CHLOROPOLYSPORINS A, B AND C, NOVEL GLYCOPEPTIDE ANTIBIOTICS FROM *FAENIA INTERJECTA* SP. NOV.

II. FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL CHARACTERIZATION

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New antibiotics, chloropolysporins A, B and C, were found in the culture broth of an actinomycete identified as *Faenia interjecta* sp. nov. They were isolated from the culture filtrate by column chromatography on various resinous adsorbents, followed by preparative reverse phase HPLC. Chloropolysporins A, B and C possessed all the same new aglycone composed of actinoidic acid, 3-chloro-4-hydroxyphenylglycine, *N*-methyl-*p*-hydroxyphenylglycine and vancomycinic acid. From elementary analyses and mass spectroscopic measurements, the molecular formulae of chloropolysporins A, B and C appear to be $C_{89}H_{99}O_{39}N_8Cl_3$ (MW 2,008), $C_{83}H_{89}O_{34}N_8Cl_3$ (MW 1,846) and $C_{77}H_{79}O_{30}N_8Cl_3$ (MW 1,700), respectively. Their physico-chemical characterizations including molecular formulae revealed that chloropolysporins A, B and C were new members of glycopeptide antibiotics.

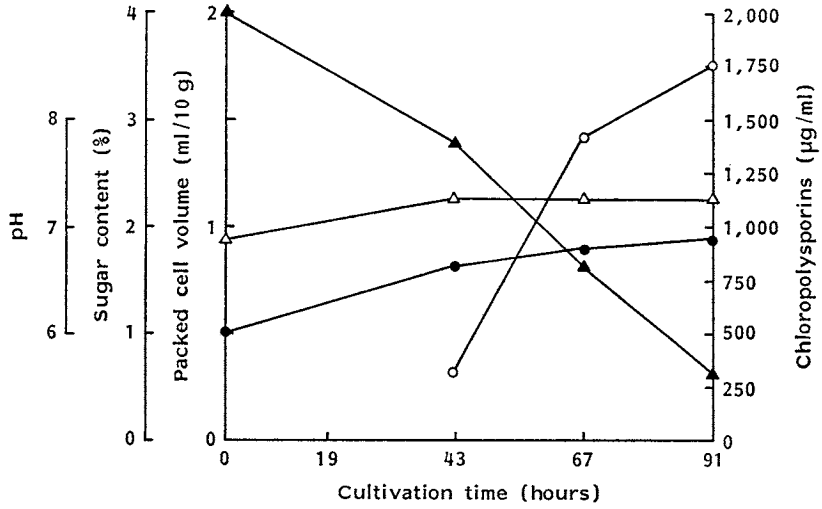
As described in the preceding paper[1], chloropolysporins A, B and C, novel glycopeptide antibiotics, are produced by a new species of actinomycete designated as *Faenia interjecta* sp. nov. SANK 60983.

In this paper, we report fermentation, isolation, physico-chemical properties and degradation studies of chloropolysporins A, B and C.

Fermentation

One loopful growth of strain SANK 60983 was inoculated into a 500-ml Erlenmeyer flask containing 80 ml of a medium composed of glucose 5.0%, pork meat powder 1.0%, NaCl 0.25%, $CaCO_3$ 0.5% and Nissan Disfoam CB 442 0.02%. The pH of the medium was adjusted to 7.2 before sterilization. The flask was cultivated on a rotary shaker at 28°C for 72 hours. A 25-ml aliquot of the culture from the Erlenmeyer flask was inoculated into six 2-liter Erlenmeyer flasks each containing 500 ml of the medium described above and cultivated on a rotary shaker at 28°C for 24 hours. A 3-liter aliquot of the culture was transferred into an 100-liter fermentor containing 60 liters of the same medium and this seed cultivation was conducted at 28°C for 24 hours. After inoculation of 15 liters of the culture into a 600-liter fermentor containing 300 liters of the medium described above, fermentation was carried out for 96 hours under agitation of 125 rpm, aeration of 300 liters per minute and inner pressure of 1.0 kg/cm^2. Mycelial growth was expressed as a packed cell volume (ml) after centrifugation of 10 g of the culture broth at 3,000 rpm for 15 minutes. The maximal potency of chloropolysporins, 1,750 μg/ml, was obtained after 96 hours of fermentation. Antibiotic production during fermentation was monitored by the HPLC method described later. A typical time course of the fermentation in the 600-liter fermentor is shown in Fig. 1.

Fig. 1. Fermentation process of chloropolysporin complex.
○ Chloropolysporins, ● packed cell volume, ▲ sugar content, △ pH.



Isolation

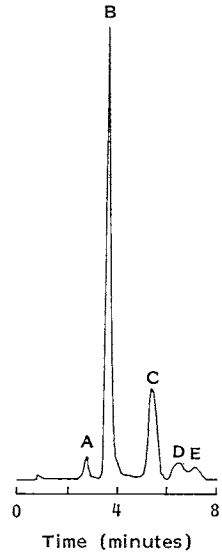
The filtrate (480 liters) from the culture broth (580 liters) was adsorbed on a Diaion HP-20 column (Mitsubishi Chemical Ind. Ltd., Japan, 60 liters), washed with water (300 liters), and the active substances were eluted with 50% aqueous acetone (600 liters). The active eluate was adjusted to pH 3.8 with concentrate HCl, concentrated to 290 liters and then washed two times with butanol in each portion of 200 liters. The aqueous layer was concentrated to 20 liters *in vacuo* and was adjusted to pH 7.5 with 1 N NaOH and applied to a Polyamide column (Woelm Co., West Germany, 12 liters). The column was washed with deionized water (70 liters) and eluted with 50% aqueous methanol (65 liters). After adjusting to pH 4.0 with 1 N HCl, the active eluate was concentrated *in vacuo* and lyophilized to afford 280 g of crude brownish powder (**1**: Yield 28%).

Fig. 2. HPLC retention pattern of chloropolysporin complex.

Column: YMC Pack A-312 ODS (6 mm × 15 cm). Solvent: Sodium heptane sulfonate 1.7% (w/v), conc AcOH 2.0%, conc NH_4OH 0.4%, CH_3CN 16%. Flow rate: 1.5 ml/minute.

Detection: UV at 280 nm.

A: Chloropolysporin A, B: chloropolysporin B, C: chloropolysporin C, D: chloropolysporin D, E: chloropolysporin E.



The HPLC pattern of chloropolysporin complex, which were designated as chloropolysporins A, B, C, D and E in the order of their elution, is shown in Fig. 2.

Two hundred g of **1** was dissolved in deionized water (10 liters), adjusted to pH 5.8 with 1 N NaOH and re-chromatographed on a Polyamide column (8.3 liters) by developing with deionized water to resolve two fractions. The first fraction (1.4 liters) and the second fraction (1.4 liters) contained chloro-

polysporins A and B, respectively. Each of them was adjusted to pH 4.0 with 1 N HCl, concentrated *in vacuo* and lyophilized to yield 26 g of crude powder containing chloropolysporin A (**2**: Yield 90%, purity of chloropolysporin A 27%, chloropolysporin B 45%) and 84 g of partially purified powder containing chloropolysporin B (**3**: Yield 72%, purity 83%).

Main component, chloropolysporin B, was further purified from **3** (2 g) by HPLC on a preparative reverse phase column (Yamamura Co., Japan, YMC-Pack S-343 I15 ODS, 20×250 mm) developed with 16% aqueous acetonitrile solution containing 0.2% trifluoroacetic acid. The collected fractions containing chloropolysporin B were adjusted to pH 7.0 with 1 N NaOH, evaporated to remove acetonitrile and charged on a Diaion HP-20 column (100 ml) to change its salt form from trifluoroacetic acid salt to hydrochloride. For this purpose, the column was washed with distilled water (200 ml), subsequently with 0.1 N HCl (200 ml) and again with distilled water (200 ml) and eluted with 50% aqueous acetone. Finally, the eluate was concentrated *in vacuo* to 4 ml, adjusted to pH 4.0 with 1 N HCl and lyophilized to yield 1.2 g of purified chloropolysporin B hydrochloride (yield 72%).

Two minor components, chloropolysporins A and C were purified as follows: For purification of chloropolysporin A, 320 mg of **2** dissolved in 50% aqueous methanol (6 ml) was applied to a TSK gel Toyopearl HW40-F column (Toyo Soda Co., Japan, 400 ml) equilibrated with the same solvent mixture. Although separation between chloropolysporins A and B was still not complete, the fractions containing chloropolysporins A and B in 1 : 1 ratio were combined, concentrated *in vacuo* and lyophilized to afford brownish powder (53 mg, yield 30%, purity 48%). A final purification was done by the same procedure as in the final purification step of chloropolysporin B except for a concentration of acetonitrile (10%). The pure fractions showing a single peak in the HPLC system were collected, desalted on a Diaion HP-20 column and lyophilized to yield 20 mg of chloropolysporin A hydrochloride (yield 78%).

For purifying chloropolysporin C, 6 g of **1** dissolved in 1 liter of buffer solution of 0.01 M sodium heptane sulfonate containing 2.5% acetic acid and 0.5% concentrate NH_4OH was pumped on a Prep Pak-500 C_{18} column of Waters Prep LC/System (Waters Co., U.S.A.). Elution from this column was achieved with the same buffer solution except for addition of 16% acetonitrile[2]. The chloropolysporin C rich fractions were collected, evaporated to remove acetonitrile, adjusted to pH 7.0 with 6 N NaOH and desalted on a Diaion HP-20 column (100 ml). After washing with deionized water (200 ml), the antibiotic was eluted with 50% aqueous acetone (200 ml), concentrated and lyophilized to obtain 840 mg of powder (yield 70%, purity 53%). Further purification was done on a Lobar column RP-18 size B (Merck Co., West Germany) using the same solvent system. The fractions containing chloropolysporin C were combined, concentrated *in vacuo*, desalted on a Diaion HP-20 column and lyophilized to afford 500 mg of white powder (yield 70%, purity 62%). In order to remove chloropolysporin D, the lyophilized powder was applied to a Toyopearl HW40-F column (400 ml) and developed with 50% aqueous methanol. The pure fractions were combined, evaporated to remove methanol *in vacuo* and lyophilized to yield 247 mg of chloropolysporin C heptane sulfonate (yield 80%). The purification procedure described above was summarized in Fig. 3.

To elucidate primary physico-chemical properties of these pure materials, they were changed to sulfate salts by the method reported by McGAHREN *et al.*[2].

Two minor components, chloropolysporins D and E, were determined to be the artificial epimers of B and C, respectively[3].

Physico-chemical Properties

The sulfates of chloropolysporins A, B and C are all amphoteric, off-white powder, soluble in water and dimethyl sulfoxide, but insoluble in lower alcohols, acetone, ethyl acetate and ethyl ether. These antibiotics showed positive reactions for ferric chloride, iodine, Rydon-Smith reagent, ninhydrin and sulfuric acid on silica gel TLC plate. The molecular formulae of chloropolysporins A, B and C were established to be $C_{89}H_{99}O_{39}N_8Cl_3$ (MW 2,008), $C_{83}H_{89}O_{34}N_8Cl_3$ (MW 1,846) and $C_{77}H_{79}O_{30}N_8Cl_3$ (MW 1,700), respectively, by elementary analyses as well as their quasi molecular ion $(M+H)^+$ by fast atom bombardment mass spectra (FAB-MS) in agreement with those of calculated nominal values. The UV spectra of these three compounds showed a characteristic absorption of glycopeptide antibiotics at 280 nm and their IR spectra indicated the presence of hydroxyl groups at 3400 cm^{-1} and amide bonds between 1560 cm^{-1} to 1650 cm^{-1}. These results as well as other physico-chemical properties of these compounds are summarized in Tables 1, 2 and 3. It is very difficult to assign the signals in the ^1H NMR spectra (270 MHz, DMSO-d_6) of these compounds (Figs. 4, 5 and 6) because of their broadened line width and highly complicated resolution. But these spectra suggest the presence of an *N*-methyl group (br s δ 2.2~2.8) and one (C) or two (A and B) methyl group (s) (br d δ 1.0~1.3) in every compound.

Fig. 3. Isolation procedure of chloropolysporins A, B and C.

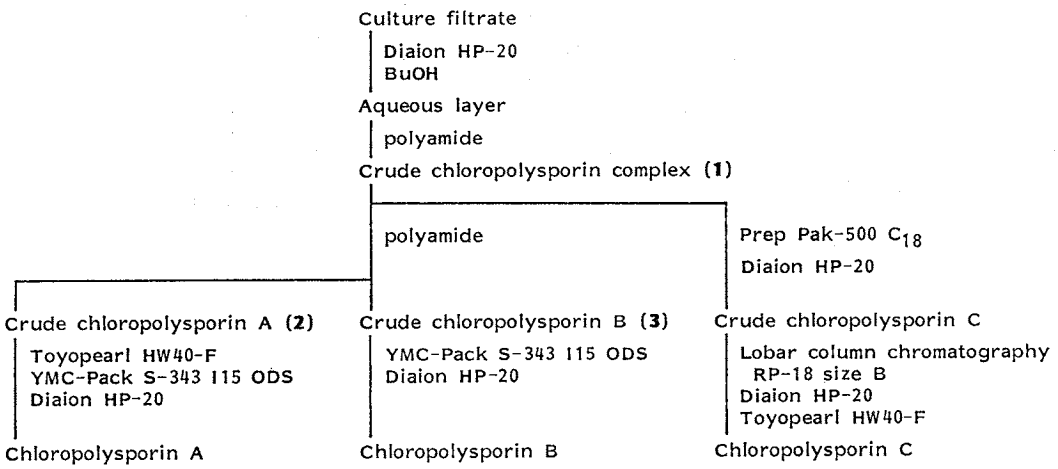


Table 1. Physico-chemical properties of chloropolysporin A.

FAB-MS (*m/z*)	2,009 $(M+H)^+$
UV $\lambda_{max}^{0.01N\text{-}HCl}$ nm ($E_{1cm}^{1\%}$)	280 (39)
$[\alpha]_D^{25}$	−63° (*c* 0.99, 0.1 N HCl)

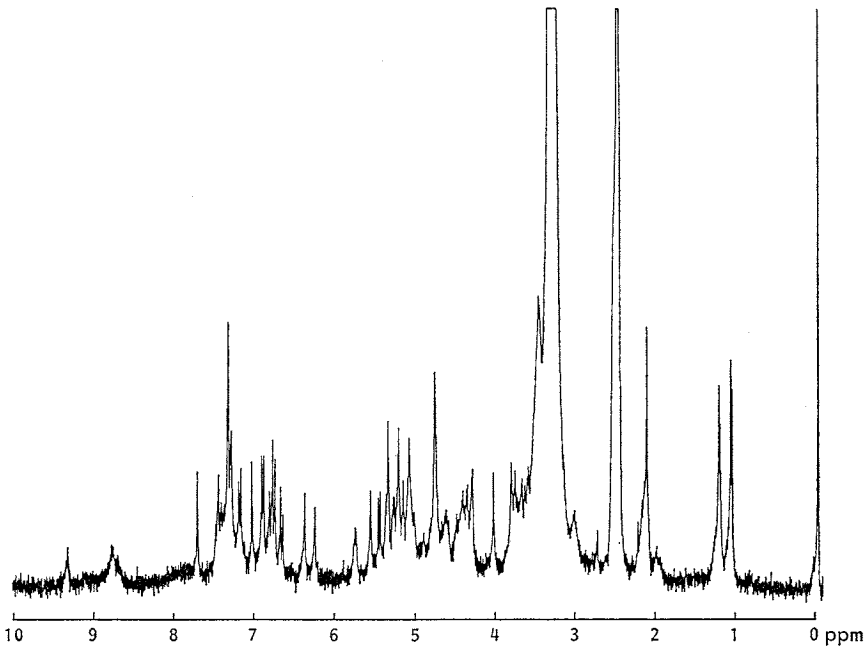
Table 2. Physico-chemical properties of chloropolysporin B.

Elementary analysis	
Calcd for $C_{83}H_{89}O_{34}N_8Cl_3 \cdot \frac{1}{2}H_2SO_4 \cdot 10H_2O$ (2,078):	C 47.97, H 5.34, N 5.39, Cl 5.12, S 0.77.
Found:	C 48.33, H 5.05, N 5.48, Cl 5.11, S 1.00.
FAB-MS (*m/z*)	1,847 $(M+H)^+$
UV $\lambda_{max}^{0.01N\text{-}HCl}$ nm ($E_{1cm}^{1\%}$)	280 (51)
$[\alpha]_D^{25}$	−64.5° (*c* 1.04, 0.1 N HCl)

Table 3. Physico-chemical properties of chloropolysporin C.

Elementary analysis	
Calcd for $C_{77}H_{79}O_{30}N_8Cl_3 \cdot \frac{1}{2}H_2SO_4 \cdot 5H_2O$ (1,842):	C 50.21, H 4.92, N 6.08, Cl 5.77, S 0.87.
Found:	C 50.53, H 4.69, N 6.14, Cl 5.62, S 1.12.
FAB-MS (*m/z*)	1,701 $(M+H)^+$
UV $\lambda_{max}^{0.01N\text{-}HCl}$ nm ($E_{1cm}^{1\%}$)	280 (57)
$[\alpha]_D^{25}$	−64.4° (*c* 1.08, 0.1 N HCl)

Fig. 4. ^1H NMR spectrum of chloropolysporin A.



Degradation Studies

^1H NMR and mass spectral analyses of the amino acid constituents obtained from the acid hydrolysate of all these three compounds (concentrate HCl - CH_3COOH, 1 : 1, at 105°C 20 hours and/or HI/P, at 105°C, 20 hours[4]) indicated the presence of actinoidic acid, 3-chloro-4-hydroxyphenylglycine, *N*-methyl-*p*-hydroxyphenylglycine and dideoxyvancomycinic acid (Fig. 7). These unusual amino acids are typical constituents in glycopeptide antibiotics[5].

The presence of 1 mol of glucose, mannose, rhamnose and galactose in chloropolysporin A, glucose, mannose and rhamnose in chloropolysporin B and glucose and mannose in chloropolysporin C were detected by gas chromatographic analysis of their hydrolysate (5% HCl in absolute MeOH, 105°C, 16 hours) followed by their trimethylsilylation. Ristosamine was detected from each hydrolysate of chloropolysporins A, B and C (5% HCl in absolute MeOH, 105°C, 5 hours).

Further details of the degradation studies will be described in the subsequent paper on their structure elucidations[3].

Comparative Studies

More than fifteen groups of glycopeptide antibiotics have been reported as shown in Table 4.

Fig. 5. ^{1}H NMR spectrum of chloropolysporin B.

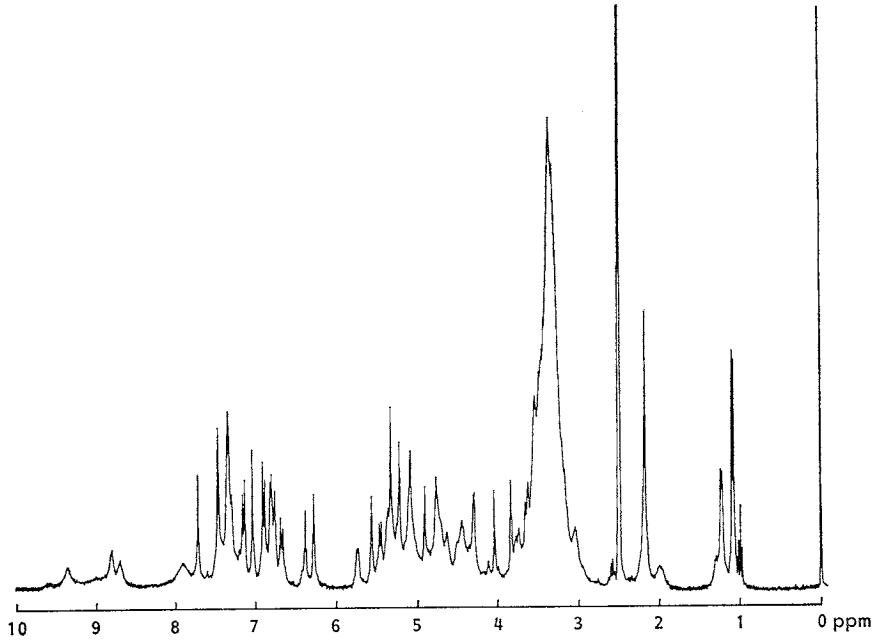


Fig. 6. ^{1}H NMR spectrum of chloropolysporin C.

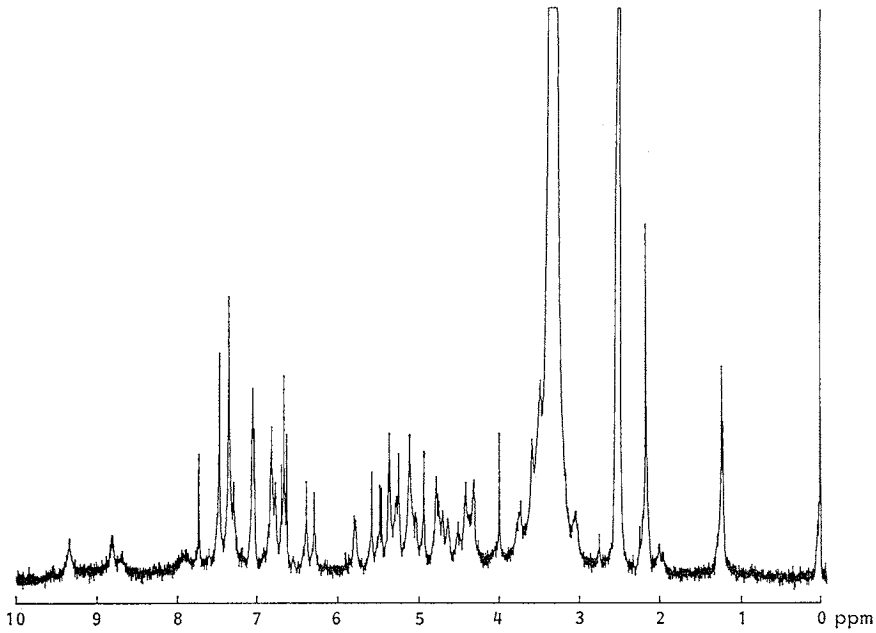
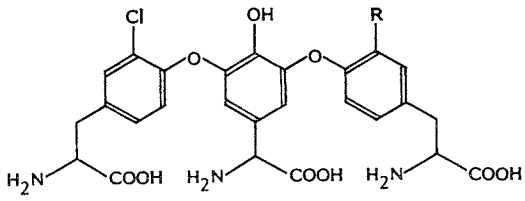
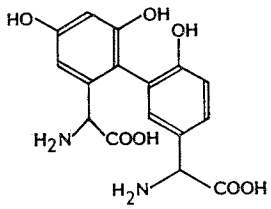


Fig. 7. Degradation products from chloropolysporins and β-avoparcin.

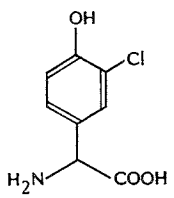


Dideoxyvancomycinic acid R = Cl

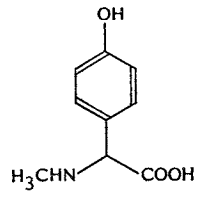
Monodechlorodideoxy-vancomycinic acid R = H



Actinoidic acid



3-Chloro-4-hydroxy-phenylglycine



N-Methyl-*p*-hydroxy-phenylglycine

Table 4. Characteristic chemical constituents of glycopeptide antibiotics.

	Amino acid*	Amino sugar	Chlorine
A477[7]	Not reported	Not reported	2
A35512B[8]	Demethylristomycinic acid	*epi*-Vancosamine	1
A41030[9]	Demethylristomycinic acid	None	1~3
A47934[10]	Sulfate ester of demethyl-ristomycinic acid	None	3
AB-65[11]	Not reported	Unknown sugar	2
Actaplanin[12,13]	Ristomycinic acid	Ristosamine	1
Actinoidin[14]	(3-Chloro-)4-hydroxyphenylglycine, phenylalanine	Acosamine, actinosamine	1~2
Aridicin[15,16]	*N*-Methyl derivative of demethyl-ristomycinic acid	None	4
Avoparcin[17]	(3-Chloro-)4-hydroxyphenylglycine, *N*-methyl-*p*-hydroxyphenylglycine	Ristosamine	1~2
LL-AM374[18]	Not reported	Not reported	1
Mannopeptin[19]	Glycine, (β-methyl)phenylalanine, serine, tyrosine	None	0
OA-7653[20]	Glutamic acid	Not reported	Not reported
Ristocetin[21,22]	Ristomycinic acid	Ristosamine	0
Teicoplanin[23~25]	Demethylristomycinic acid	Glucosamine	2
Vancomycin[26,27]	Aspartic acid, *N*-methylleucine	Vancosamine	2
Chloropolysporin	3-Chloro-4-hydroxyphenylglycine, *N*-methyl-*p*-hydroxyphenylglycine	Ristosamine	3

* Actinoidic acid and vancomycinic acid moieties are omitted.

The variety of their structures was mainly observed by their constituents of amino acids or amino sugars. In the point of the composition of amino acids, it was revealed that chloropolysporins were closely related to β-avoparcin[2,6], but chloropolysporins were clearly differed from β-avoparcin in the

existence of vancomycinic acid moiety in the former and monodechlorovancomycinic acid in the latter (Fig. 7). Therefore, chloropolysporins A, B and C were demonstrated as new antibiotics.

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