*, opened and the residue dissolved in 2% sodium citrate buffer (pH 2·0) and then analysed using a Beckman Model 6300 Amino Acid Analyzer. Moles of each amino acid were first determined using molar absorption values derived from amino acid standards.

Spectroscopic analyses

After isolation of the antifungal compounds, each was subjected to electrospray mass spectroscopy analysis by dissolving the sample in methanol : water : acetic acid (50 : 50 : 1 v/v/v). The samples were injected into Montana State University's custom-built instrument with a spray flow of 2 μl min^{-1} and a spray voltage of 2·2 kV via the loop injection method.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was applied on antifungal compounds in a Brucker 500 MHz instrument with the sample dissolved in 100% deuterated methanol. Each sample was subjected to 2048 scans with a sweep width of 6024 and 8 k real points.

Bioassays

To demonstrate the general antifungal activity of *Ps. viridiflava*, semi-purified preparations of *Ps. viridiflava* extracts were dissolved at 10 μg μl^{-1} in methanol in 0·1% TFA and 10 μl were spotted onto PDA (Table 1) and allowed to dry. Each plate was overlaid with an aqueous suspension of the test fungus in water (about 10^4–10^6 spores or hyphal fragments ml^{-1}), sealed with Parafilm (Chicago, IL, USA) and incubated accordingly (Table 1). In each case, a positive control (solvent-extracted medium without bacterium) was tested.

Ecomycin assays

Minimum inhibitory concentrations (MICs) of the ecomycins against human fungal pathogens were determined. Serial dilutions of semi-purified ecomycin and purified ecomycin B in CH_3OH, ranging in concentration from 50 ng ml^{-1} to 50 μg ml^{-1}, were dispensed into microtitre plates. Each well (10^4 cells ml^{-1}) was then inoculated with a standardized suspension of test fungi in RPMI-1640 in 0·165 mol l^{-1} 4-morpholinepropanesulphonic acid. The microplates were incubated at 37 °C. Growth in the experimental wells was assessed against a positive control (broth and fungi without ecomycin B).

In vitro mammalian toxicity was measured on both semi-purified and purified ecomycin preparations. Serial dilutions of test preparations were added to Jurkat human leukaemia T-cells and murine spleen cells and assayed for toxicity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Green *et al*. 1984).

In solvent tests, ecomycin activity was quantified in unit measurements, a unit being defined as the concentration required to produce a clear zone of inhibition on a 15 × 100 mm yeast-mannitol (YM) agar plate overlaid with some 5 ml of 4% agar containing *C. albicans* at 0·1 O.D. (625 nm). Sensitivity of the ecomycins to elevated pHs was ascertained using the methods described by Harrison *et al*. (1991).

Table 1 Electrospray mass spectral analysis purified Peaks A, B and C from high-performance liquid chromatography (Fig. 3)

Molecular species	Peak A (ecomycin A)	Peak B (ecomycin B)	Peak C (ecomycin C)
$[M+H]^+$	1137·7	1153·5	1181·8
$[M+2H]^+ +$	569·3	577·3	591·4
Mass (assigned)	1137	1153	1181

The compounds were analysed by electrospray mass spectroscopy as described in Materials and Methods. Masses are those of the $[M+H]^+$ and $[M+2H]^+ +$ species.

RESULTS AND DISCUSSION

Effects of solvents and pH

Ecomycins were tested for their solubilities in various solvents prior to final purification. Semi-purified ecomycins were placed in test solvents, mixed thoroughly for 10 min and centrifuged to remove insoluble material. Semi-purified pseudomycins were used in these tests as comparisons (Harrison *et al*. 1991). Serial dilutions were made and the preparation assessed for antimycotic activity on the YM with *C. albicans* plate test. Ecomycins appear to be soluble in water and a number of other relatively polar organic solvents (Fig. 1). The difference in fungal inhibition from solvent to solvent was assumed to be a direct reflection of solubility and not solvent-induced loss of activity, as activity did not appear to decrease with extended exposures to the test solvents. These results support the possibility of using the ecomycins in various solvent-based applications, especially between water and methylene chloride on the polarity index (Fig. 1). One of the major limitations on the industrial or medical uses of the syringomycins and pseudomycins is their lability at pH above 6·5 (Harrison *et al*. 1991). In extensive tests with semi-purified ecomycin preparations using the *C. albicans* bioassay, the ecomycins retained complete biological activity at a pH of 7·5 or below for at least 1 week. Furthermore, they retained bioactivity for at least 3 d at pH 7·75 and 8·0 and up to 12 h at pH 9·0 (Fig. 2).

Purification of the ecomycins

At least three major peaks of antimycotic activity appeared in the effluent of the HPLC-Altima C-18 column (Fig. 3). The first bioactive peak, eluting with a retention time of 19·6 min,

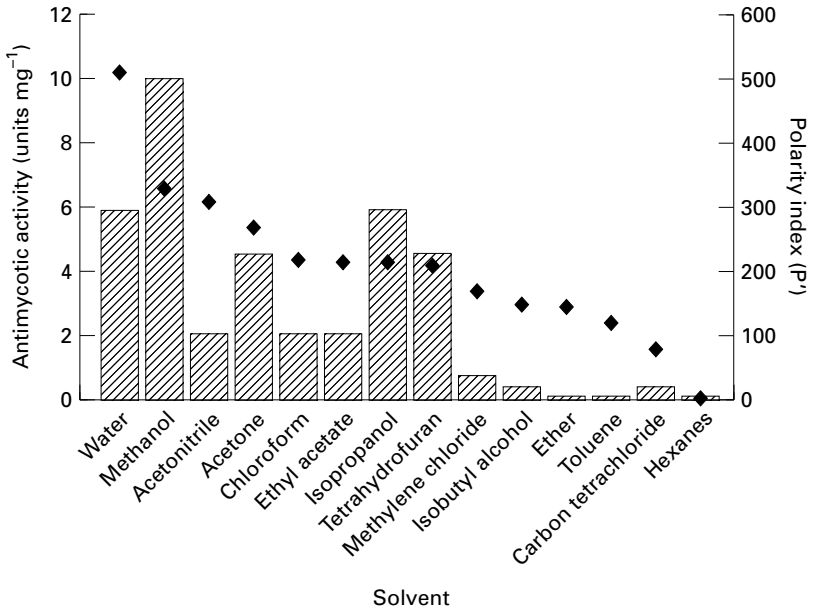

Fig. 1 The solubility of a semi-purified ecomycin preparation in various solvents. solubility (▨) was measured in the antimycotic assay and the polarity index (◆) is taken from standard literature values. See Materials and Methods for experimental details

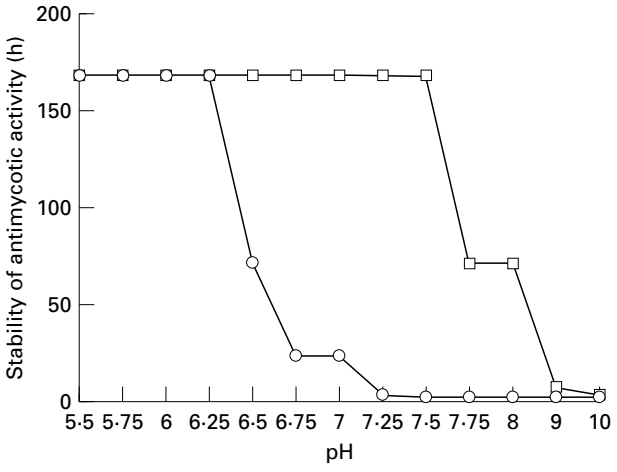

Fig. 2 The effect of pH on a semi-purified ecomycin preparation (□) *vs* a semi-purified pseudomycin preparation (○). Stability of the antimycotics was measured as a function of their bioactivity against *Candida albicans* (see Materials and Methods)

was designated peak A, peak B had a retention time of 20·4 min and peak C a retention time of 23·8 min (Fig. 3). Each compound was rechromatographed on the Altima C-18 column using the second solvent system and programmed elution schedule as on the first run. Each ecomycin eluted as a distinct peak (at 214 nm) with a unique retention time. The only bioactivity present in the column fractions coincided with the ecomycin peaks. This is best illustrated by the elution of peak B from the second HPLC column (Fig. 4). The approximate yield of peak A was 400 μg l^{-1}, peak B 200 μg l^{-1} and peak C about 100 μg l^{-1}.

Purity of ecomycins

In addition to HPLC, the purity of each of the peaks A, B and C was also evaluated using thin layer chromatography (TLC) on Merck silica gel plates, as previously described (Harrison *et al.* 1991). Each designated ecomycin appeared on the plate as a single ninhydrin-positive spot at an R_F value similar to but unique from syringomycin and the pseucomycins (Harrison *et al.* 1991). Furthermore, the H-NMR spectra obtained on these compounds appeared free of any major contaminating compounds and comparable to the published spectra of other lipopeptide antimycotics (Ballio *et al.* 1990, 1994). Each of the compounds was sufficiently pure for further chemical and biological analysis.

Mass spectral analysis

Electrospray mass spectrometry on each of the peaks was performed as a first step towards defining their structures and determining their uniqueness since other methods, including TLC, were equivocal. Each compound produced a predominant, protonated molecular ion as well as a doubly charged species (Table 1). None of the compounds produced potassium $[M+39]^+$ or sodium $[M+23]^+$ adducts. A representative spectrum of ecomycin B illustrates this and shows the high degree of purity of the preparation (Fig. 5). The peak at 1153·9 is assigned to $[M+H]^+$, while that at 577·3 is

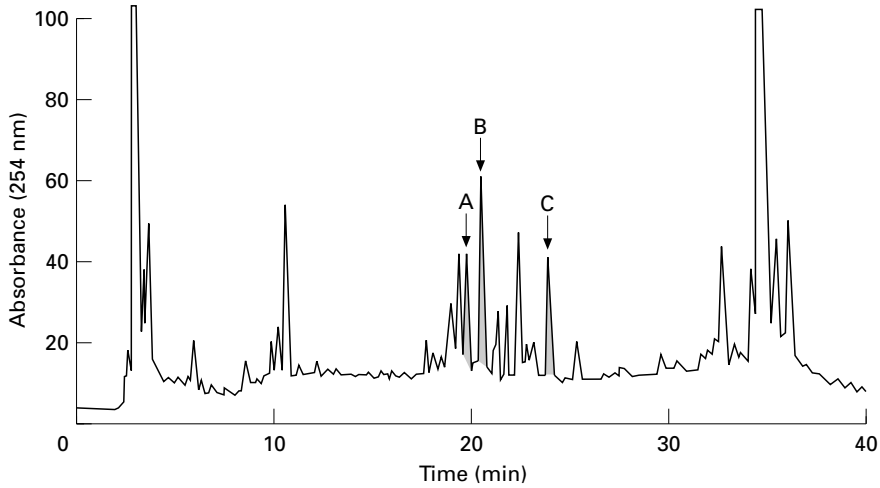

Fig. 3 Elution profile of the antifungal compounds of *Pseudomonas viridiflava* (labelled peaks) from an Altima C-18 high-performance liquid chromatography column. The column was monitored at 254 nm and each tube was checked for antifungal activity against *Candida albicans* (shaded peaks)

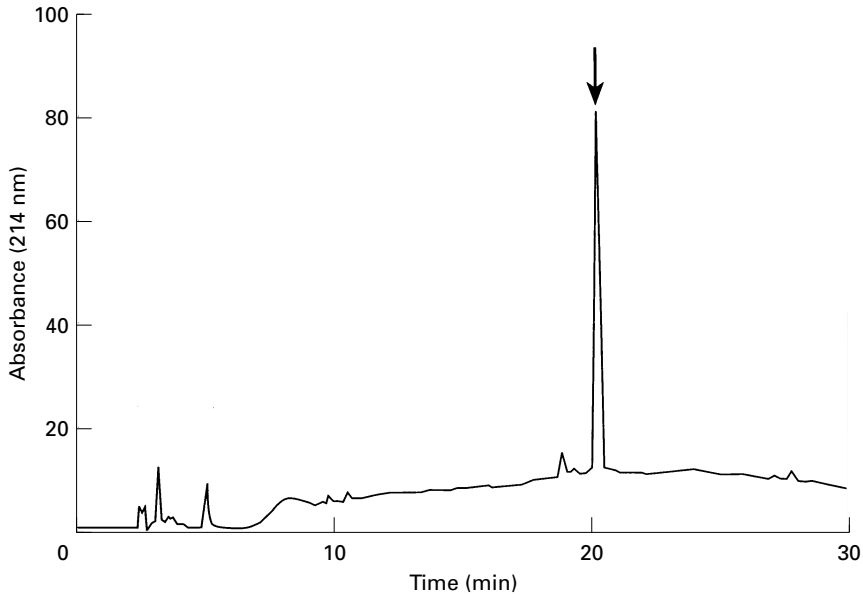

Fig. 4 The elution of ecomycin B from an Altima C-18 column. The column was monitored at 214 nm and the fractions were assayed for activity against *Candida albicans*. The only bioactivity was present in the peak designated by the arrow

$[M+2H]^{+}+$. The other small peaks in the spectrum are unidentified.

The compound eluting as Peak A (Table 1) had a mass nearly equivalent to that of syringotoxin ($MH^{+} = 1136$) (Ballio *et al.* 1990). However, peaks B and C, having masses of 1153 and 1181, were unique from the other pseudomonad lipopeptide antimycotics (Segre *et al.* 1989; Ballio *et al.* 1990, 1991, 1994; Isogai *et al.* 1990; Harrison *et al.* 1991). Based on the similarities of the masses of peaks B and C (Table 1), it seems likely that they represent a novel family of lipopeptides, the ecomycins. Structural differences probably occur between the lipid side-chains as in the pseudomycins and syringomycins (Ballio *et al.* 1991, 1994; Harrison *et al.* 1991). For example, the 28 Da differences between ecomycins B and C could arise from the equivalent difference of two methylene groups in the lipid side-chain.

Constituent residues of the ecomycins

Each ecomycin contained β-hydroxy aspartic acid, threonine, serine, homoserine, glycine, alanine and an unknown amino acid (Table 2) and with one exception (syringotoxin) distinguishes them from all other antifungal lipopeptides. However, ecomycins A and B yielded low amounts of asx, but differed significantly in the level of alanine. Ecomycin A was the only member of the family containing 2,4-diaminobutyric

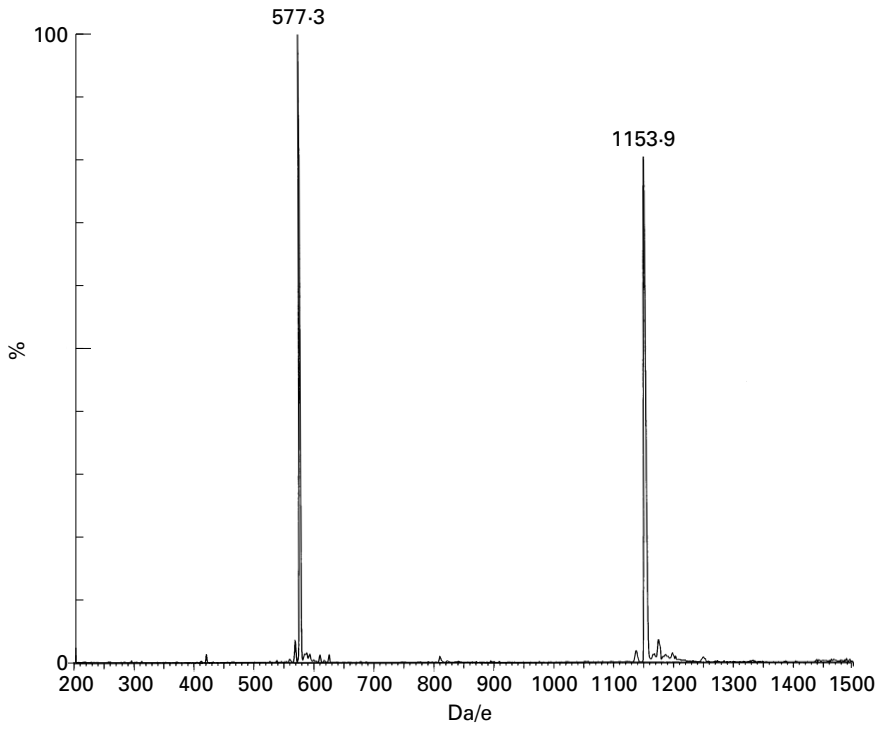

Fig. 5 An electrospray mass spectrum of ecomycin B. Conditions for the experiment are in Materials and Methods. The peak at 1153·9 is $[M+H]^+$ while that at 577·3 is $[M+2H]^{++}$

Table 2 Amino acid compositions of ecomycins B and C*

Amino acid	Ecomycin A	Ecomycin B	Ecomycin C
3(OH) Asp	16·7	20·0	28·9
Asx	2·0	1·6	ND
Threonine	11·9	12·8	13·3
Serine	11·2	12·0	13·3
Homoserine	4·9	5·6	4·4
Glx	ND	ND	8·9
Glycine	16·3	16·8	13·3
Alanine	7·5	17·6	6·7
Unknown	+	+	+
Ornithine	13·8	ND	ND
Diaminobutyric acid	15·8	ND	ND

* The samples were hydrolysed and analysed as described in Materials and Methods. Mole per cents [(moles of each amino acid)/(moles of all amino acids) × 100] are presented. Unusual amino acids include 3-hydroxyaspartic acid (3 (OH) Asp), homoserine, an unknown eluting between histidine and ornithine, ornithine (Dm) and 2,4-diaminobutyric acid (Dab). Amino acids present at levels <1 mole per cent are not reported. ND, Not detected; +, present but not quantifiable since no standard is available.

acid and ornithine. Ecomycin C produced a modest glx peak and had a level of alanine similar to that of ecomycin A.

Analysis of amino acid compositions of pseudomonad-derived lipopeptides is complicated by the presence of unusual amino acids including 4-chlorothreonine,2-amino,2,3-dehydrobutyric acid and allothreonine. Residues such as these are often difficult to resolve from the common amino acids, may not be commercially available and can readily decompose during hydrolysis. All of these factors make their identification and quantitation problematic. For example, it is possible that ecomycin A is syringotoxin (Ballio *et al.* 1990), since the amino acid composition of ecomycin A is similar to that of syringotoxin. Only those unusual amino acids mentioned above and a 2 amu total mass difference distinguish syringotoxin from ecomycin A. A standard syringotoxin preparation was not available for comparison purposes. However, based on the molecular weight and amino acid compositional data, ecomycins B and C represent a unique set of related lipopeptides not possessing phenylalanine, lysine, arginine, ornithine or diaminobutyric acid, which are constituents of such compounds as the pseudomycins, syringomycins, syringostatins and syringotoxin (Segre *et al.* 1989; Ballio *et al.* 1990; Isogai *et al.* 1990; Harrison *et al.* 1990).

Preliminary data have been acquired on the fatty acid content of ecomycin B. Analyses were made after methylation of samples followed by gas chromatography/mass spectroscopy (Alltech 1993). Ecomycin B possessed a compound whose spectral characteristics and retention time were identical to methyl sterate. Ecomycin C yielded a compound whose

retention time and spectrum were not identical to any standard straight-chain fatty acid. Theoretically, the ecomycins, having as many as eight or nine amino acid residues (Table 2), possess enough mass difference from the total molecular weight (Table 1) to account for at least one fatty acid residue per molecule, as do each of the other known lipopeptide antimycotics from the pseudomonads (Harrison *et al.* 1991; Ballio *et al.* 1994). A variation in the structure of the fatty acid side-chain would account for the different forms of ecomycin as, for instance, in the case of the pseudomycins (Ballio *et al.* 1994).

Biological activities of the ecomycins

Ecomycin preparations killed a number of human pathogenic fungi including *C. albicans* and *Cryptococcus neoformans* (Table 3). These microbes are two of the most important fungal pathogens of man. Some of the most important fungal pathogens of plants were also tested; all were sensitive to ecomycin B (Table 3). Of those listed, *Fusarium oxysporum*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani* are some of the most destructive and widespread crop pathogens. In addition, such critically important fungi as *Cladosporium resinae* (jet fuel destruction) and *Portia placenta* (wood degradation) were also sensitive to the ecomycins. However, *Aspergillus niger*, *A. fumigatus* and *Trichoderma* spp. were insensitive (Table 3). Purified ecomycin B was tested for its specific bioactivity against several human pathogens using the MIC test. Of these tested, *Crypt. neoformans* was the most sensitive to ecomycin B (MIC = 4·0 μg ml^{-1}) and *Crypt. glabrata* was also quite sensitive to ecomycin B (MIC = 8 μg ml^{-1}) (Table 4). This is in contrast to 0·1 μg ml^{-1} sensitivity of *Crypt. neoformans* to pseudomycin A (Strobel, unpublished). However, in each case, amphotericin B was more potent, but it is extremely toxic to human cells. The broad spectrum activity of the ecomycins combined with their lack of toxicity make them attractive alternatives to amphotericin B.

Table 3 Sensitivity of some plant pathogenic fungi and fungi important in home, industrial or military situations to the semi-purified ecomycins*

Organism	Attribute	Sensitivity*
Aspergillus fumigatus	Human pathogen	I
Candida albicans	Human pathogen	S
Candida kefir	Human pathogen	S
Candida krusei	Human pathogen	S
Candida glabrata	Human pathogen	S
Candida parasilopsis	Human pathogen	S
Candida tropicalis	Human pathogen	S
Cryptococcus neoformans	Human pathogen	S
Alternaria solani	Plant pathogen	S
Diplodia viticola	Plant pathogen	S
Drechslera sorokiniana	Plant pathogen	S
Fusarium spp. (*Capsicum*)	Plant pathogen	S
Fusarium avenaceum	Plant pathogen	S
Fusarium lateritium	Plant pathogen	S
Fusarium oxysporum	Plant pathogen	S
Geotrichum citri-aurantii	Plant pathogen	S
Rhizoctonia solani	Plant pathogen	S
Sclerotinia sclerotiorum	Plant pathogen	S
Sclerotium rolfsii	Plant pathogen	S
Stemphylium citri	Plant pathogen	S
Aspergillus niger	Plastics degradation (ATCC 9642)	I
Cladosporium resinae	Jet fuel degradation (ATCC 38834)	S
Postia placenta	Wood degradation (ATCC 11538)	S
Trichoderma spp.	Mildew, Florida household	I

S, Sensitive at 100 μg ml^{-1} or less of crude extract; I, insensitive; ATCC, American Type Culture Collection.
* The semi-purified ecomycins were tested on potato dextrose agar plates (see Materials and Methods).

The ecomycins

We have identified and partially characterized three antifungal lipopeptides produced by *Ps. viridiflava* EB273. These molecules, of which at least two are novel, constitute a new family of antimycotics, the ecomycins. Comparable studies to these have also been performed with *Ps. viridiflava* EB274 (California, USA) and EB227 (Israel). Each of these isolates also produces antifungal lipopeptides whose masses are identical to those of ecomycins B and C (Table 1). More recently, however, a new antimycotic, of molecular weight 1167, has been obtained as a minor component of EB274 (semi-purified preparation). It appears to be another of the ecomycins. Further structural and chemical characterization of this and the other ecomycins is currently underway.

The role of the ecomycins in the ecology/pathology of *Ps. viridiflava* is currently not clear. However, many of the other pseudomonad-derived antifungal compounds also possess phytotoxicity (Ballio *et al.* 1990; Harrison *et al.* 1991). Given the information presented in this report we are now in a position to not only ascertain the biological role of the ecomycins in nature, but to determine if the *Ps. viridiflava* group may be a source for other novel antimycotics.

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Table 4 Minimal inhibitory concentration (MIC) values of ecomycins against several human fungal pathogens

Pathogenic species (ATCC)	MIC (μg ml^{-1})*		
	Semi-purified ecomycins	Ecomycin B	Amphotericin B
Candida albicans 90028	39	31	0·6
Candida albicans 90029	20	31	0·6
Candida glabrata 90030	39	8	0·6
Candida kefir 46764	16	NT	0·1
Candida parasilopsis 90018	39	31	0·6
Cryptococcus neoformans 90112	19	4	0·1
Cryptococcus neoformans 90113	19	4	0·3

* The MIC test was carried out as indicated in the Materials and Methods.
NT, No tests were conducted; ATCC, American Type Culture Collection.

Antonio, TX, USA). Pharmagenesis, the parent company of Ecopharm, performed the animal cell toxicity tests.

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