PARVODICIN, A NOVEL GLYCOPEPTIDE FROM A NEW SPECIES, *ACTINOMADURA PARVOSATA*: DISCOVERY, TAXONOMY, ACTIVITY AND STRUCTURE ELUCIDATION

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An extensive taxonomic investigation identified strain SK&F-AAJ-271 as a new species, designated *Actinomadura parvosata*. Fermentations of this organism produce a complex of acidic, lipophilic glycopeptide antibiotics, the parvodicins. Structures for seven of the isolated components were derived from a combination of mass spectral, high-field NMR and chemical techniques. The *O*-acetyl functionality present in two of the isolated components is a structural feature unique among the known members of this class of antibiotics. The parvodicins are active *in vitro* against a range of Gram-positive bacteria. The most active parvodicin, C_1, produces high serum levels *in vivo* and has the potential for a long duration of action.

The increasing importance of vancomycin for the treatment of methicillin-resistant staphylococcal infections[1,2] has stimulated the search for novel members of this class of glycopeptide antibiotics. Central to our effort in this area has been a mechanism-based screen[3] in which a mimetic of the bacterial cell wall receptor[4] for glycopeptides is used to antagonize selectively the activity of these antibiotics in the complex mixture of culture products, the implementation of efficient isolation and purification procedures,[5,6] as well as the development of fast atom bombardment mass spectrometry (FAB-MS) techniques[7] and modern two-dimensional (2D) NMR methods[8] for structural characterization. This strategy has led to the discovery, isolation and structure elucidation of the aridicins,[8~10] the kibdelins[11,12] and as reported in detail here, the parvodicins.[13]

Materials and Methods

Chemotaxonomy, Physiological and Biochemical Evaluation

Strain SK&F-AAJ-271 was isolated from a soil sample collected on the shore of a pond or stream near Myittanyunt, Burma. Because SK&F-AAJ-271 sporulates poorly, the culture was grown in shake flasks and maintained as frozen stock in a mechanical freezer at −70°C or in the vapor phase of liquid nitrogen. Slant cultures were maintained on thin inorganic salts - starch agar (Difco inorganic salts - starch agar (ISP 4) 12 g, Difco Bactoagar 15 g, distilled water (1 liter) supplemented with yeast extract (2 g/liter)). Morphological observations were made on plates of modified thin inorganic salts - starch agar (Difco inorganic salts - starch agar (ISP 4) 12 g, Difco Bactoagar 15 g, distilled water 800 ml, soil extract 200 ml, thiamine hydrochloride 0.5 mg, biotin 0.25 mg). Color descriptions were chosen by comparison to color chips from the ISCC-NBS Color Charts[14] or the Methuen Handbook of

Colour.[15]

A Jeol T300 scanning electron microscope was used for microscopic evaluation. Coverslip cultures, prepared by the method of KAWATO and SHINOBU,[16] were fixed in glutaraldehyde, post-fixed with osmium tetroxide, critical point dried and sputter coated with gold.

Purified cell preparations were analyzed by the methods of BECKER *et al.*[17] Whole-cell hydrolysates and cell wall phospholipids were examined using the techniques of LECHEVALIER[18] and LECHEVALIER *et al.*,[19] respectively.

The physiological and biochemical tests used to characterize SK&F-AAJ-271 were those of GORDON[20,21], GORDON and MIHM,[22] and GOODFELLOW *et al.*[23] The tests used to determine melanoid pigments were those of SHIRLING and GOTTLIEB.[24] Phosphatase activity was determined by the methods of KURUP and SCHMITT.[25] The modified carbon utilization medium of PRIDHAM and GOTTLIEB (ISP 9)[26] proved unsuitable for the characterization of SK&F-AAJ-271. The medium used to determine sole carbon source utilization was the C-2 medium of NONOMURA and OHARA.[27]

Susceptibility of SK&F-AAJ-271 to antibiotics was examined by placing BBL susceptibility disks on nutrient agar plates seeded with SK&F-AAJ-271. Plates were held at 4°C for 1 hour to permit diffusion of the antibiotics. Diameters of the zones of inhibition were measured after incubation for 1 week at 28°C.

High Performance Liquid Chromatography (HPLC) Assay of Parvodicins

A C_{18} Sep-Pak cartridge (Waters Associates) was used for pretreatment of broth samples and crude extracts. All other samples were analyzed directly. HPLC analyses[28] of samples were conducted on a Beckman 345 binary liquid chromatograph using an Altex Ultrasphere-ODS column (5 μm, 4.6 i.d. $\times$150 mm) with detection at 220 nm. Analyses were performed using: a) 14→37% acetonitrile (8 minutes) in 0.01 M potassium phosphate buffer (pH 3.2), flow rate 1.5 ml/minute or b) 30% acetonitrile (1 minute), then 30→35% acetonitrile (15 minutes) in 0.01 M potassium phosphate buffer (pH 3.2), flow rate 1.5 ml/minute.

Fermentation

A 14-day old agar slant of *A. parvosata* dispersed in 10 ml of sterile water was used to inoculate 500 ml of seed medium 13H[9] contained in a 4-liter aspirator bottle. After incubation at 28°C on a rotary shaker (New Brunswick Model G53) at 250 rpm (5 cm throw) for 4 days, the first seed was transferred to 15 liters of seed medium 13H in a 20-liter fermentor which was maintained at 28°C, aerated at 0.4 vvm and agitated at 300~400 rpm. After 4 days, 5 liters of the second seed were transferred to 50 liters of seed medium 13H contained in a 75-liter Chemap fermentor. This third seed, incubated at 28°C, aerated at 0.5 vvm and agitated at 250~300 rpm for 4 days, was inoculated into 500 liters of production medium E1 (glucose 20 g, soy peptone 10 g, yeast extract 1 g, $CaCO_3$ 1 g and $CoCl_2$ 0.001 g in 1 liter of distilled water) contained in a 750-liter ABEC fermentor. This production stage was incubated at 28°C, aerated at 0.3 vvm and agitated at 120 rpm. Production of parvodicin C_1, measured by analytical reversed-phase HPLC (RPHPLC), peaked at 60 μg/ml by 90 hours. This was accompanied by a decline in the level of parvodicin C_3, which was the major component observed earlier in the fermentation.

Isolation and Purification of the Parvodicin Complex

A: The crude fermentation broth (*ca.* 600 liters) was clarified by rotary drum filtration (Komline-Sanderson, Laboratory Scale Model) using Hyflo Supercell (Johns-Manville Products Corp.). After the filtrate (400 liters) was chilled (4°C), 40 liters of IRA-68 anion exchange resin (Rohm and Haas) was added with stirring and with continuous addition of concentrated hydrochloric acid to maintain the pH between 6 and 8. The resin was allowed to settle, the broth was decanted and the resin was washed twice with water (40 liters). The complex was batch-eluted with ammonium hydroxide (1 M, 270 liters). The solution was neutralized with concentrated phosphoric acid and applied to two columns (8 liters) of Diaion HP-20 resin (Mitsubishi Chem. Inc.). Each column was washed with water (20 liters) and the complex was eluted with 60% acetonitrile in water (20 liters). The combined eluates were concentrated *in vacuo* to 10 liters, which was treated in 1 liter portions with Affigel-10-D-Ala-D-Ala

(300 ml).[6,29] The gel was washed with sodium phosphate buffer (0.02 M, pH 7, 1 liter) and the complex was eluted with 600 ml of 50% acetonitrile in ammonium hydroxide (0.1 M). The combined eluates were neutralized with phosphoric acid, diluted to *ca.* 10% acetonitrile and applied to Diaion HP-20 resin. After the water wash, elution and concentration as described above, lyophilization of the concentrate yielded 4 g of solid.

Isolation of the individual components was effected by preparative RPHPLC of portions of the crude complex on a Vydac C18 column (HP Chemicals, 5.1 i.d.×50 cm, 15~20 μm, 300 *A*) using a gradient of acetonitrile (15→20%, 10 minutes; 20→24%, 17 minutes; 24→25%, 48 minutes) in potassium phosphate buffer (0.01 M, pH 6.0) at a flow rate of 100 ml/minute with detection at 280 nm, as shown for a typical 650 mg load in Fig. 2. Upon completion of the chromatographic runs, like fractions were pooled and desalted on Diaion HP-20 resin to yield parvodicins A (16 mg), B_1 (28 mg), B_2 (68 mg), C_1 (300 mg) and C_2 (230 mg).

B: A portion of fermentation broth (15 liters) was clarified by filtration with Celite and the filtrate (11 liters) was applied to a column (4 liters) of Diaion HP-20 resin. The column was washed with water (20 liters) and the complex was eluted with 60% acetonitrile in water (10 liters). The eluate was concentrated *in vacuo* to 3 liters and applied in two portions to a column (200 ml) of Affigel-10-D-Ala-D-Ala.[6,29] Each column was washed with sodium phosphate buffer (0.02 M, pH 7.0, 1 liter) and water (400 ml) prior to elution of the complex with 50% acetonitrile in ammonium hydroxide (0.1 M, 500 ml). The eluates were neutralized immediately with dilute hydrochloric acid, combined and lyophilized.

The crude complex was chromatographed on a Whatman Magnum 20 column (2.2 i.d.×50 cm) packed with Whatman Partisil-10 ODS-3 using a step-gradient of acetonitrile in potassium phosphate buffer (0.1 M, pH 3.2) at a flow rate of 15 ml/minute with detection at 280 nm. The individual components eluted at 29% acetonitrile. Like fractions were pooled, diluted with six volumes of water and applied to Diaion HP-20 resin. After washing with water, the components were eluted with 60% acetonitrile in water. The acetonitrile was removed *in vacuo* and the water by lyophilization to yield components C_1 (24 mg), C_2 (6 mg), C_3 (8 mg) and C_4 (4 mg). In a similar isolation procedure conducted subsequently, the affinity-bound complex was eluted with 5% acetonitrile in water (1 liter) prior to elution of the bulk of the complex with 50% acetonitrile in ammonium hydroxide. This eluate was desalted and lyophilized, providing a sample of complex (125 mg) enriched in minor components (RPHPLC determination).

General Procedures

Isoelectric focusing was performed using an LKB Multiphase apparatus and activity against *Bacillus subtilis* ATCC 6633 for detection.[30] Carbohydrate analysis and UV spectroscopy were conducted as described previously.[10] FAB-MS data was obtained with a VG Analytical ZAB-1F mass spectrometer equipped with a high-field magnet using thioglycerol-oxalic acid as solvent matrix.[7] ^1H NMR data was obtained at 500.1 MHz using a Jeol GX500 spectrometer, utilizing the techniques described in detail elsewhere.[8]

Aglycone Production

A sample of Affigel-isolated complex enriched in minor components (105 mg) was dissolved in 5 ml of a solution of hydrochloric acid (0.3 M) in 10% aqueous dimethylsulfoxide. The mixture was heated under nitrogen at 100~105°C for 75 minutes, cooled to room temp and concentrated *in vacuo*; RPHPLC analysis revealed two species present in a 4 to 1 ratio. After a standard course of affinity chromatography, the isolated material was subjected to semi-preparative RPHPLC (Partisil-10 ODS-3, Magnum 20, 2.2 i.d.×50 cm) using an isocratic system of acetonitrile (17%) in potassium phosphate buffer (0.01 M, pH 6.0) at a flow rate of 10 ml/minute with detection at 220 nm. Like fractions were pooled and desalted (HP-20) to yield aglycone I (26 mg; isoelectric point 5.3; $E^{1\%}_{1cm}$ (280 nm; CH_3CN - H_2O, 1:1) 68) and aglycone II (5 mg; isoelectric point 5.3; $E^{1\%}_{1cm}$ (280 nm; CH_3CN - H_2O, 1:1) 70).

Pseudoaglycone Production

A mixture of components C_1 and C_2 (2.5 g, isolated by preparative RPHPLC) was dissolved in 50% aqueous acetonitrile (160 ml) and the apparent pH was adjusted to 3 with hydrochloric acid. The

mixture was heated at reflux for 20 hours, cooled to room temp and concentrated *in vacuo*. The product was subjected to a standard course of affinity chromatography and desalting, yielding 1.8 g. A portion (250 mg) of this was chromatographed and desalted as described for the aglycones to provide the mannosyl aglycone (131 mg; isoelectric point 5.2; $E^{1\%}_{1cm}$ (280 nm; CH_3CN - H_2O, 1 : 1) 70).

Fatty Acid Analysis

Acidic methanolysis of individual components was conducted and the derived fatty acid methyl esters were identified by comparisons with the gas chromatography mass spectrometry (GC-MS) data obtained from standards as described previously[12,31] using a DB-17 fused silica capillary column (0.25 mm i.d. ×15 m) temperature-programed from a) 75 to 250°C at a rate of 12°C/minute or b) 90 to 275°C at a rate of 10°C/minute (see Table 2).

Antimicrobial Activity

In vitro (MIC, μg/ml) activity was determined using methods described previously.[9]

Pharmacokinetic Studies

The pharmacokinetics of parvodicin C_1 were determined by means of techniques described in detail elsewhere[32] except that serum concentrations of antibiotic were determined also at 480, 960, 1,080 and 1,440 minutes after drug administration.

Results

Morphological and Cultural Characteristics

Strain SK&F-AAJ-271 has the general morphological features that were described by LECHEVALIER and LECHEVALIER[33] for the genus *Actinomadura*. It is a Gram-positive, filamentous organism that formed a substrate mycelium and, on a few media, an aerial mycelium. No motile elements were observed in either the aerial or substrate mycelium.

The substrate mycelium was well-developed with moderately to densely branched, long hyphae that were approximately 0.5 to 1.0 μm in diameter. No spores were produced on the substrate mycelium and mycelial fragmentation was never observed.

The aerial mycelium produced long, branching aerial hyphae that bore chains of spores following five to seven weeks incubation on modified thin inorganic salts - starch agar. Chains of more than 30 spores were common, but short chains of ten spores or less were also usually present. A few of the spore chains appeared to be sessile but most were borne on short to relatively long sporophores. An occasional spore chain was hooked, irregularly curved or formed a tightly closed spiral that resembled a pseudo-sporangium. The most common spore chain morphology, however, was one in which the upper half to two-thirds of the spore chain formed a spiral of two to six turns. These spirals could be very regular and open or tightly closed. The spirals could also be somewhat irregular and have a knot-like appearance. The spores varied in size and shape (round to almost square), particularly when only a few tufts of spore-bearing hyphae were produced following prolonged incubation on suboptimal media. On modified thin inorganic salts - starch agar, a typical spore was smooth-walled, ovoid to rectangular, and approximately 0.5 to 2.5 μm long and 0.5 to 1.3 μm in diameter.

Plates for the determination of cultural characteristics of SK&F-AAJ-271 were incubated at 28°C in closed petri dish cans and observed at intervals up to 21 days. As summarized in Table 1, the substrate mycelium of SK&F-AAJ-271 varied from yellow-brown to orange to purple. The aerial mycelium was white to pink. Soluble pigments varied from yellow-brown to orange to violet-red. In yeast extract - malt extract agar and oatmeal agar, the reverse side pigments and soluble pigments were affected by pH.

Table 1. Cultural characteristics of SK&F-AAJ-271.

Medium		Growth and morphology[a]
Yeast extract - malt extract agar (ISP 2)	G:	Excellent, violet-red to dark reddish brown
	AM:	None to sparse, white, sterile
	SP:	Dark reddish orange to strong reddish brown
Oatmeal agar (ISP 3)	G:	Good, dark reddish brown (Methuen 10 E6 violet-brown) to violet-red (Munsell 10 R/P 6/6)
	AM:	Sparse, white, sterile
	SP:	Pale reddish brown to violet-red
Inorganic salts - starch agar (ISP 4)	G:	Fair to good, yellow-brown to light orange
	AM:	None
	SP:	Pale orange
Modified thin inorganic salts - starch agar	G:	Good, yellow-brown to light orange
	AM:	None to sparse, white, spores none to sparse
	SP:	None
Glycerol - asparagine agar (ISP 5)	G:	Fair to good, yellow-brown to reddish brown (ICSS-NBS 43, moderate reddish brown)
	AM:	Moderate white bloom, sterile
	SP:	Pale yellow-brown, variably present
GAUZE's mineral agar I	G:	Fair to good, yellow-brown
	AM:	None to sparse, white, sterile
	SP:	None
GAUZE's organic agar 2	G:	Fair, reddish orange (ISCC-NBS 35, strong reddish orange)
	AM:	None
	SP:	None
Peptone - yeast extract - iron agar (ISP 6)	G:	Fair, yellow-brown
	AM:	None
	SP:	None
Glucose - yeast extract agar (ISP 6)	G:	Fair to good, yellow-brown to brownish orange (ICSS-NBS 54)
	AM:	None
	SP:	None
Synthetic agar	G:	Poor to fair, orange (ISCC-NBS 48, vivid orange to ISCC-NBS 50, strong orange)
	AM:	None
	SP:	None
C-2 agar with glucose	G:	Good, violet-red to purple (ISCC-NBS 243, very dark reddish purple)
	AM:	Moderate, white to pale pink, sterile
	SP:	Pale lavender to purplish red (ISCC-NBS 262, grayish purplish red)

[a] G: Growth of substrate mycelium, AM: aerial mycelium, SP: soluble pigment.

Chemotaxonomy, Physiological and Biochemical Characteristics

Purified cell wall preparations contained *meso*-diaminopimelic acid, alanine, glutamic acid, glucosamine, muramic acid, a major amount of glucose and a trace of mannose. Whole-cell hydrolysates contained galactose, glucose, mannose, ribose and madurose. Phospholipids present were phosphatidyl ethanolamine, phosphatidyl methylethanolamine, diphosphatidyl glycerol, phosphatidyl inositol, traces of phosphatidyl inositol mannosides and an unknown glucosamine-containing phospholipid. Thus, SK&F-AAJ-271 has a type III cell wall with a type B sugar pattern[34] and a phospholipid pattern of type PIV.[19]

The organism does not grow under anaerobic conditions. Temperature range for growth was

15 to 45°C; growth at 15 and 45°C usually was poor. The following tests were positive: reduction of nitrate to nitrite; hydrolysis of adenine (0.5%), casein, elastin, gelatin, hypoxanthine and L-tyrosine; decomposition of allantoin, arbutin, esculin and urea; production of catalase, phosphatase, deoxyribonuclease and ribonuclease. Hydrolysis of starch was weak and partial. Decomposition of hippurate was positive following prolonged incubation (8 weeks). Hydrogen sulfide production was weakly positive. Negative results were obtained for: production of melanin; liquefaction of gelatin; hydrolysis of cellulose (Avicel) and xanthine. No growth occurred in lysozyme broth or in 4% NaCl. SK&F-AAJ-271 was resistant to discs containing rifampicin (5 μg) and benzylpenicillin (10 U).

Acid was produced from adonitol, L-arabinose, D-cellobiose, dextrin, glucose, D-fructose, D-galactose, glycerol, *i*-inositol, inulin, lactose, maltose, D-mannitol, D-mannose, melibiose, α-methyl-D-glucoside, α-methyl-D-mannoside, raffinose, rhamnose, D-ribose, salicin, D-sorbitol, starch, sucrose, trehalose and D-xylose. No acid was produced from dulcitol, *i*-erythritol, D-melezitose and L-sorbose. Citrate, malate, succinate, oxalate, lactate, acetate, pyruvate and formate were utilized; benzoate, propionate, tartrate and mucate were not utilized.

On the C-2 medium of NONOMURA and OHARA,[27] SK&F-AAJ-271 utilized adonitol, L-arabinose, D-cellobiose, glucose, D-fructose, D-galactose, *i*-inositol, D-mannitol, raffinose, rhamnose, salicin, sucrose and D-xylose as sole carbon sources; inulin was poorly utilized. The vitamins in this medium were not essential for the growth of SK&F-AAJ-271, but they did enhance substrate mycelium growth and pigment production.

Identification and Classification

The description of SK&F-AAJ-271 was compared with those of the *Actinomadura* species listed on the Approved Lists of Bacterial Names. The type cultures of those species having a red, reddish brown or violet-red substrate mycelium with a pink aerial mycelium (*Actinomadura luzonensis* ATCC 31491, *Actinomadura roseola* ATCC 33579, *Actinomadura roseoviolacea* ATCC 27297, *Actinomodura rubra* ATCC 27031, *Actinomadura salmonea* ATCC 33580, *Actinomadura vinacea* ATCC 33581) were grown on a series of 15 media used in the morphological characterization of SK&F-AAJ-271. These direct comparisons of morphology and color, along with biochemical and physiological data from the taxonomic literature, permitted easy differentiation of *A. luzonensis*, *A. roseoviolacea*, *A. roseola*, *A. rubra*, *A. salmonea* and *A. vinacea* from SK&F-AAJ-271.

A. roseola and *A. vinacea* both produce spore chains which are straight, hooked or slightly wavy. The spore surface of these two species is warty.[35] *A. roseoviolacea* produces spore chains which are predominantly tightly closed spirals that form pseudo-sporangia. On peptone - yeast extract - iron agar, *A. roseoviolacea* produces a reddish black to black substrate mycelium, no aerial mycelium and a grayish-reddish brown soluble pigment. *A. roseoviolacea* is resistant to lysozyme and does not degrade adenine or elastin.[23] It neither grows in the presence of 0.4% adenine nor produces allantoinase or urease.[36]

A. luzonensis produces only pale orange-yellow to yellow-brown soluble pigments and does not utilize sucrose, raffinose or salicin as sole carbon sources.[37] *A. rubra* is a much more intensely pigmented culture than SK&F-AAJ-271. On oatmeal agar (ISP 3), yeast extract - malt extract agar (ISP 2), GAUZE's mineral agar I and glucose - yeast extract agar, *A. rubra* produces a bright reddish orange to dark reddish brown substrate mycelium with a bright red-orange to scarlet soluble pigment. In addition, *A. rubra* does not produce allantoinase or urease.[36]

Of the six cultures compared directly with SK&F-AAJ-271, the spore chains of *A. salmonea* most closely resembled those found in SK&F-AAJ-271. The spore surface of *A. salmonea*, however, was warty.[35] On synthetic agar, *A. salmonea* grew poorly and the substrate mycelium was translucent to pale yellow-brown, with no aerial mycelium or soluble pigment present. *A. salmonea* neither produced allantoinase or urease nor degraded ribonucleic acid or L-tyrosine.[36]

Our efforts to obtain *Actinomadura carminata* for comparative taxonomic purposes were unsuccessful. However, patent literature indicates[38] that *A. carminata* formed spore chains arranged monopodially along the hyphae that, more often than not, were twisted in a tightly closed spiral, doughnut or dense ball. The number of spores per chain was 6 to 14 and the spores were round and smooth-walled. On yeast extract - malt extract agar (ISP 2), *A. carminata* produced a brownish violet substrate mycelium, a well-developed, fluffy pink aerial mycelium and no soluble pigment. Taxonomic literature[39] indicates that the spore chains of *A. carminata* were predominantly tightly closed spirals that were embedded in slime to form pseudo-sporangia. On oatmeal agar, growth of *A. carminata* was good with a pale lilac to reddish lilac to red-violet substrate mycelium, a well-developed pink aerial mycelium and a weak soluble pigment. On GAUZE's organic agar 2, *A. carminata* produced a brownish violet or blackish violet substrate mycelium with no aerial mycelium or soluble pigment. Thus, SK&F-AAJ-271 was readily distinguishable from *A. carminata* on the basis of spore chain morphology and length, as well as pigmentation and growth characteristics on various media.

Strain SK&F-AAJ-271 is therefore regarded as a new species of the genus *Actinomadura* for which we propose the name *Actinomadura parvosata* (*parvus* L. adj., small in number; *satus* L. adj., seeded). The specific epithet refers to the few, if any, spores produced by this culture on media normally used to characterize actinomycetes. Strain SK&F-AAJ-271, the type strain of *A. parvosata*, has been deposited in the American Type Culture Collection, Rockville, MD, under the accession number ATCC 53463.

Production and Isolation of the Parvodicins

Production-stage fermentations of *A. parvosata* were conducted under conditions similar to those reported previously for *Kibdelosporangium aridum*.[9,11] Antibiotic production, which was coincident with a purple pigmentation of the broth, occurred during growth and peaked by 4 days, declining after biomass had reached a maximum.

Partially-purified samples of the *A. parvosata* fermentation broth, examined by RPHPLC and isoelectric focusing,[30] contained a complex of at least ten lipophilic components of low isoelectric point (3.8). The two major components were designated C_1 and C_3 on the basis of their RPHPLC retention times relative to those of the aridicin "C" components[10] (Fig. 1a); the remaining components were denoted analogously. Upon repeated analysis of samples collected during the course of the fermentation, it was observed that the level of components C_3 and C_4 in each sample decreased with a concomitant increase in the level of components C_1 and C_2. This apparent conversion was accelerated by brief treatment of the samples with base (Fig. 1b), and was accompanied by an increase in the *in vitro* biological activity of the antibiotic complex. Consequently, two similar but distinct isolation schemes were followed.

The primary isolation procedure began with adsorption of the complex from clarified broth onto the weak anion exchange resin IRA-68, followed by elution with dilute ammonium hydroxide; this step was necessitated by the low fermentation titer. The neutralized eluate was desalted and subjected to a standard course of Diaion HP-20 and Affigel-10-D-Ala-D-Ala[6,29] chromatography. Concentra-

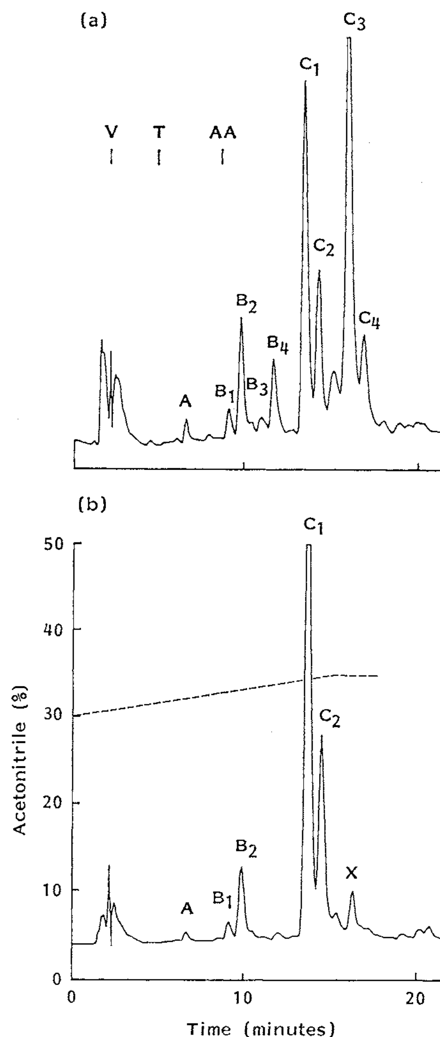
tion of the Affigel eluate using Diaion HP-20 resin provided 4 g of solid material containing primarily components A, B_1, B_2, C_1 and C_2.

While analytical RPHPLC separation[28] of these components was achieved routinely, attempted scale-up to a large-particle (>40 μm) semi-preparative column[5,10] resulted in a loss of resolution of the C_1 and C_2 components. This very difficult separation problem was overcome by use of a shallow gradient of acetonitrile on a large column of small-particle reversed-phase packing (Fig. 2). At higher loadings, this system effectively differentiated C_1 and C_2 despite the subtle nature of their structural difference (*vide infra*), allowing 30~50 mg of each to be obtained in pure form per chromatographic run after desalting. Quantities of pure A, B_1 and B_2 (6~20 mg) also were obtained per run after desalting.

For isolation of components C_3 and C_4 the IRA-68 step was omitted and the affinity chromatography was conducted with only brief exposure to dilute ammonia. Semi-preparative RPHPLC on a small-particle column (Whatman Partisil ODS-3, 2.2×50 cm, 10 μm) using a step-gradient of acetonitrile in potassium phosphate buffer provided components C_1, C_2, C_3 and C_4, which eluted at 29% acetonitrile.

Fig. 1. Analytical RPHPLC chromatogram of affinity-purified parvodicin complex before (a) and after (b) treatment with ammonia.

Altex Ultrasphere-ODS, 5 μm, 4.6×150 mm; 30~35% acetonitrile in 0.01 M phosphate (pH 3.2); 1.5 ml/minute flow rate; UV detection at 220 nm. The relative retention times of vancomycin (V), teicoplanin A2-2 (T) and aridicin A (AA) are noted in (a).



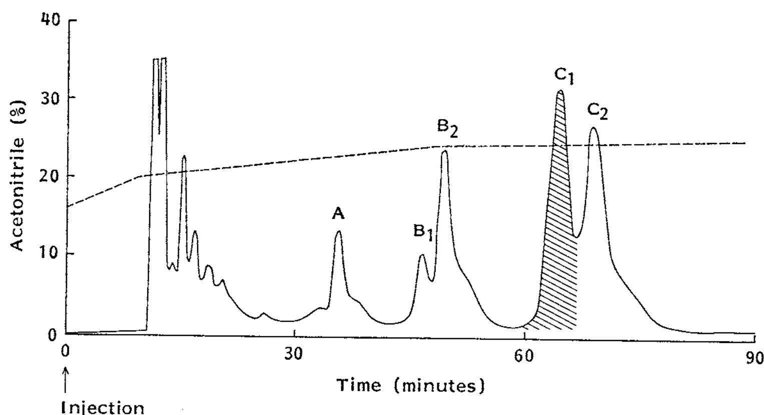
Mass Spectral and Chemical Characterization

Detailed information on the isolated components (Table 2) was provided by FAB-MS,[7] which revealed from relative intensities of peaks in the molecular ion clusters that two atoms of chlorine were present in each component.

Components C_1 and C_2 each had a nominal mass of 1,730 daltons. An exact mass measurement of the MH^+ of C_1 was obtained at a resolution of 10,000 by peak matching against the MH^+ (1,758 daltons) of renin substrate, providing a determined accurate mass of 1,731.496±0.01 daltons. Given the constraint of two chlorine atoms in the molecule, the number of possible elemental compositions was narrowed[7] and, in conjunction with chemical and additional spectral data (*vide infra*), a molecular formula of $C_{83}H_{88}Cl_2N_8O_{29}$ (calculated mass: m/z 1,730.503) was established. Major fragment ions in the spectrum of each component indicated the presence of both a hexose (m/z 1,569, MH^+-162) and an *N*-dodecanoyl hexuronic acid (m/z 1,374,

Fig. 2. Preparative RPHPLC chromatogram of ammonia-treated, affinity-purified parvodicin complex (650 mg).

Vydac C18, 15~20 μm, 300 Å, 5.1×50 cm; 15~25% acetonitrile in 0.01 M phosphate (pH 6.0); 100 ml/minute flow rate; UV detection at 280 nm.



MH^+-357) moiety at terminal locations.[7] An N-dodecanoyl glucuronic acid moiety had been observed previously as a constituent of aridicin C.[31]

The 1,772 dalton nominal mass determined from the FAB-MS of components C_3 and C_4 suggested the presence of an additional acetyl group (42 daltons) in these molecules. Fragment ions observed at m/z 1,416 (MH^+-357) indicated that the acetyl function did not reside on the N-dodecanoyl hexuronic acid moiety of these components. Brief ammonolysis collapsed component C_3 to C_1 and component C_4 to C_2, each identified by RPHPLC and confirmed by FAB-MS analysis; this base lability of the acetyl group suggested that it was attached at an oxygen rather than a nitrogen atom.

Similarly, FAB-MS analysis revealed a nominal mass of 1,716 daltons for both components B_1 and B_2, with fragment ions indicating the presence of a hexose (m/z 1,555, MH^+-162) and an N-undecanoyl hexuronic acid (m/z 1,374, MH^+-343) moiety in each component. FAB-MS analysis of component A indicated a nominal mass of 1,702 daltons and the presence of a hexose (m/z 1,541, MH^+-162) and an N-decanoyl hexuronic acid (m/z 1,374, MH^+-329) moiety.

Upon mild acid hydrolysis under two different sets of conditions, each of these components liberated the same aglycone (aglycone I, MH^+ m/z 1,212, derived molecular formula $C_{59}H_{47}Cl_2N_7O_{18}$) and hexosyl aglycone ($MH^+$ m/z 1,374); the hexose was identified by carbohydrate analysis[10] as D-mannose. Therefore, with the structural difference between the A, B and C_1/C_2 components and the structural difference within the isomeric pairs B_1/B_2, C_1/C_2 and C_3/C_4 apparently confined to the glycolipid moiety, fatty acid analysis was conducted. Samples were subjected to total methanolysis and organic extracts of the products were analyzed by GC and GC-MS[12,31] (Table 2). Methyl 10-methylundecanoate and methyl *n*-dodecanoate were identified by comparison with authentic standards as the respective methanolysis products from C_1/C_3 and C_2/C_4. Similarly identified were methyl 9-methyldecanoate from B_1, methyl *n*-undecanoate from B_2 and methyl *n*-decanoate from A.

These data indicate that parvodicins A~C, like aridicins A~C, are based upon a common mannosyl aglycone with individual members differentiated by the nature of the N-acyl group in the aminodeoxy hexuronic acid moiety.[8,10,31] Additional variation is introduced into some parvodicin components by a base-labile O-acetyl group, a functionality unique among the known glycopeptide

Table 2. Characterization of parvodicins.

Component	Empirical formula	FAB-MS (*m/z*)			Derived fatty acid methyl ester			UV ($E^{1\%}_{1cm}$, 280 nm)	
		MH^+	Fragment 1	Fragment 2		MH^+ (*m/z*)	GCRT[a] (minutes)	CH_3CN - H_2O (1 : 1)	(0.1 M HCl)
A	$C_{81}H_{84}Cl_2N_8O_{29}$	1,703	1,541	1,374	*n*-Decanoate	187	5.26[b]	61	—
B_1	$C_{82}H_{86}Cl_2N_8O_{29}$	1,717	1,555	1,374	9-Methyl decanoate	201	6.10[b]	60	—
B_2	$C_{82}H_{86}Cl_2N_8O_{29}$	1,717	1,555	1,374	*n*-Undecanoate	201	6.56[b]	57	—
C_1	$C_{83}H_{88}Cl_2N_8O_{29}$	1,731	1,569	1,374	10-Methyl undecanoate	215	6.90[c]	58	51
C_2	$C_{83}H_{88}Cl_2N_8O_{29}$	1,731	1,569	1,374	*n*-Dodecanoate	215	7.34[c]	—	45
C_3	$C_{85}H_{90}Cl_2N_8O_{30}$	1,773	—	1,416	10-Methyl undecanoate	215	6.90[c]	—	46
C_4	$C_{85}H_{90}Cl_2N_8O_{30}$	1,773	—	1,416	*n*-Dodecanoate	215	7.34[c]	—	45

[a] GC retention times. [b] 90~275°C, 10°C/minute. [c] 75~250°C, 12°C/minute.

antibiotics of this class.[40] Within the heptapeptide core of the parvodicin aglycone, mass spectral data indicate the absence of one oxygen and two chlorine atoms relative to aridicin aglycone[8] and the presence of one additional methylene unit relative to teicoplanin aglycone.[41]

Structure of the Parvodicins

The structure of the parvodicin aglycone, as well as the form and sites of carbohydrate and acetyl group attachment in parvodicins C_1 and C_3, was derived from comparison of the 2D NMR spectral patterns from C_1 and C_3 with those from aridicin A[8] and teicoplanin A2-2.[42] The spectral analysis was facilitated by the high resolution observed for individual resonances in spectra recorded at 500 MHz in D_2O - DMSO-d_6 (1 : 1) solvent mixtures at 45~50°C and pH 6. Under these conditions, signals for the amine, amide, carboxyl and hydroxyl protons are not observed due to exchange with the D_2O cosolvent.

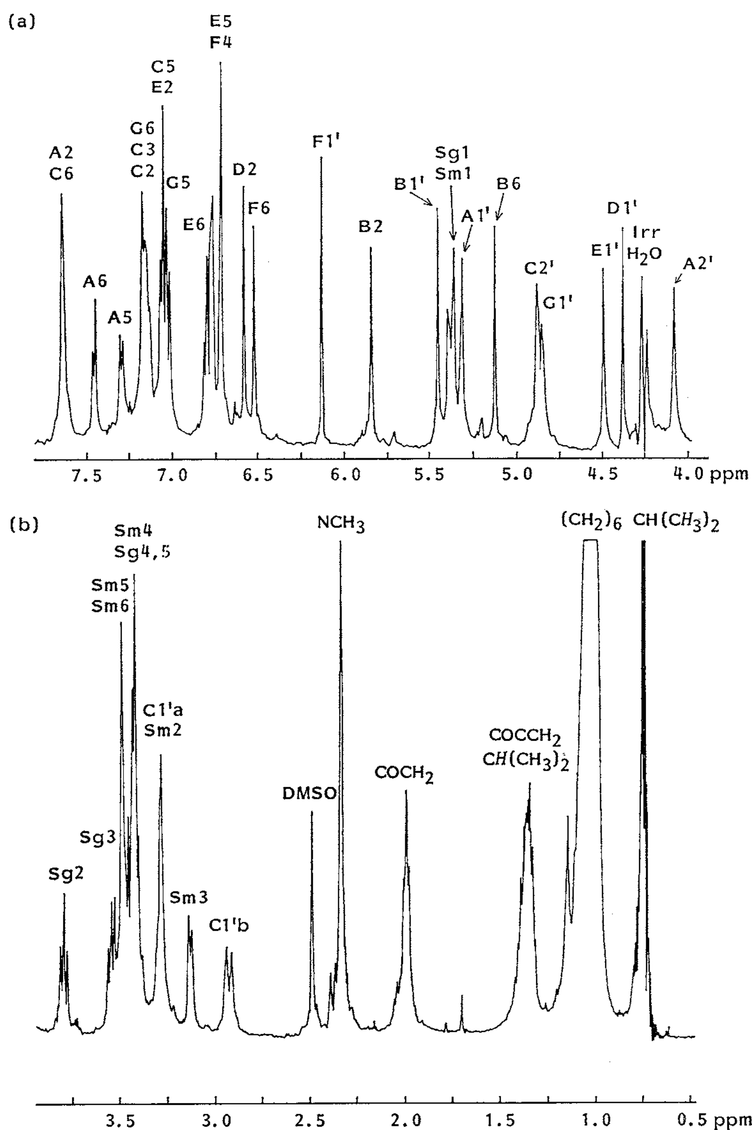
Immediately apparent at highest field in the unenhanced one-dimensional spectrum of parvodicin C_1 (Fig. 3) are the six-proton methyl doublet,* the methine and the eight methylene proton signals expected for the 10-methyl undecanoyl portion of the glycolipid residue. Also, an *N*-methyl singlet (2.34 ppm) is obvious. The remaining signal assignments (Table 3) are derived from 2D proton correlation spectroscopy (COSY) and nuclear Overhauser spectroscopy (NOESY) experiments,[8] which provide information on the bond connectivities and short, through-space relationships among individual protons. These data are summarized in Table 4, with the following spectral highlights instrumental in the elucidation of parvodicin C_1.

The 2D delayed-COSY spectrum of parvodicin C_1 revealed crosspeaks representative of proton-proton coupling connectivities for seven aromatic spin systems. The crosspeak patterns observed for the rings designated B, D, E, F and G[43] were identical to those in the corresponding spectra of aridicin A recorded under similar conditions, suggesting a structural homogeneity in these portions of the two molecules. The 2D NOE spectral patterns observed for the B, D, E and F rings of the two molecules also were extremely similar, as was the set of strong crosspeaks among the resonances A2, E2, A1′, B6, E1′ and A2′. This pattern is a common feature in the 2D NOE spectrum of all biologically active glycopeptide antibiotics of this class analyzed to date[8,42,44,45] and is a direct consequence of the stereochemical features within the A~E framework required for binding of these molecules to the L-Lys-D-Ala-D-Ala terminus of the cell wall peptidoglycan chain.[4,46,47] Within ring A, the appearance of the A2 resonance as a doublet (δ 7.56, $J_{A2,A6}$=2.0 Hz), as in the spectra of both aridicin A and teicoplanin A2-2, indicated the presence of a chlorine atom at A3. The resonance at δ 7.21 (d, $J_{A5,A6}$ =8.3 Hz) was assigned as A5 on the basis of the NOE observed to A6, despite the absence of an A5/A6 COSY crosspeak. Under the conditions of this particular delayed-COSY experiment (optimized for 2 Hz couplings), the weak crosspeak expected for these *ortho*-paired spins was absent because of excessive line-broadening in the A5 and A6 resonances.[42] The ABMX coupling pattern, with each resonance appearing as a doublet of doublets, observed for ring C in the 2D COSY spectrum of parvodicin C_1 revealed the absence of chlorine on this ring. This was strengthened by the presence of a strong C3/B2 NOE signal which was not observed in the spectrum of either aridicin A or teicoplanin A2-2. The strong NOE crosspeak pattern involving C6, C2, C2′, C1′a and C1′b, as in the teicoplanin spectrum, indicated the absence of a C1′ hydroxyl function in parvodicin C_1.

* As a consequence of the y-axis expansion used for ease of visualization of the one-proton resonances in Fig. 3, sidebands bracketing this doublet are magnified, giving it the appearance of a multiplet.

Fig. 3. One-dimensional ^1H NMR spectrum of parvodicin C_1 at 500 MHz in D_2O - DMSO-d_6 (1 : 1) at 45°C.

Irr H_2O: Residual signal from water suppression.



The absence of F2/F4 and F2/F6 COSY crosspeaks and F2/F1′ NOE allowed placement of the second chlorine at F2, as in the aridicins. The F3 hydroxyl was inferred from the appearance of the F4 resonance as a doublet (6.68 ppm, 3.0 Hz) coupled to F6. The strong F6/G2 NOE confirmed the F5-G3 ether linkage. Finally, the strong NOEs observed between the methyl resonance and G1′ and G6 allowed placement of the methyl group on the terminal nitrogen atom of the nucleus.

In spite of the absence of crosspeaks to the NH protons in the D_2O - DMSO-d_6 solvent mixture, the large number of correlations which existed in the 2D NOE maps suggested that the stereochemistries at the eight chiral centers and six peptide bonds of parvodicin C_1 were the same as those in aridicin A and teicoplanin A2-2. Furthermore, it was apparent that all three glycopeptides maintained the

Table 3. Proton chemical shift assignments of parvocidin C_1.[a]

Assignment	δ	J (Hz)	Assignment	δ	J (Hz)
A Residue			F Residue		
A2	7.56	d, 2.0	F4	6.68	d, 3.0
A5	7.21	d, 8.3	F6	6.44	d, 3.0
A6	7.37	dd, 2.0 and 8.3	F1′	6.05	s
A1′	5.23	br	G Residue		
A2′	4.00	d, 2.3	G2	6.63	d, 2.3
B Residue			G5	6.94	d, 8.2
B2	5.76	d, 2.3	G6	7.09	dd, 2.3 and 8.2
B6	5.04	d, 2.3	G1′	4.77	br s
B1′	5.37	s	NCH_3	2.34	s
C Residue			Glycolipid (Sg) Residue		
C2	7.05	dd, 2.0 and 8.2	Sg1	5.31	d, 8.2
C3	7.08	dd, 2.0 and 8.2	Sg2	3.80	dd, 8.2 and 10
C5	6.98	dd, 2.0 and 8.5	Sg3	3.55	dd, 8.0 and 10
C6	7.56	dd, 2.0 and 8.5	Sg4	3.42	Unresolved
C1′a	3.28	br d	Sg5	3.45	Unresolved
C1′b	2.93	br d, 13	$COCH_2$	1.99	t, 7.5
C2′	4.80	dd, 3.5 and 5.0	$COCCH_2$	1.37	m
D Residue			$(CH_2)_6$	1.04	m
D4	6.63	d, 2.2	$CH(CH_3)_2$	1.37	m
D2	6.50	d, 2.2	$CH(CH_3)_2$	0.75	d, 6.5
D1′	4.30	s	Mannose (Sm) Residue		
E Residue			Sm1	5.28	d, 1.8
E2	6.97	d, 2.5	Sm2	3.28	dd, 1.8 and 3.5
E5	6.68	d, 8.5	Sm3	3.13	dd, 3.5 and 9.0
E6	6.72	d, 2.5 and 8.5	Sm4	3.45	Unresolved
E1′	4.41	s	Sm5,6	3.49	Unresolved

[a] Assignments in solvent D_2O - DMSO-d_6 (1 : 1) at 45°C and pH 6. Coupling constants are derived from first-order analysis of spin systems.

same three-dimensional solution conformation in this solvent mixture.

Definitive evidence for the placement of mannose at the D5 phenol of parvodicin C_1 was the COSY crosspeak between the protons at D4 and mannose C-1 (Sm1) indicative of long-range coupling through the glycosidic oxygen atom. This was supported by the strong NOEs D4/Sm1 and D4/Sm2. The small Sm1-Sm2 coupling constant (1.8 Hz), as well as the strong NOE pattern Sm1/Sm2, Sm2/Sm3 and Sm3/Sm4, established the α-anomeric linkage of the sugar.[8,42] The $J_{3,4}$ coupling of 9 Hz expected for the $^4C_1$ pyranoside form of the D-mannose ring was observed.[8,48,49]

Finally, the weak NOE crosspeak observed from the anomeric proton glycolipid C-1 (Sg1) to proton C5 in the Sg1 cross section of the 2D NOE spectrum of parvodicin C_1 established attachment of the glycolipid at the B4 phenolic oxygen. The appearance of this resonance as a doublet ($J_{1,2}$= 8.1 Hz) coupled to Sg2 was indicative of attachment *via* a β-anomeric linkage. The strong *syn*-axial NOE pattern Sg1/Sg3, Sg1/Sg5, Sg3/Sg5 and Sg2/Sg4, along with the 8 to 10 Hz ^3J coupling constants, supported the $^4C^1$ conformation of a β-D sugar.[48,49] The extreme similarity of the sugar resonances in the parvodicin C_1 spectra to those in aridicin A spectra supported assignment of the 2-[10-methyl-undecanamido]-2-deoxy-β-D-glucopyranosiduronic acid structure.[8,31]

The one-dimensional and 2D COSY spectra of parvodicin C_3 were virtually identical to the corresponding spectra of component C_1 with four exceptions. The first of these was the 0.26 ppm relative

Table 4. Scalar and 2D NOE connectivities of parvodicin C_1.

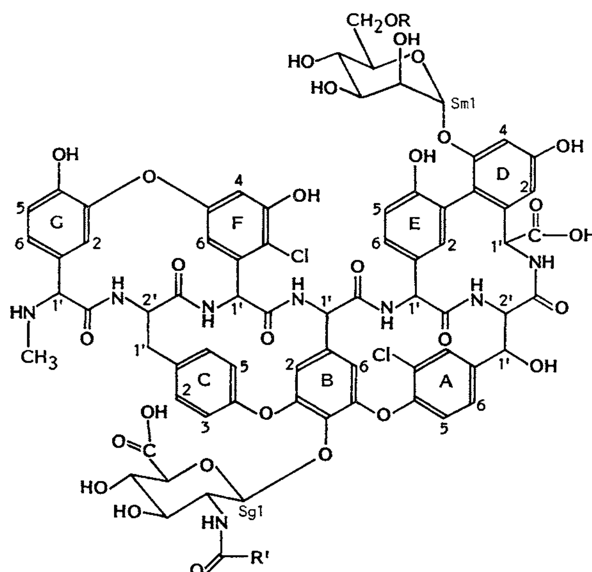
COSY crosspeaks	COSY crosspeaks	NOESY crosspeaks	NOESY crosspeaks
A Residue	F Residue	A Residue	D Residue
A2, A6	F4, F6	A2′, A2	D1′, D2
A1′, A2′	F1′, F4	A2′, E2	D4, Sm1
B Residue	F1′, F6	A2′, A1′	D4, Sm2
B2, B6	G Residue	A2′, E1′	E Residue
B1′, B2	G2, G6	A1′, A2	E1′, E2
B1′, B6	G5, G6	A1′, E1′	F Residue
C Residue	Glycolipid	A1′, E2	F6, G2
C5, C6	Sg1, Sg2	A5, A6	G Residue
C2, C6	Sg2, Sg3	A5, B6	G1′, G6
C2, C3	Sg3, Sg4	A2′, B6	G1′, NCH_3
C5, C3	$CH(CH_3)_2$	A2, E1′	G5, G6
D Residue	Mannose	B Residue	G6, NCH_3
D2, D4	Sm1, D4	B1′, B2	Glycolipid
D4, Sm1	Sm1, Sm2	B1′, B6	Sg1, Sg2
D1′, D2	Sm2, Sm3	B2, C2	Sg1, Sg3
E Residue	Sm3, Sm4	B2, C3	Sg1, Sg5
E2, E6		B6, E6	Sg2, Sg3
E5, E6		B6, E1′	Sg2, Sg4
		B2, F1′	Sg3, Sg5
		C Residue	Mannose
		C2′, C1′a	Sm1, D4
		C2′, C1′b	Sm1, Sm2
		C1′a, C1′b	Sm2, Sm3
		C2, C1′a	Sm3, Sm4 and 5
		C2, C1′b	
		C6, C1′b	
		C6, C5	

downfield shift (to 5.03 ppm) of the G1′ resonance of parvodicin C_3. This resonance, adjacent to the amine terminus of the peptide chain, is strongly affected by changes in the charge density at the nitrogen atom, which varies with sample pH.[8] The observed chemical shift differences between these resonances, therefore, can be attributed simply to a slight pH difference between the two samples rather than to a structural difference in this region of the two molecules.

The three remaining differences are structurally significant. An additional three-proton singlet (1.90 ppm), consistent with the presence of an *O*-acetyl function, is observed in the spectrum of component C_3. Also, the resonances for the two protons of the mannose C-6 (Sm6, 4.08 ppm) and the proton of the mannose C-5 (Sm5, 3.66 ppm) are shifted downfield by 0.59 and 0.17 ppm from their respective positions in the spectrum of component C_1. These differences are consistent with the presence of the *O*-acetyl function at the C-6 oxygen of the mannose residue,[50] with the remainder of the molecule identical in structure to component C_1.

NMR spectral analysis of the remaining parvodicin components was not conducted. However, since mass spectral data and hydrolysis experiments indicated that components A, B_1, B_2, C_1, C_2, C_3 and C_4 are based on a common mannosyl aglycone, it is inferred, by analogy to the aridicin,[8,10,31] kibdelin[12] and teicoplanin[41,51] series of glycopeptides, that the *N*-acylglucuronic acid moiety of each component is attached at the B-4 phenol. Component C_4, converted to component C_2 under basic conditions, is assumed to be acetylated at the mannose C-6 oxygen by analogy to component C_3. On

Fig. 4. Structure of the parvodicins.



Parvodicin A	R=H	$R'=n\text{-}C_9H_{19}$
Parvodicin B_1	R=H	$R'=(CH_2)_7CH(CH_3)_2$
Parvodicin B_2	R=H	$R'=n\text{-}C_{10}H_{21}$
Parvodicin C_1	R=H	$R'=(CH_2)_8CH(CH_3)_2$
Parvodicin C_2	R=H	$R'=n\text{-}(CH_2)_{10}CH_3$
Parvodicin C_3	$R=COCH_3$	$R'=(CH_2)_8CH(CH_3)_2$
Parvodicin C_4	$R=COCH_3$	$R'=n\text{-}(CH_2)_{10}CH_3$

the basis of the data and these assumptions, therefore, the major components of the parvodicin complex are assigned the structures of Fig. 4.

Minor Components of the Complex

From the analytical chromatogram of Fig. 1a, it is apparent that several minor components also are present in the complex. None of these components were isolated or characterized. However, the components labeled B_3 and B_4 (Fig. 1a) are, almost certainly, the *O*-acetylated analogues of components B_1 and B_2, respectively, based upon their conversion to these components under basic conditions (*cf* Fig. 1b).

The species labeled X in Fig. 1b, also never isolated, is representative of a third structural variation within the complex. Component X had an analytical RPHPLC retention time similar or identical to that of component C_3, with which it co-eluted at the semi-preparative level. FAB-MS of component C_3 revealed a minor molecular ion peak cluster at *m/z* 1,745. This cluster remained unchanged in the mass spectra of samples in which C_3 had been converted entirely to C_1 by saponification of the acetate ester. While the 14 dalton mass increase of X relative to C_1/C_2 initially was suggestive of a homolog differing in the glycolipid, a minor mass spectral fragment ion cluster at *m/z* 1,388 indicated that the source of heterogeneity resided in the mannosyl aglycone.

This was confirmed with the isolation of a second aglycone (aglycone II) from the acid hydrolysate of a sample of complex enriched in minor components. Aglycone II, slightly more lipophilic than aglycone I, was determined by FAB-MS to be a two-chlorine-containing species of nominal mass 1,225

Table 5. Proton chemical shift assignments of aglycones.[a]

Assignment	Aglycone I		Aglycone II	
	δ	J (Hz)	δ	J (Hz)
ANH	6.71	12.2	6.72	11.7
A1′	5.17		5.17	
A2′	4.18		4.18	
A2	7.84		7.84	
A6	7.51		7.52	
A5	7.28		7.29	
BNH	7.18	7.2	7.17	7.5
B1′	5.63		5.64	
B2	5.83		5.84	
B6	5.13		5.13	
CNH	8.06	8.4	8.26	8.1
C2′	5.01		4.96	
C1′a	3.41	14.4 and 5.7	3.41	14.4 and 6.0
C1′b	2.97	14.4 and 2.7	3.03	
C2	7.17		7.17	
C3	7.25		7.23	
C5	7.09		7.09	
C6	7.78		7.77	
DNH	8.48	6.3	8.48	6.8
D1′	4.51		4.51	
D2	6.34		6.36	
D4	6.47		6.47	
ENH	8.50	6.0	8.50	6.8
E1′	4.41		4.43	
E2	7.16		7.17	
E5	6.74		6.74	
E6	6.78		6.78	
FNH	7.63	10.6	7.60	10.2
F1′	6.13		6.15	
F4	6.68		6.68	
F6	6.58		6.57	
G1′	5.39		5.50	
G2	6.84		6.99	
G5	7.09		7.11	
G6	7.26		7.28	
N-Methyl 1	2.56		2.68	
N-Methyl 2	—		2.89	

[a] Assignments in DMSO-d_6 containing 3% trifluoroacetic acid at 40°C.

daltons, 14 daltons higher in mass than aglycone I.

One-dimensional ^{1}H NMR spectra were recorded at 500 MHz in D_2O - DMSO-d_6 (1 : 1) at pH 6~7 and 40°C for both species. Resonances in the spectrum of aglycone I displayed chemical shifts and couplings which were virtually identical to those observed for the protons of the heptapeptide core in the spectrum of component C_1 and were assigned on this basis. Exceptions were the resonances for G1′, shifted 0.23 ppm upfield from its position in the spectrum of component C_1, presumably because of sample pH differences (*vide supra*), and the resonances for D2 and D4, which were shifted upfield 0.21 and 0.28 ppm, respectively, as is expected when mannose is removed.[8,52] In the spectrum of aglycone II, the additional methylene/methyl resonance expected on the basis of the mass spectral

Table 6. Antibacterial activity of the parvodicins.

Compound	MIC (μg/ml)								
	S.a. HH127	*S.a.* 910	*S.a.* 209P	*S.a.* 675[a]	*S.e.* 2479	*S.h.* 651[a]	*E.f.* 34358[a]	*E.f.* 657[a]	*S.s.* 2657
Parvodicin complex	3.1	1.6	0.8	3.1	12.5	25	0.1	0.1	6.3
Parvodicin C_4	3.1	3.1	1.6	6.3	25	25	0.4	0.4	12.5
Parvodicin C_3	1.6	1.6	0.4	3.1	25	25	0.1	0.05	6.3
Parvodicin C_2	0.8	0.8	0.4	1.6	12.5	50	0.4	0.2	0.8
Parvodicin C_1	0.8	0.4	0.4	0.8	6.3	25	0.2	0.2	0.4
Aglycone I	0.4	0.4	0.4	0.8	0.8	3.1	1.6	1.6	0.8
Aglycone II	0.4	0.4	0.4	0.4	0.8	6.3	1.6	1.6	0.8
Aridicin A	6.3	3.1	1.6	6.3	50	50	1.6	0.8	25.0
Teicoplanin	3.1	0.8	0.4	3.1	12.5	12.5	0.1	0.1	1.6
Vancomycin	1.6	1.6	1.6	1.6	1.6	3.1	1.6	1.6	1.6
Parvodicin A	3.1	N.T.	N.T.	6.3	12.5	50	3.1	3.1	N.T.
Parvodicin B_1	1.6	N.T.	N.T.	3.1	50	50	1.6	3.1	N.T.
Parvodicin B_2	6.3	N.T.	N.T.	6.3	50	50	3.1	3.1	N.T.

[a] Methicillin-resistant strain.

S.a.: *Staphylococcus aureus*, *S.e.*: *Staphylococcus epidermidis*, *S.h.*: *Staphylococcus hemolyticus*, *E.f.*: *Enterococcus faecalis*, *S.s.*: *Staphylococcus saprophyticus*.

N.T.: Not tested.

data was not observed, but careful integration of the *N*-methyl singlet revealed a relative six-proton intensity, suggesting that two methyl groups were present on the terminal nitrogen atoms of this aglycone.

Proof of this simple structural difference was provided by analysis of one- and two-dimensional NMR data obtained at 40°C in DMSO-d_6 containing 3% trifluoroacetic acid, conditions which allow the amide proton resonances to be observed. As summarized in Table 5, the chemical shifts and amide couplings displayed by the two species were virtually identical, with only the CNH (+0.2 ppm), G2 (+0.15 ppm) and G1′ (+0.11 ppm) resonances affected by the presence of the second methyl group. Also, upon amine protonation under the acidic conditions of this experiment, the two resonances expected for the diastereotopic *N*-methyl groups were observed. In the 2D NOE spectrum of aglycone II, the observed crosspeak patterns were extremely similar to those in the spectrum of aridicin aglycone recorded under comparable conditions,[8] providing unambiguous evidence that the stereochemistries at the chiral centers of these two glycopeptides were identical.

Antibacterial Activity

Comparison of the *in vitro* activity of the Affigel-purified parvodicin complex (*ca.* 85% w/w) with the isolated components was informative (Table 6). Parvodicins C_1 and C_2 were found to be considerably more potent than their acetylated analogs C_3 and C_4 against selected strains of Staphylococci *in vitro*. This explained the observed increase in the *in vitro* activity of the complex during the purification process as components C_3 and C_4 were saponified to C_1 and C_2.

Parvodicin C_1 is the most active lipid-containing compound isolated by us to date. MIC values against *Staphylococcus aureus* strains are 1~4-fold lower than those of teicoplanin complex and 2~4-fold lower than those of vancomycin in our tests. Activity against coagulase-negative Staphylococci, though better than that of aridicin A, is inferior to that of vancomycin. In common with most of the lipid-containing glycopeptides, activity against Enterococci was high. As observed previously[53] in the

aridicin series of glycopeptides, the nature and length of the fatty acid portion of the glycolipid moiety are subtle determinants of activity *in vitro*. Terminal branching of the lipid chain appears to result in improved activity ($C_3>C_4$, $C_1>C_2$, $B_1>B_2$). Activity appears to peak at a chain length of twelve carbons ($C_2>B_2\simeq A$; $C_1>B_1$). Removal of the sugars leads to a considerable increase in activity against coagulase-negative Staphylococci.

Pharmacokinetics

Pharmacokinetic studies of parvodicin C_1 in mice indicated the potential for a long duration of action. Intravenous administration (20 mg/kg) resulted in a very high peak serum concentration (143 μg/ml), which declined in a mono-exponential manner with an elimination half-life of 11 hours. This was considerably longer than the β elimination half-life determined in parallel studies[32] for vancomycin (0.33 hour), teicoplanin complex (2.5 hours) and aridicin A (3.7 hours), and was attributed to the low systemic clearance (0.14 ml/minute/kg) of the compound. This, in turn, may be the result of the high serum binding (>98%) of the molecule due to its acidity and greater lipophilicity relative to aridicin A. These findings are consistent with the pharmacokinetic structure-activity relationships developed previously[32] for this class of antibiotics.

Conclusions

Upon thorough investigation of its morphology, chemotaxonomy and physiology, strain SK&F-AAJ-271 was determined to be a new species, which we have designated *Actinomadura parvosata*. Isolated from fermentations of this organism was a novel complex of acidic, lipophilic antibiotics of the vancomycin-ristocetin class,[40] the parvodicins. Mass spectral studies, in conjunction with chemical studies, revealed that parvodicins A~C were based upon a common mannosyl aglycone.

The structure within the heptapeptide core of this species, as well as the form and sites of carbohydrate attachment, were determined from 2D NMR studies of parvodicin C_1. Similar studies of parvodicin C_3 allowed determination of the mannose C-6 oxygen as the site of attachment of the *O*-acetyl group, a functionality unique among the known members of this class of antibiotics. The remaining isolated components differed from C_1 or C_3 only in the length and/or nature of the acyl portion of the *N*-acyl-aminodeoxyglucuronic acid residue. One or more minor components of the complex which were not isolated are *N*-dimethyl analogs of some major components.

Parvodicin C_1 is the component most active *in vitro* against staphylococcal strains. The acidity and lipophilicity of the molecule lead to high serum binding and a long *in vivo* β elimination half-life, suggesting the potential for a long *in vivo* duration of action.

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