COMPONENTS AND DEGRADATION COMPOUNDS OF THE AVOPARCIN COMPLEX

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The isolation and characterization of many of the components of the avoparcin complex are described. A number of mild degradations products from this complex are similarly treated. Conditions for the analytical and preparative HPLC resolution of these materials are outlined.

Avoparcin is a member of the same glycopeptide family of antibiotics as vancomycin and ristocetin[1,2,3]. While there is increased interest in vancomycin in the medical field in recent times avoparcin is also of growing importance in the agricultural industry as a feed additive[4]*. The avoparcin complex is composed of two major antibiotics called α- and β-avoparcin along with a number of minor components as indicated by analytical HPLC. We have already reported on the structures of the major components[5,6]. In this paper we describe the isolation of most of the components as well as the characterization of several of the minor antibiotics. The information provided should lead to a better understanding of structure activity relationships in this glycopeptide class.

Scheme 1 illustrates the structures of components frequently found in fermentation broth as well as some degradation products. The aromatic rings are labelled A through G with positions on each being designated by the numerals 1 through 6. Consequently any aromatic proton can be designated using a capital letter with a number subscript. The methine protons of the amino acids are numbered 1′ through 7′ starting with the terminal *p*-hydroxyphenylsarcosine. The two remaining benzylic protons of the aglycone are logically labelled A_{bz} and C_{bz}. The attached sugars are referred to using the first three letters of the name with the ristosamine of the disaccharide being primed. Hence carbohydrate protons can be described by the use of a three-letter abbreviation followed by H with a numeral subscript such as glu H_1 for the anomeric proton of glucose. Table 1 shows the relative retention times of the compounds of Scheme 1 as detected by analytical HPLC on a C_{18} μBondapak column.

Nos. **1** and **2**, The Deristosaminyl Compounds

The α- and β-deristosaminyl avoparcins have been isolated as a mixture of the two in the ratio of about one to four.** They are present to the extent of about a percent or less in all normally recovered batches. These materials have been shown to lack the ristosamine sugar of the attached disaccharide[5]. Very mild treatment such as standing at room temperature in dilute acetic acid solution will cause α- and β-avoparcin to be converted to the corresponding deristosaminyl compounds. The mixture of these two compounds exhibits about 50% or less of the activity of avoparcin against Gram-positive bacteria

* Avoparcin is licensed as a growth promoter in animal feed in a total of 25 countries to date.

** A mixture of 20% deristosaminyl-α-avoparcin and 80% of the corresponding β-compound had been isolated by the late Mr. J. H. Martin using CM-Sephadex chromatography. We failed to resolve these two components by preparative HPLC.

Scheme 1. Components of avoparcin complex.

(**1**) Deristosaminyl-α-avoparcin: R, R^5=H; R^1=*O*-β-D-glucose (**17**); R^2=*O*-α-L-ristosamine (**14**); R^3=*O*-α-D-mannose (**16**); R^4=*O*-α-L-rhamnose (**15**).

(**2**) Deristosaminyl-β-avoparcin: R=Cl; R^5=H; R^1, R^2, R^3, R^4 as in (**1**).

(**3**) D-Galactosyl-β-avoparcin: R, R^2, R^3, R^4 as in (**2**); R^1=*O*-2-L-(ristosaminyl)-*O*-β-D-glucose (**13**); R^5=*O*-α-D-galactose (**18**).

(**4**) α-Avoparcin: R, R^2, R^3, R^4, R^5 as in (**1**); R^1=*O*-2-L-(ristosaminyl)-*O*-β-D-glucose (**13**).

(**5**) β-Avoparcin: R, R^2, R^3, R^4, R^5 as in (**2**); R^1=*O*-2-L-(ristosaminyl)-*O*-β-D-glucose (**13**).

(**6**) *epi*-α-Avoparcin: R, R^1, R^2, R^3, R^4, R^5 as in (**4**); *S* configuration of 1′.

(**7**) *epi*-β-Avoparcin: R, R^1, R^2, R^3, R^4, R^5 as in (**5**); *S* configuration of 1′.

(**8**) ε-Avoparcin: R, R^1, R^2, R^4, R^5 as in (**5**); R^3=H.

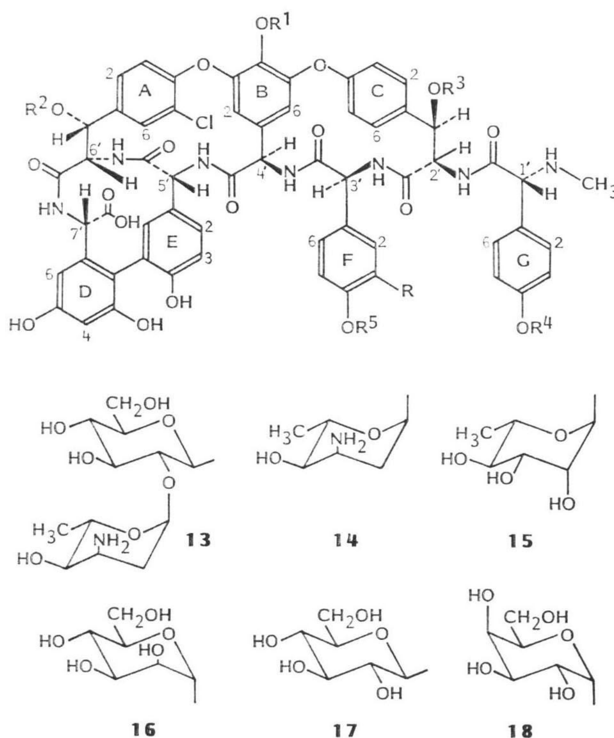
Degradation compounds

(**9**) α-CDP-I avoparcin: R, R^1, R^4, R^5=H; R^2, R^3 as in (**4**).

(**10**) β-CDP-I avoparcin: R=Cl, R^1, R^4, R^5=H; R^2, R^3, as in (**5**).

(**11**) Mannosyl-α-avoparcin aglycone: R, R^1, R^2, R^4, R^5=H; R^3=*O*-α-D-mannose (**16**).

(**12**) Mannosyl-β-avoparcin aglycone: R=Cl; R^1, R^2, R^4, R^5=H; R^3=*O*-α-D-mannose (**16**).



(Table 2). The ¹H NMR spectrum of the mixture lacks the doublet signal at δ 1.17 hence this signal in the spectrum of the α- and β-avoparcin compounds can with confidence be attributed to the methyl group of ristosamine of the disaccharide. The glycosidic linkage of this ristosamine is the most labile of the glycosidic bonds in avoparcin. Indeed it is so labile that at this stage we cannot with certainty state that the deristosaminyl compounds are true metabolites but may arise rather because of very mild acidic conditions either in the fermentative or refining stages.

No. 3, D-Galactosyl-β-avoparcin

Compound No. **3** with an HPLC retention time of 0.6 relative to β-avoparcin is a true metabolite. It is not always observed in fermentations and when observed, it is usually present to the extent of about

Table 1. Components found in fermentations of avoparcin.
Listed also are a number of degradation products.

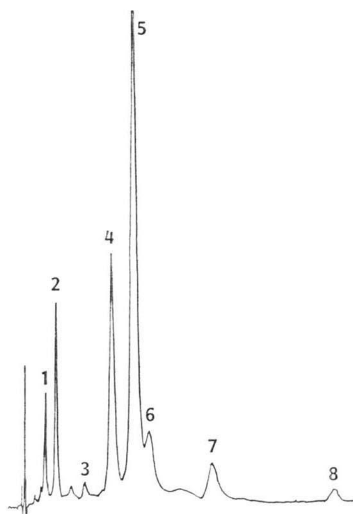
Compound number	HPLC* retention time Relative to β-avoparcin (1.0)	Name or designation
1	0.3	α-Deristosaminyl avoparcin
2	0.4	β-Deristosaminyl avoparcin
3	0.6	D-Galactosyl-β-avoparcin
4	0.8	α-Avoparcin
5	1.0	β-Avoparcin
6	1.2	*epi*-α-Avoparcin
7	1.6	*epi*-β-Avoparcin
8	2.5	Demannosyl-β-avoparcin or ε-avoparcin
Degradation compounds		
9	2.5	α-CDP-I avoparcin
10	3.6	β-CDP-I avoparcin
11	0.35	Mannosyl aglycone of α-avoparcin
12	0.57	Mannosyl aglycone of β-avoparcin

* Analytical HPLC was carried out using a C_{18} μBondapak column at 35°C and an aqueous system which was 2.5% in acetic acid, 0.01 M in sodium heptane sulfonic acid, 11.8% in acetonitrile, and buffered at pH 4.0 using ammonium hydroxide. An HPLC trace of an avoparcin preparation is shown below.

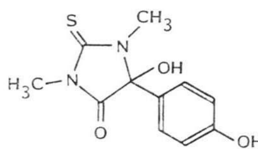
half a percent or less of the major β-component. The structure of this compound was determined using ^{1}H NMR studies and by gas chromatographic analyses of the hydrolyzed alditol peracetates. The key assignments amongst the chemical shifts of the β-avoparcin as well as other components are given in Table 3. When compared with the spectrum of compound No. **3**, the following differences are noted. The anomeric region of this spectrum had an extra signal at δ 5.50 which indicated a possible extra sugar. This notion was substantiated by comparison of integration values of the δ 3~4 region in the spectra of β-avoparcin and compound No. **3**.

The extra sugar was identified as galactose by comparing the gas chromatographic elution profile of the alditol peracetates obtained from this compound with those obtained from the β-compound and by using authentic sugar alditol peracetate controls[7]. The extra peak in the spectrum derived from compound No. **3** hydrolysate was matched exactly with that of galactitol peracetate. The galactose was shown to be of the D-configuration by the enzymatic procedure outlined by STURGEON.[8]

Fig. 1. Analytical HPLC trace of an avoparcin preparation.



Attachment of the galactose by an α-glycosidic linkage (the gal H_1 signal is a broad singlet



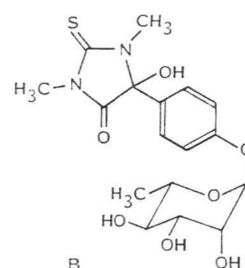


Table 2. Antibacterial activities* of β-avoparcin, D-galactosyl-β-avoparcin, *epi*-β-avoparcin, ε-avoparcin, unresolved α-,β-deristosaminyl avoparcins and the mannosyl aglycone of unresolved α-,β-avoparcins.

Organism	No. of strains	Range of minimal inhibitory concentration (μg/ml)					
		β-Avoparcin	D-Galactosyl-β-avoparcin	*epi*-β-Avoparcin	ε-Avoparcin	Deristosaminyl α-, β-avoparcin	Mannosyl aglycone of α-,β-avoparcin
Klebsiella	2	>256	>256	>64	>256	>256	>256
Enterobacter	2	>256	>256	64	>256	>256	>256
Serratia	2	>256	>256	>256	>256	>256	>256
Proteus	2	>256	>256	>128	>256	>256	>256
Escherichia coli	3	16~>256	32~>256	64~>256	4~>256	32~>256	>256
Salmonella	2	>256	>256	64~256	>256	>256	>256
Pseudomonas	2	>256	>256	>256	>256	>256	>256
Enterococcus	2	1	4	>256	1	2~4	16
Staphylococcus	2	0.5~2.0	4~16	32	1	4~16	2~4
Micrococcus	1	0.5	1	32	0.25	0.5	2
Staphylococcus Smith	1	2	4	128	0.25	8	2
Actinobacter	2	>256	>256	64~256	>256	>256	>256

* Mueller-Hinton agar dilution method.

Table 3. Proton chemical shift* assignments (δ) for D-galactosyl-β-, β-, *epi*-β- and ε-avoparcins.

Positions	D-Galactosyl-β-avoparcin	β-Avoparcin	*epi*-β-Avoparcin	ε-Avoparcin
NCH_3	2.18 s	2.22 s	2.33 s	2.30 s
1′	4.06 s	4.08 s	4.06 s	4.06 s
6′	4.32 d (12; α-NH)	4.33 d (12; α-NH)	4.33 d (12; α-NH)	4.30 d (12; α-NH)
7′	4.39 d (5; α-NH)	4.39 d (5; α-NH)	4.41 d (5; α-NH)	4.38 d (5; α-NH)
5′	4.58 d (4.5; α-NH)	4.63 d (5; α-NH)	4.66 d (5; α-NH)	4.57 d (5; α-NH)
Rha H_1	4.78 s	4.78 s	4.78 s	4.78 s
Man H_1	4.93 s	4.93 s	4.89 s	—
2′	5.01 d (4; C_{bz})	5.05 d (4.4; C_{bz})	5.19 d (4.5; C_{bz})	4.82 d (2.5; C_{bz})
A_{bz}	5.12 brs	5.11 s	5.11 s	5.11 brs
B_2	5.27 brs	4.25 s	4.21 s	5.20 brs
3′	5.36 (unclear)	5.32 d (9; α-NH)	5.28 d (9; α-NH)	5.24 (unclear)
Ris H_1	5.36 brs	5.34 brs	5.33 brs	5.33 d (~2)
C_{bz}	5.41 d (4.5; 2′)	5.40 d (4.5; 2′)	5.33 brs	5.13 (unclear)
Ris′ H_1	5.47 brs	5.47 brs	5.46 brs	5.49 brs
Gal H_1	5.50 brs (2)	—	—	—
4′	5.63 (unclear)	5.65 d (8; α-NH)	5.65 d (7.5; α-NH)	5.61 d (7.5; α-NH)
Glu H_1	5.63 d (8; Glu H_2)	5.65 d (8; Glu H_2)	5.65 (unclear)	5.63 d (8; Glu H_2)
B_6	5.72 brs	5.75 s	5.80 s	5.76 s
D_6	6.31 d (~2)	6.29 d (2.2)	6.33 d (2)	6.29 d (~2)
D_4	6.41 d (~2)	6.42 d (2.2)	6.43 d (2)	6.41 d (~2)
E_3	6.70 d (8.4)	6.71 d (8)	6.71 d (8.4)	6.71 d (8)
E_2	6.81 dd (8, 2)	6.88 dd (8, 2)	6.82 dd (8, 2)	6.87 dd (8, 2)
F_6	7.00 (unclear)	6.83 dd (8, 2)	6.82 (unclear)	6.82 d (8)
$G_{3,5}$	7.00 dd (8.5, 2)	6.95 dd (8.5, 2)	6.89 dd (8.5, 2)	6.96 d (8.5)
F_2	7.12 d (~2)	7.04 d (~2)	7.08 d (~2)	7.06 d (~2)
F_5	7.16 d (8)	6.80 d (8)	6.76 d (8)	6.82 d (8)
$G_{2,6}$	7.27 d (~9)	7.22 dd (8.5, 2)	7.15 dd (8.5, 2)	7.31 d (8.5)
C_6	7.64 d (~9)	7.61 dd (8.5, 2)	7.84 d (8.5)	7.78 d (8.5)
A_6	7.73 brs	7.73 brs	7.73 brs	7.72 brs

* 270 MH_z ^1H NMR spectra in DMSO-d_6 at concentration of *ca* 15 mM at 75°C.

Table 4. Glycosylation deshielding of aromatic protons.

Compounds	Chemical shifts, multiplicities, and coupling constants of				
	Protons 2 and 6	Protons 3 and 5	F_2	F_6	F_5
A	7.12 d (9)	6.80 d (9)			
B	7.20 d (9)	7.06 d (9)			
β-Avoparcin			7.04 d (1.5)	6.83 dd (8,2)	6.80 d (8)
D-Galactosyl-β-avoparcin			7.12 d (1.5)	7.00*	7.16 d (8)

* Due to signal overlap the *J* values are not clear.

with a coupling constant of less than 2 Hz) to the F ring phenolic group is clearly indicated as shown by the following ^{1}H NMR analysis. Table 4 compares glycosylation deshielding of *ortho-* and *meta-* aromatic protons in going from the thiohydantoin A to B with similar observed effects concerning the F ring in β-avoparcin as opposed to galactosyl-β-avoparcin. The ^{1}H NMR assignments of the protons of A and B have previously been reported[5].

The deshielding of protons 3 and 5 in going from A to B is δ 0.26 while for protons 2 and 6 it is δ 0.08. In comparing β-avoparcin and galactosyl-β-avoparcin we observed deshielding effects of δ 0.36, 0.08 and 0.17 for protons F_5, F_2 and F_6, respectively in the spectrum of the latter compound with reference to the β-spectrum. The presence of chlorine in the ring modifies the picture somewhat but the overall deshielding pattern is unmistakably the same for both sets of compounds. Hence the F ring phenolic group in compound No. **3** is glycosylated by D-galactose. D-Galactosyl-β-avoparcin exhibits about 50% or less of the *in vitro* Gram-positive activity of β-avoparcin (Table 2). Recently we obtained a mass spectrum on this material by fast atom bombardment (FAB)*. A quasi molecular ion $[M+H]^+$ was observed at *m/z* 2,104 in agreement with the calculated nominal value of 2,104.

Nos. **4** and **5**, α- and β-Avoparcins

The heptane sulfonic acid ion-pairing system used to resolve several of the minor components from the avoparcin complex was ineffective for separating the α- and β-avoparcin compounds. Another system was devised consisting of acetic acid as the ion-pairing agent, ammonia as the buffering system and low concentrations of ethyl acetate as the resolving solvent. This system was quite useful for separating gram quantities of the α- and β-compounds. Surprisingly it did not resolve the deristosaminyl impurities from either component consequently the preparations obtained had to be re-chromatogaphed in the heptane sulfonic acid system to obtain highly purified entities. The α-compound obtained by this procedure was 99% pure as measured by UV area percent integration using analytical HPLC. The β-compound exhibited 96% purity under similar conditions. Parameters used to test the quality of these preparations were specific rotation, $E^{1\%}_{1cm}$ at 280 nm and microanalysis. All the data obtained bolstered the HPLC results indicating that the two preparations were very high-quality materials. Nevertheless the proton NMR spectra on both preparations indicated the presence of triethylamine as a very minor impurity. All preparations of avoparcin entities that involved a triethylamine sulfate precipitation step contained the triethylamine signals in their ^{1}H NMR spectra. Heptane sulfonic acid salt solutions of an avoparcin component could be adjusted to a pH above the isoelectric point (pH 6.8) using a weak anion exchanger in the hydroxyl form and then converted to the sulfate salt by treatment with a strong cation

* This work was done by Dr. HOWARD MORRIS of Imperial College, London, England. The quasi mol ion determination was made by Dr. M. SEGAL of Lederle Laboratories.

exchanger in the sulfate form. Such preparations were free of triethylamines but the overall yield was in the 40% range and the method was time consuming.

Nos. **6** and **7**, α- and β-*epi*-Avoparcins

Compound No. **6**, *epi*-α-avoparcin while detected in some fermentations was never isolated by us since it was present to only a very minor extent. The peaks representing both the α- and *epi*-α-avoparcins in an analytical trace of an avoparcin sample could be dramatically enhanced by the addition of a small amount of an epimerized solution of pure α-avoparcin (containing α- and *epi*-α-avoparcin). Compound No. **7** was detected in many fermentations to the extent of about a quarter of a percent or so of the total complex. However, it was realized that by heating a solution of crude avoparcin from such fermentations to 70°C or so for a short period the amount of compound No. **7** present could be increased to an isolable amount. It was from such treated preparations that we first isolated *epi*-β-avoparcin. The structure of the compound has been previously reported[6]. The unequivocable proof of this structure as well as further details on the epimerization phenomenon will be given in a subsequent paper.

No. **8**, ε-Avoparcin

Compound No. **8**, ε-avoparcin is found in some fermentations to the extent of up to two and a half percent of the β component. Since the relative retention time of the ε-compound is so much greater than that of β the resolution of this compound from the complex was relatively straightforward.

Equal amounts of ε and β compounds were hydrolyzed separately and the carbohydrate hydrolysates were compared by TLC which showed the absence of mannose from the ε material. In the 270 MHz ^{1}H NMR spectrum of ε, the anomeric mannosyl proton signal observed at δ 4.93 in the α and β spectra is clearly missing. Proton C_{bz} of ε-avoparcin is observed at δ 5.13, as compared to δ 5.40 in the β-spectrum (Table 3). This shift is consistent with the absence of mannose since C_{bz} in both vancomycin and ristocetin is at δ 5.19 and both of these glycopeptides have free hydroxyl groups at the corresponding benzylic positions. Consistent with these results is the fact that one less signal appears in the anomeric region in the ^{13}C NMR spectrum of ε as compared to that of α or β. Finally the appearance of a pseudomolecular ion $[M+Na]^+$ at m/z 1,805$\pm$2 in the ^{225}Cf plasma desorption mass spectrum of ε is in agreement with a molecular weight of 1,780 for the material*. Hence ε is the compound demannosyl-β-avoparcin. This metabolite is somewhat more active than β-avoparcin against Gram-positive organisms as measured by minimal inhibitory concentrations on agar plates (Table 2). We should also mention at this stage that we did not detect the corresponding α compounds of either demannosyl or galactosyl-β-avoparcin in fermentation broths.

Degradation Products of Avoparcin

We had previously reported that the aglycone of avoparcin could be obtained by refluxing the complex for one hour in 1 N HCl solution[5]. While there is little doubt that such treatment does remove all the carbohydrates from the aglycone the chances are that the reaction does not completely stop there since even under milder acidic conditions the benzylic hydroxyl groups show instability. In any event we have never isolated and characterized the authentic aglycone of avoparcin. Refluxing avoparcin complex in very mild acid (0.14 N HCl) yielded a microcrystalline material which was designated avoparcin CDP-I (crystalline degradation product one). By preparative HPLC using a sulfonic acid ion-pairing system CDP-I was resolved into at least three components namely, CDP-I α-avoparcin, CDP-I

* This work was done by Professor R. D. MacFarlane of Texas A&M University.

β-avoparcin and a third material which was a mixture of the α- and β-mannosyl aglycones of avoparcin. It has already been reported that the CDP-I compounds retain only the benzylic sugars ristosamine and mannose.[5] In the ^1H NMR spectra of the purified CDP-I compounds there is a split methyl signal at δ 1.24 which must belong to the methyl of the remaining benzylic ristosamine. By elimination then the remaining split methyl signal at δ 1.12 in the spectrum of β-avoparcin belongs to the methyl group of rhamnose.

The analytical HPLC trace on the third material isolated from the CDP-I preparation showed it to be a mixture of 18% and 76% of two components with retention times of 0.35 and 0.57 respectively relative to β-avoparcin (1.0). A ^{13}C NMR spectrum on the material (20,000 scans) showed clear distinct carbon peaks at 107.2, 105.4, 104.0, 102.1 and 98.1 ppm. The first four of these signals belong to aromatic carbons D_4, D_6, B_2 and B_4 which are either *ortho-ortho* or *ortho-para* to oxygen substituents while the last peak is that of the anomeric carbon of mannose. In the ^1H NMR spectrum of the material the only anomeric signal remaining is that of mannose at δ 4.93. Hence the only remaining sugar is mannose which is bound by an α-glycosidic linkage since the coupling constant ^1J ($^{13}CH_1$) was determined to be 170 Hz from the undecoupled ^{13}C NMR spectrum[9,10,11]. When tested against a number of Gram-positive organisms, the material displayed about 25 to 50% of the activity of the β-compound. It should be restated that these degradation compounds are obtained by mild acid hydrolysis and are not observed in HPLC traces of fermentation isolates.

The isolation of the mannosyl aglycones defines the resistance of the glycosidic linkages in avoparcin to mild acid hydrolysis. The weakest bond is that of the ristosamine in the disaccharide. From a large number of TLC studies during earlier hydrolytic experiments on avoparcin it was found that rhamnose was released more rapidly than glucose under a given set of conditions. Presumably galactose would be released from compound No. **3** at about the same time as the other two phenolic bound sugars. The first of the benzylic sugars to yield to mild hydrolysis is obviously the 2-deoxysugar ristosamine and hence the mannose is the most tenaciously held sugar of the antibiotic.

Experimental

General

The modular analytical HPLC used consisted of a Spectra Physics 740 model pump, a Micromeritics 725 autoinjector and a model 770 Spectrophotometric Schoeffel detector. The μBondapak C_{18} column used was kept at a temperature of 35°C using a 731 Micromeritics column compartment. When long retention time components such as the CDP-I avoparcin were being analyzed, the oven temperature was raised to 50°C and the pump speed was increased from the normal 2.0 ml to 2.4 ml per minute. Components were usually run at 1 mg/ml concentration.

The preparative HPLC used was the Waters Prep LC/System 500 fitted with Prep Pak-500 C_{18} columns. All solutions pumped through the columns were filtered through Millipore filter paper and degassed by swirling under vacuum. A column was usually equilibrated using about 1.5 liters of buffer and the charge which varied from 2 to 5 g dissolved in 1 liter of buffer was pumped on. This was followed by another 1 liter of buffer before elution commenced. The concentration of eluting solvent was often chosen so that the desired component was eluted off by a volume of less than 9 liters.

Monitoring of the elution was carried out using a Pharmacia Dual Path UV-2 system combined with a Linear Corporation dual-pen recorder. Sensitivities were selected where the desired component gave less than full-scale deflection on the shorter 1-mm path detector. Detector wave length was arbitrarily set at 254 nm.

When the desired component was located and checked for purity by analytical HPLC, the fractions were desolventized, concentrated and pumped back on the PrepPak Column to remove salt. This was

done using 2 to 3 liters of H_2O and following that the component was eluted off using an aqueous solution which was 25% in CH_3CN and 0.2% in CH_3COOH. Again the eluate was desolventized and concentrated. At this stage the compound in question was in the undesirable heptane sulfonic acid salt form. In all we used three methods to eliminate this salt. The first method involved adding 10% sodium dodecyl sulfate solution until precipitation was complete or nearly complete. The dodecyl sulfate salt of the antibiotic was recovered, washed with water and dissolved in MeOH. The compound as the sulfate salt was precipitated from the MeOH solution by the addition of 0.5 M triethylamine sulfate [$(TEA)_2H_2SO_4$]. The latter solution was usually prepared by adding about 7 ml of TEA to 50 ml of MeOH and adding approximately 1.4 ml of conc. H_2SO_4 until the "pH" was about 4.0. The precipitate was recovered, washed well with MeOH, dissolved in H_2O, filtered and the filtrate freeze-dried.

Since sodium dodecyl sulfate failed to precipitate fully those avoparcins with two or less amino groups a second method was developed. The concentrated heptane sulfonic acid salt solution was freeze-dried. The freeze-dried solid could be taken up in MeOH and from this point on handled as described in the first method. The third procedure was used only a few times. The heptane sulfonic acid salt of avoparcin can be adsorbed on the PrepPak column from aqueous solution. If the loaded column is then washed with copious amounts (12 to 15 liters) of 0.1 M Na_2SO_4 solution, surprisingly the free base of avoparcin can be eluted off the column using CH_3CN solution. While this is a viable method of undesirable salt removal, many of the minor components we were dealing with are insoluble in aqueous solution in the free base form and under these conditions the filters and check valves of the Waters instrument can become clogged. Analytical HPLC was carried out using an aqueous system: 11.8% in CH_3CN buffered at pH 4.0 using KH_2PO_4 and using 0.01 M sodium heptane sulfonic acid as the counter ion. The column used was a Waters Associates μBondapak C_{18}.

Components were sometimes checked by TLC on Brinkmann F_{254} silica gel thin layer plates. A useful developing system consisted of 50% MeOH, 40% 0.35 M NaOAc and 10% acetone by volume. Typical Rfs were found to be: α- and β-avoparcin 0.47, ε-avoparcin 0.36, *epi*-β-avoparcin 0.16 and α- and β-CDP-I avoparcin 0.72. Detection was by UV absorption or by spraying the developed thin layer with dilute aqueous *p*-nitrobenzenediazonium fluoborate solution. Gas chromatography on alditol peracetates was carried out using a Varian Model 3700 fitted with a 183-cm glass column, I. D. 6.4 mm containing 3% OU-225 on 110/120 Gas Chrom Q (Supelco Inc.). Detection was by flame ionization.

^1H NMR spectra of avoparcin components and degradation compounds were recorded on a 270 MHz Bruker Instrument in DMSO-d_6 at 75°C. The ^{13}C NMR spectrum of the mannosyl aglycone of avoparcin was recorded in DMSO-d_6 at 25°C on a Bruker CXP 50.3 MHz (20,269 scans). This latter spectrum was kindly supplied by Prof. I. M. ARMITAGE of Yale University. Water content of all compounds was determined by the Karl Fisher method.

D-Galactosyl-β-avoparcin

An avoparcin preparation in the form of the sulfate salt was shown by analytical HPLC to have about 0.3% of a material present whose retention time was 0.6 relative to 1.0 for β-avoparcin. About 5 g of this material was dissolved in 1 liter of buffer consisting of 25% HOAc, 0.01% sodium heptane sulfonic acid (HSA) and with the pH adjusted to 4.0 using conc. NH_4OH and charged to a preparative HPLC column. The column was then equilibrated with another 1 liter of buffer solution and elution was carried out using 12.5% CH_3CN in the same buffer system. The desired component was observed by an inflection point on the front of the major α-,β-avoparcin peak. Samples from the beginning of the peak up to the beginning of the inflection point from five such columns were combined, desolventized, desalted on the preparative column and following elution concentrated to about 40 ml. This concentrate was treated dropwise with 10% sodium dodecyl sulfate solution to yield a precipitate. The precipitation was not carried to completion but filtered and the residue after washing with H_2O was taken up in 30 ml of MeOH, filtered and the filtrate treated with a slight excess of 0.5 M $(TEA)_2H_2SO_4$ in MeOH. The resultant precipitate was recovered, rinsed with MeOH, dissolved in 25 ml H_2O, filtered and freeze-dried to 157 mg of white powder which subsequent testing showed was D-galactosyl-β-avoparcin. Analytical HPLC gave the following profile.

Avoparcin compound	α-Deristosaminyl	β-Deristosaminyl	D-Galactosyl-β	α	β
Retention time (seconds)	224	464	599	744	909
Area % by UV	2.34	2.2	86.4	4.95	2.78

$[\alpha]_D^{25}$ $-73\pm2°$ (*c* 0.50, H_2O).

Anal. Calcd for $C_{95}H_{111}O_{41}N_9Cl_2\cdot1.5H_2SO_4\cdot12H_2O$ (2,469): C 46.22, H 5.59, N 5.11, Cl 2.87, S 1.95.
Found: C 45.98, H 5.27, N 5.33, Cl 2.77, S 2.02.

Gas Chromatography of Alditol Peracetates of Carbohydrates of β and Compound No. **3**

A 50-mg portion of compound No. **3** was refluxed for 35 minutes in 20 ml of 2 N HCl. After cooling, the solution was adjusted to pH 6.8 using 1 N NaOH. To this neutralized suspension was added one pellet of $NaBH_4$ and the flask was allowed to stand at room temperature overnight. The suspension was adjusted to pH 6.5 using 1 N HCl and freeze-dried. The resultant powder was stirred with pyridine - Ac_2O overnight. The solvent was evaporated and the solid triturated with toluene and taken to dryness three times. Finally the residue was extracted with CH_2Cl_2 which was evaporated to 14 mg of residue. A 50-mg sample of compound No. **3** was handled in similar fashion. The β-avoparcin residue by gas chromatography exhibited four peaks which had retention times of 0.279, 0.793, 0.853 and 1.00 (relative to 1.00 for glucitol peracetate). By peak matching with authentic samples the early peaks were identified as rhamnitol, ristosaminitol and mannitol peracetates respectively. Chromatography of compound No. **3** peracetates gave the same four peaks and also an additional peak with a relative retention time of 0.931. This latter peak was matched exactly with authentic galactitol peracetate.

Configuration of Galactose from Compound No. **3**

In the presence of D-galactose and the enzyme D-galactose dehydrogenase, nicotinamide adenine dinucleotide (NAD) is converted to NADH. This change is measured spectrophotometrically at 340 nm. Approximately 24 mg (10 μmol) of compound No. **3** was refluxed in 1 N HCl for 1.5 hours and the solution neutralized to pH 6.6 using IR-45 (OH^-). The filtrate was concentrated to 1 ml and 0.2 ml of this solution was diluted to 2 ml (solution X). The following solutions were prepared: Solution A, 0.1 M Tris buffer pH 8.6, solution B, 0.017 M NAD, solution C, 5 units/ml D-galactose dehydrogenase and solution D, 1 mM D-galactose. Into a 1-cm fused silica cell, 2.68 ml of solution A, 0.1 ml of solution B and 0.2 ml of solution D were mixed. The initial absorbance was measured at 340 nm. Then 0.02 ml of solution C was added to start the reaction. The change in absorbance was checked to a constant value. The amount of D-galactose in μmol is given by the change in absorbance multiplied by $3/6.22=0.482$. We found a value of 0.21 μmol for D-galactose. Using solution X instead of D-galactose our determined value of D-galactose was 0.16 μmol which was 80% as compared to the expected theoretical 86% value. Consequently the galactose of compound No. **3** has the D configuration.

Isolation of High-quality α- and β-Avoparcin

A charge of 4 g of avoparcin was dissolved in 1 liter of buffer solution consisting of a 1% HOAc solution with pH adjusted to 4.5 by adding conc. NH_4OH. It usually took about 6.2 ml of NH_4OH per liter of solution. This charge was pumped on the column and after equilibration with another 1 liter of buffer the antibiotics were eluted off with 1.65% EtOAc in the same buffer solution. The detector trace showed good resolution of α- and β-avoparcins with only very slight overlap. Several such charges were resolved and the α and β fractions were combined separately. Overlap fractions were discarded. After being desolventized each component solution was re-applied to a column which had been equilibrated with HSA buffer. After the charge was on the column about 3 liters of HSA buffer was pumped through to ensure proper equilibration. The column was eluted with 13.5% CH_3CN in HSA buffer. The detector trace showed that the deristosaminyl components had been separated out. There was a hint of a shoulder on the downside of the main peak in the case of the β-avoparcin. Of the α-component we recovered 0.75 g material which by analytical HPLC was 99% pure by UV area % integration. Other analytical data were as follows: $[\alpha]_D^{25}$ $-96\pm2°$ (*c* 0.62, 0.1 N HCl); $E_{1cm}^{1\%}$ at 280 nm 42.0 in 0.1 N HCl; Ash content 0.14%.

Anal. Calcd for $C_{89}H_{102}O_{36}N_9Cl_9\cdot1.5H_2SO_4\cdot12H_2O$ (2,272): C 47.07, H 5.72, N 5.55, Cl 1.56, S 2.12.
Found: C 47.81, H 5.31, N 5.75, Cl 1.68, S 2.67.

The β-component was processed in identical fashion to yield 1.5 g of freeze-dried white solid which by UV area % was 96% pure. Analytical data were as follows: $[\alpha]_D^{25}$ $-102\pm2°$ (*c* 0.65, 0.1 N HCl); $E_{1cm}^{1\%}$ at 280 nm 44.0 in 0.1 N HCl; Ash 0.001%.

Anal. Calcd for $C_{89}H_{101}O_{36}N_9Cl_2 \cdot 1.5H_2SO_4 \cdot 12H_2O$ (2,307): C 46.34, H 5.23, N 5.46, Cl 3.07, S 2.08.
Found: C 46.60, H 5.07, N 5.51, Cl 3.02, S 2.28.

Isolation of *epi*-β-Avoparcin

About 30 g of avoparcin together with 20 g of NaOAc in 250 ml of H_2O with the pH adjusted to 7.5 using 5 N NaOH was left standing overnight on a steam bath. The temperature of the solution was about 65°C. The solution was percolated through 700 g of granular carbon in an open column. After washing the column with H_2O the antibiotic complex was eluted using acetone - H_2O, 50: 50 solution. The concentrated eluate was freeze-dried to 14 g of off-white solid. A 3 g charge of this material was applied to a preparative column in 1 liter of HSA buffer and after equilibration the column was eluted with 17.5% CH_3CN in the same buffer system. In the early eluate fractions were substantial amounts of α- and β-avoparcins. At about the fifth liter of eluate the monitor showed a significant peak. The fractions of this peak were combined, desolventized, desalted and freeze-dried. By treatment of a methanolic solution of the freeze-dried material with $(TEA)_2H_2SO_4$ the HSA salt was replaced by the sulfate salt to yield after freeze-drying 380 mg of faintly off-white material which subsequent testing showed to be *epi*-β-avoparcin. HPLC analytical data on the preparation were as follows:

Avoparcin compound	β	*epi*-β	ε
Retention time (seconds)	1,708	1,704	2,264
Area % by UV	1.6	94.8	3.8

$[\alpha]_D^{25}$ $-107\pm3°$ (*c* 0.35, H_2O); ash 0.0%.

Anal. Calcd for $C_{89}H_{101}O_{36}N_9Cl_2 \cdot 1.5H_2SO_4 \cdot 12H_2O$ (2,307): C 46.34, H 5.59, N 5.46, Cl 3.07, S 2.08.
Found: C 45.25, H 5.29, N 5.39, Cl 3.06, S 2.10.

Isolation of ε-Avoparcin

Starting material for this isolation was the sulfate salt of a relatively crude avoparcin preparation which the HPLC analytical trace showed had half a percent or so of ε-avoparcin present. About 4 g of this solid was dissolved in 1 liter of HSA buffer and charged to a preparative column. After reequilibration with fresh buffer solution the column was eluted with 16% CH_3CN in the same buffer solution. Under these conditions the 20-mm path detector usually reached saturation sensitivity at several areas of high substrate elution but the 1-mm path detector could, in most cases, be kept on scale by sensitivity adjustment. This extra "eye" was invaluable to us in many separations. Holdback volumes on the preparative columns in our hands were about 600 ml. ε-Avoparcin eluted off between the twelfth and fourteenth holdback volumes. Combined fractions were desolventized under vacuum at 40~45°C and recharged to the stripped column. Column stripping was normally carried out using 0.5 to 1.5 liters of 30% CH_3CN in HSA buffer. Excess strip solution was washed off using 2 to 3 liters H_2O. The desolventized ε-avoparcin was then applied followed by H_2O wash to remove buffer salts and eluted off using 25% CH_3CN and 0.25% HOAc in aqueous solution.

The eluate was desolventized and concentrated to about 50 ml and 10% sodium dodecyl sulfate solution was added carefully dropwise without going to completion. The precipitate was washed with H_2O, taken up in MeOH processed with $(TEA)_2H_2SO_4$ and freeze-dried as described previously to yield 415 mg of white powder which by analytical HPLC was 97% pure ε-avoparcin: $[\alpha]_D^{25}$ $-117\pm2°$ (*c* 0.61, 0.1 N HCl).

Anal. Calcd for $C_{83}H_{91}O_{31}N_9Cl_2 \cdot 1.5H_2SO_4 \cdot 9H_2O$ (2,091): C 47.68, H 5.40, N 6.03, Cl 3.39, S 2.30.
Found: C 47.45, H 5.07, N 6.03, Cl 3.40, S 2.45.

The filtrate from the sodium dodecyl sulfate precipitation was treated with a slight excess of the same reagent to get a second precipitate which was worked up in the regular fashion to obtain an ε-avoparcin preparation (100 mg) which had about 20% of deristosaminyl-ε-avoparcin present.

Comparison of Carbohydrate Hydrolysates of β- and ε-Avoparcins

A 90-mg aliquot of β-avoparcin was taken up in 25 ml of MeOH which had been saturated with dry

HCl gas and refluxed gently overnight. The solvent was evaporated to dryness and the residue dissolved in 10 ml H_2O and the pH adjusted to 7.0 using IR-45 (OH^-). The resin was removed by filtration, rinsed twice with H_2O and the rinsings and filtrate were freeze-dried. This freeze-dried material was taken up in H_2O again and stirred with Dowex 50W-X4 (NH_4^+). The filtrate from this resin was freeze-dried to 18 mg. Similarly, 90 mg of ε-avoparcin was processed by methanolysis and resin treatments to an analogous freeze-dried end-product. TLC of both end-products on a F_{254} Brinkmann silica gel plate was carried out using the system EtOAc - isopropyl alcohol - H_2O as 10: 6: 4. Detection of spots on the developed thin layer was made by spraying with methanolic H_2SO_4 followed by heating. The β-hydrolysate showed four spots with Rf's at 0.75, 0.65, 0.52 and 0.25. By comparison ε-avoparcin hydrolysate exhibited only three spots; the one at 0.65 was missing. The missing spot was shown to be methylmannoside by comparison with an authentic sample.

Preparation of CDP-I Avoparcin

About 40 g of avoparcin was refluxed for two hours in 600 ml of 0.14 N HCl solution. The cooled filtrate was neutralized to pH 6.9 using IR-45 (OH^-). The washed, filtered resin was stirred twice with 600 ml of MeOH and the combined filtrates were diluted to 2 liters with acetone and allowed to stand overnight in the cold room. This procedure yielded a first crop of 12 g of microcrystalline CDP-I avoparcin. A second crop of 7.5 g was also obtained.

Resolution of CDP-I Avoparcin

A charge of 4 g of the CDP-I avoparcin was chromatographed on the Waters PrepPak 500 C_{18} column using 17.5% CH_3CN in HSA buffer as the resolving agent. The detector trace indicated a minor, early peak followed by a major peak which showed a point of inflection in the ascending portion of the curve. A total of three such columns were developed. The fractions with the minor peak were combined, desolventized, desalted and freeze-dried. The freeze-dried powder was taken up in MeOH and the HSA was replaced by sulfate using 0.5 M $(TEA)_2H_2SO_4$ in MeOH. The insoluble sulfate salt was washed with MeOH, dissolved in H_2O, filtered and freeze-dried to 530 mg of white powder. Using a flow speed of 2.4 ml per minute and a column temperature of 50°C an analytical trace was obtained which indicated the following:

Retention time (seconds)	207	319	387	580	1,390
Area % by UV	16.2	70.0	3.5	2.9	2.9

The two early major components turned out to be α- and β-mannosyl aglycones of avoparcin. $[\alpha]_D^{25}$ $-58\pm3°$ (*c* 0.47, 0.1 N HCl); $E_{1cm}^{1\%}$ at 280 nm 53.7 in 0.1 N HCl.

Anal. Calcd for β-mannosyl aglycone of avoparcin: $C_{65}H_{59}O_{23}N_7Cl_2 \cdot 0.5H_2SO_4 \cdot 6H_2O$ (1,534): C 50.89, H 4.73, N 6.39, Cl 4.62, S 1.04.
Found: C 50.29, H 4.49, N 5.97, Cl 3.96, S 1.52.

The fractions of the major peak up to the vicinity of the inflection point were combined from all three columns and processed to the freeze-dried sulfate salt as described for the mannosyl aglycone component to yield 290 mg of white powder which was 68% α-CDP-I avoparcin. Analytical HPLC under normal running conditions gave the following results:

Retention time (seconds)	301	319	1,354	1,957	2,065
Area % by UV	2.5	5.3	68.3	8.2	10.3

$[\alpha]_D^{25}$ $-40\pm3°$ (*c* 0.38, 0.1 N HCl); $E_{1cm}^{1\%}$ at 280 nm 55.9 in 0.1 N HCl.

Anal. Calcd for $C_{71}H_{71}O_{25}N_8Cl \cdot H_2SO_4 \cdot 7H_2O$ (1,696): C 50.28, H 5.17, N 6.60, Cl 2.09, S 1.89.
Found: C 50.28, H 4.78, N 6.75, Cl 2.17, S 1.87.

Fractions of the major peak well beyond the vicinity of the inflection point were combined from the three runs and processed as described above to yield 1.5 g of β-CDP-I avoparcin. Analytical HPLC gave the following results:

Retention time (seconds)	1,350	1,975
Area % by UV	10.7	84.3

$[\alpha]_D^{25}$ $-51\pm2°$ (*c* 0.72, 0.1 N HCl); $E_{1cm}^{1\%}$ at 280 nm 51 in 0.1 N HCl.

Anal. Calcd for $C_{71}H_{70}O_{25}N_8Cl_2 \cdot H_2SO_4 \cdot 9H_2O$ (1,766): C 48.29, H 5.14, N 6.34, Cl 4.01, S 1.81.
Found: C 48.37, H 4.78, N 6.21, Cl 3.55, S 2.18.

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