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

V. COMPARATIVE STUDIES OF THE BIOLOGICAL PROPERTIES

TOSHIO TAKATSU, TOSHIAKI KATAYAMA, MUTSUO NAKAJIMA, SHUJI TAKAHASHI and TATSUO HANEISHI

Fermentation Research Laboratories,

TERUO MAGARIBUCHI and MASAZO TAJIMA

Biological Research Laboratories,
Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

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Chloropolysporins A, B and C, as well as derivatives prepared from this group and α- and β-avoparcins by enzymatic and mild acid hydrolysis, were active against Gram-positive bacteria including clinically isolated methicillin-resistant Staphylococci (MIC 0.39~6.25 μg/ml) and anaerobic enterobacteria (MIC 0.10~1.56 μg/ml). Derhamnosyl and demannosyl derivatives from both groups of antibiotics showed stronger activities than the parent compounds. The MIC and MBC values against Staphylococci were similar and were not effected by the presence of serum. Moreover, chloropolysporin C exhibited very strong synergistic effects with various β-lactam antibiotics against methicillin-resistant strains of *Staphylococcus aureus*. Some of these compounds also protected mice from experimental infection with *S. aureus*. Acute toxicities of chloropolysporins by intravenous administration ranged from 215~290 mg/kg in mice. Chloropolysporin B as well as other glycopeptide antibiotics, showed distinctive growth promoting activity in broiler chickens.

As reported in the preceding papers[1~3], the novel glycopeptide antibiotics, chloropolysporins A, B and C were produced by *Faenia interjecta* sp. nov. Their physico-chemical characteristics and structural elucidation revealed that they can be distinguished from other members of the glycopeptide antibiotic family. Some new deglycosylated derivatives were prepared by enzymatic and partial hydrolysis[4].

This report describes the comparison of the biological properties of chloropolysporins A, B and C, as well as some new deglycosylated derivatives, along with vancomycin[5] and α- and β-avoparcins[6].

In Vitro Studies

The minimal inhibitory concentrations (MICs) of chloropolysporins A, B and C against various species of bacteria were determined by a serial 2-fold agar dilution method using Mueller-Hinton agar medium. Vancomycin[5], which has been mainly used in the treatment of infectious diseases caused by methicillin-resistant Staphylococci and pseudomembranous colitis caused by *Clostridium difficile*, and β-avoparcin[6], a feed additive for growth promotion of livestock, were used as references. The results are presented in Table 1.

Chloropolysporins were strongly active against aerobic Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and also strongly inhibited anaerobic Gram-positive enterobacteria, whereas they were inactive against Gram-negative bacteria.

Generally, chloropolysporin C was the most active component of the chloropolysporin complex

Table 1. Antimicrobial spectra of chloropolysporins A, B and C.

Organism	MIC (μg/ml)				
	CPS-A	CPS-B	CPS-C	β-AVP	VCM
Staphylococcus aureus FDA 209P JC-1	1.56	1.56	1.56	1.56	0.78
S. aureus SANK 70175 (multi-resistant)	1.56	3.13	1.56	1.56	0.39
S. aureus SANK 71183 (MRSA)	3.13	3.13	3.13	3.13	0.78
S. aureus SANK 71283 (MRSA)	3.13	3.13	1.56	3.13	0.78
S. aureus Smith	3.13	12.5	6.25	6.25	1.56
S. epidermidis 180	NT	3.13	3.13	3.13	1.56
Enterococcus faecalis SANK 71778	1.56	1.56	1.56	1.56	3.13
Bacillus subtilis PCI 219	0.78	0.78	0.78	0.39	0.20
Micrococcus luteus PCI 1001	NT	0.78	0.20	0.39	0.78
Mycobacterium smegmatis ATCC 607	NT	25	12.5	25	6.25
Escherichia coli NIHJ JC-2	>100	>100	>100	>100	>100
Proteus vulgaris OX 19	NT	>100	>100	>100	>100
Propionibacterium acnes ATCC 11828*	NT	0.39	0.20	0.20	0.78
Peptococcus magnus ATCC 14956*	NT	0.78	0.39	0.39	0.39
Clostridium difficile ATCC 9689*	NT	1.56	0.78	0.78	1.56
C. perfringens ATCC 13123*	NT	0.20	0.10	0.20	1.56
Bacteroides fragilis ATCC 25285*	NT	>100	>100	>100	100

Medium: Mueller-Hinton agar.
* GAM agar.
Inoculum: 10^6 cfu/ml, 37°C, 24 hours.
CPS-A: Chloropolysporin A, CPS-B: chloropolysporin B, CPS-C: chloropolysporin C, β-AVP: β-avoparcin, VCM: vancomycin.
NT: Not tested.

and its activity was slightly stronger than that of β-avoparcin. Vancomycin was the most active agent in the five antibiotics tested against Gram-positive aerobes. However, it was noteworthy that chloropolysporin C had two to four times stronger activities than vancomycin against Gram-positive anaerobes, especially against *Clostridium perfringens* and *C. difficile*.

Table 2 shows the MICs of the several deglycosylated derivatives of chloropolysporins B and C, and α- and β-avoparcins. In *in vitro* activity, demannosylchloropolysporin C was superior to that of vancomycin. The relationship of the activities between the original compounds and their partially deglycosylated products are similar to the relationship between chloropolysporins B and C, *i.e.* almost all the converted compounds were two times or more active against Gram-positive aerobes than that of the each parent compound. Chloropolysporin pseudoaglycone (deglucosylchloropolysporin C) also showed two to eight times stronger activity than that of chloropolysporin C. It has been well-known that most of the pseudoaglycones derived from glycopeptide antibiotics indicate stronger activity than that of the parent compound *in vitro*[7~9]. Comparison of the activity of demannosylchloropolysporin B with that of chloropolysporin C (derhamnosylchloropolysporin B) showed that demannosylation of chloropolysporin B resulted in improvement of antimicrobial activities more effectively than derhamnosylation.

Table 3 shows the MICs together with the minimal bactericidal concetrations (MBCs) against some Staphylococci including methicillin-resistant strains and *Enterococcus faecalis*. The MIC by a serial two-fold dilution in Mueller-Hinton broth was defined as the lowest concentration of antibiotic which allowed no visible growth of the organism in microtiter plates. The MBC was defined as the lowest concentration of an antimicrobial agent which resulted in a 99.9%-kill of the inoculum according to the

Table 2. Antimicrobial spectra of glycopeptide derivatives.

Organism	MIC (μg/ml)									
	CPS-B	DMCPS-B	CPS-C	DMCPS-C	CPS-ϕ	α-AVP	DR-α-AVP	β-AVP	DR-β-AVP	VCM
Staphylococcus aureus FDA 209P JC-1	1.56	0.78	0.78	0.39	0.78	1.56	1.56	0.78	0.78	0.78
S. aureus SANK 70175 (multi-resistant)	6.25	0.78	3.13	0.78	0.78	6.25	3.13	3.13	1.56	0.78
S. aureus Smith	6.25	0.78	3.13	0.39	0.39	6.25	3.13	3.13	1.56	1.56
S. epidermidis IAM 1296	3.13	1.56	1.56	NT	0.78	3.13	3.13	1.56	1.56	1.56
Enterococcus faecalis NCTC 775	0.78	0.39	0.39	NT	0.39	1.56	0.78	0.78	0.78	0.78
Bacillus subtilis ATCC 6633	0.78	0.39	0.39	0.20	0.78	0.78	0.39	0.39	0.39	0.20
Micrococcus luteus ATCC 9341	0.78	0.20	0.78	0.05	0.78	1.56	0.78	0.78	0.39	0.78
Escherichia coli NIHJ JC-2	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Klebsiella pneumoniae IID 865	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Propionibacterium acnes ATCC 11828*	0.39	0.20	0.20	0.10	0.39	0.78	0.39	0.20	0.20	0.78
Peptococcus magnus ATCC 14956*	0.78	0.39	0.39	0.20	0.39	0.78	0.78	0.39	0.39	0.39
Clostridium difficile ATCC 9689*	1.56	0.78	0.78	0.20	0.78	1.56	1.56	0.78	0.39	1.56
C. perfringens ATCC 13123*	0.20	0.20	0.10	0.05	0.39	0.78	0.78	0.20	0.39	1.56
Bacteroides fragilis GAI 3025*	>100	>100	>100	100	>100	100	50	100	50	50

Medium: Mueller-Hinton agar.
* GAM agar.
Inoculum: 10^6 cfu/ml, 37°C, 24 hours.
CPS-B: Chloropolysporin B, DMCPS-B: demannosylchloropolysporin B, CPS-C: chloropolysporin C, DMCPS-C: demannosylchloropolysporin C, CPS-ϕ: chloropolysporin pseudoaglycone, α-AVP: α-avoparcin, DR-α-AVP: derhamnosyl-α-avoparcin, β-AVP: β-avoparcin, DR-β-AVP: derhamnosyl-β-avoparcin, VCM: vancomycin.
NT: Not tested.

Table 3. Antimicrobial activities of derivatives of glycopeptide antibiotics against selected strains of Gram-positive bacteria.

	S. aureus FDA 209P	*S. aureus* SANK 70175 (multi-resistant)	*S. aureus* SANK 71183 (MRSA)	*S. aureus* SANK 71486 (MRSA)	*E. faecalis* S-299
CPS-C	0.20 (0.20)*	0.20 (0.39)	0.78 (1.56)	0.39 (0.39)	0.39 (25)
CPS-ϕ	0.10 (0.10)	<0.013	0.39 (0.39)	0.20 (0.20)	0.39 (25)
α-AVP	0.39 (0.78)	1.56 (1.56)	3.13 (3.13)	1.56 (1.56)	1.56 (6.25)
DR-α-AVP	0.20 (0.39)	0.39 (0.78)	1.56 (1.56)	0.78 (0.78)	0.78 (6.25)
β-AVP	0.20 (0.39)	0.39 (0.78)	1.56 (1.56)	0.78 (0.78)	0.78 (12.5)
DR-β-AVP	0.10 (0.20)	0.39 (0.78)	0.78 (0.78)	0.39 (0.39)	0.39 (12.5)
VCM	0.39 (0.39)	0.78 (0.78)	0.39 (0.39)	0.39 (0.39)	1.56 (6.25)
CPS-C	0.39 (3.13)	0.39 (1.56)	1.56 (NT)	0.78 (0.78)	0.78 (25)
CPS-ϕ	0.20 (0.39)	0.10 (0.20)	0.39 (0.78)	0.39 (0.78)	0.39 (25)
α-AVP	0.78 (0.78)	1.56 (1.56)	3.13 (6.25)	3.13 (12.5)	1.56 (25)
DR-α-AVP	0.39 (0.78)	1.56 (3.13)	1.56 (3.13)	1.56 (3.13)	1.56 (25)
β-AVP	0.39 (1.56)	0.78 (0.78)	1.56 (NT)	1.56 (3.13)	0.78 (25)
DR-β-AVP	0.39 (0.78)	0.39 (0.39)	1.56 (3.13)	0.78 (1.56)	0.78 (25)
VCM	0.78 (0.78)	0.78 (0.78)	0.78 (NT)	0.78 (1.56)	1.56 (25)

Inoculum size: Above 10^4 cfu/ml, below 10^6 cfu/ml.
* MIC (MBC) μg/ml.
Abbreviations for the tested compounds are shown in Table 2.
NT: Not tested.

Table 4. Effect of horse serum on antimicrobial activity.

	MIC (MIC in 10% horse serum, μg/ml)				
	S. aureus FDA 209P	*S. aureus* SANK 71183 (MRSA)	*S. aureus* SANK 71486 (MRSA)	*S. aureus* SANK 70175 (multi-resistant)	*E. faecalis* S-299
CPS-C	0.20 (0.39)	0.78 (0.78)	0.39 (0.78)	0.39 (0.78)	0.39 (0.78)
α-AVP	0.78 (0.78)	3.13 (3.13)	1.56 (1.56)	1.56 (1.56)	1.56 (3.13)
DR-α-AVP	0.39 (0.78)	1.56 (1.56)	0.78 (1.56)	0.39 (0.78)	0.78 (1.56)
β-AVP	0.20 (0.39)	1.56 (1.56)	0.78 (1.56)	0.39 (0.78)	0.39 (0.78)
DR-β-AVP	0.10 (0.10)	0.39 (0.78)	0.39 (0.39)	0.20 (0.39)	0.39 (0.78)
VCM	0.39 (0.39)	0.78 (0.78)	0.39 (0.39)	0.39 (0.78)	1.56 (3.13)

Medium: Mueller-Hinton broth.
Inoculum size: 10^4 cfu/ml.
Abbreviations for the tested compounds are shown in Table 2.

method reported by SHANHOLTZER *et al.*[10]. From the point of view of MICs, derhamnosyl derivatives showed two times stronger activity than those of each parent compound. Moreover, chloropolysporin pseudoaglycone exhibited two to four times stronger activity than that of chloropolysporin C. Significant differences were not observed between MIC and MBC values against strains of *S. aureus* in both 10^6 and 10^4 cfu/ml inoculum size were not observed, but were clearly observed in the case of *Enterococcus faecalis*.

Table 4 shows the effect of horse serum on the antimicrobial activity. Addition of 10% of the serum in Mueller-Hinton broth did not cause any effects on the activities of chloropolysporins B and C.

Synergistic effects between chloropolysporin C and various β-lactam antibiotics against three strains of *S. aureus* including methicillin-resistant strains were examined. Synergistic effect is expressed in term of the fractional inhibitory concentration (FIC) index described by HALL *et al.*[11]. The

Table 5. Minimal FIC indexes in combinations of glycopeptide antibiotics and cephalosporins.

	S. aureus FDA 209P			*S. aureus* SANK 71183 (MRSA)			*S. aureus* SANK 71486 (MRSA)		
	CPS-C	β-AVP	VCM	CPS-C	β-AVP	VCM	CPS-C	β-AVP	VCM
CMZ	0.63	0.75	0.63	0.19	0.25	0.75	0.38	0.25	0.63
CER	1.00	1.00	0.63	0.16	0.25	0.63	0.25	0.25	0.63
CTX	0.63	0.75	0.75	0.16	0.38	0.53	0.31	0.50	0.63
LMOX	0.63	0.75	0.53	0.19	0.19	0.52	0.38	0.31	0.75

Judgment: <0.50; synergism, $0.50\sim1.0$; additive or indifference.
Medium: Mueller-Hinton broth.
Inoculum size: 10^4 cfu/ml.
CPS-C: Chloropolysporin C, β-AVP: β-avoparcin, VCM: vancomycin, CMZ: cefmetazole, CER: cephaloridine, CTX: cefotaxime, LMOX: latamoxef.

FIC is the sum of the MICs of drugs in combination divided by the MIC of drug alone. For two interacting drugs A and B, the MICs of drugs A and B when used alone are called Ae and Be. The concentrations that produce the same effect when used in combination are called Ac and Bc. An equation is then constructed as

$$FIC=(Ac/Ae)+(Bc/Be)\ .$$

If the sum is below 0.5, the combination is synergistic, if the sum is between 0.5 and 1.0, the combination is additive or indifference. Table 5 exhibits minimal FIC indexes which were evaluated by the checkerboard technique. Both chloropolysporin C and β-avoparcin showed very strong synergy with all four β-lactam antibiotics tested against methicillin-resistant strains of Staphylococci, whereas their combinations did not show any synergistic effect against a sensitive strain, *S. aureus* FDA 209P. No synergy was observed between vancomycin and the β-lactam antibiotics tested. It is noteworthy that chloropolysporin C showed synergism with β-lactam antibiotics, including newly developed cephalosporins which alone are only weakly active against methicillin-resistant strains of Staphylococci (minimal FIC index; $0.16\sim0.38$).

Chemotherapeutic and Toxicological Studies

Chloropolysporins were effective in mice against experimental infection with *S. aureus*. Five weeks old ICR male mice (10 mice per dose) were challenged with *S. aureus* SANK 70175 intraperitoneally (1×10^8 cells per mouse) in the presence of 5% mucin. The chloropolysporin derivatives were administered subcutaneously at 0 and 4 hours after the challenge. The ED_{50} values which were calculated by a LICHFIELD-WILCOXON's method are shown in Table 6, together with their LD_{50} values in mice. In this evaluation method, vancomycin seems to be the most effective against experimental infection with *S. aureus* SANK 70175. Modification of chloropolysporin B by derhamnosylation with Naringinase or demannosylation with α-mannosidase gave derivatives with three-fold increased *in vivo* activities compared with that of the parent compound, whereas the activities of all other modified antibiotics exhibited nearly the same or reduced activity compared to the original compounds. In similar to the pseudoaglycones of the glycopeptide antibiotics reported that of chloropolysporins also showed drastically reduced *in vivo* activities in contrast to their *in vitro* stronger activity than the parent antibiotics[9].

Growth-promoting Activity

In addition to their antimicrobial activities, growth promotion in livestock was observed with

Table 6. ED_{50} and LD_{50} values of glycopeptide derivatives (mg/kg).

Compound	CPS-B	DMCPS-B	CPS-C	DMCPS-C	CPS-ϕ	β-AVP	DR-β-AVP	VCM
ED_{50}	>200	72.0 (60~86)	64.0 (52~79)	>100	>100	19.0 (16~23)	29.0 (18~47)	6.1 (4.7~8.0)
LD_{50}	215	NT	250	NT	290	500	230	470

ED_{50}: Test organism; *Staphylococcus aureus* SANK 70175, challenge (ip); 10^8 cfu/mouse +5% mucin, treatment (sc); 0 and 4 hours after infection.
LD_{50}: Drugs were injected intravenously.
Abbreviations for the tested compounds are shown in Table 2.
NT: Not tested.
SE are shown below the ED_{50} values.

Table 7. Effect of chloropolysporin B and avoparcin on the growth of broiler chicken (% of control).

	Negative control	Avoparcin (10 mg/kg*)	Chloropolysporin B		
			5 mg/kg*	10 mg/kg*	20 mg/kg*
Body weights gain	100	104	103	103	104
Food consumption	100	102	101	102	102
Food conversion ratio	100	98	98	99	98
Viability	100	101	101	99	101

An equal number of male and female birds (50 birds each) were used for each fraction.
$P<0.05$.
* Contents in food.

chloropolysporin B as well as with avoparcin complex, which is already known[12] to have its activity and commercially available. The addition of 5~20 mg of chloropolysporin B in 1 kg of the basal diet of broilers for eight weeks resulted in improvement in body weight gain and feed conversion rate (both $P<0.05$). As shown in Table 7, the gain in body weight, feed conversion rate and growth rate were improved in chickens supplemented with chloropolysporin B. The effect of chloropolysporin B on the growth-promotion in chickens was very similar to that of the avoparcins. The feed conversion rate observed in the group treated with avoparcins was similar to the values reported in the literature[12].

Discussion

Recently, glycopeptide antibiotics have been re-investigated because of their strong activities against methicillin-resistant strains of Staphylococci[13]. Similarly, chloropolysporins A, B and C were active against Gram-positive bacteria *in vitro*, and mice were protected from the infection with *S. aureus* by intraperitoneal administration of the antibiotics. It was noted that marked synergistic effects of chloropolysporin C and β-lactam antibiotics such as cefmetazole, cephaloridine, cefotaxime and latamoxef against methicillin-resistant strains of Staphylococci were observed, but not observed against a sensitive strain, *S. aureus* FDA 209P.

Mode of action studies conducted on other members of this family, *e.g.* vancomycin[14], ristocetin[15], teicoplanin[16] *etc.*, have indicated that they inhibit cell wall biosynthesis in *S. aureus*. Moreover, it is well-known that they bind with a precursor of peptidoglycan or related compounds such as *N*-acetyl-L-Lys-D-Ala-D-Ala[17]. We observed the binding of chloropolysporins B and C to a D-Ala-D-Ala affinity gel[18], suggesting the same mechanism of action as other glycopeptide antibiotics.

Enzymatic conversion of chloropolysporins and avoparcins gave several new compounds, however, none of which was superior *in vivo* to that of vancomycin.

In addition to their antimicrobial activities, the chloropolysporin complex showed growth-promoting activity in broiler chickens as reported for other members of this family[12]. The similar growth-

promoting activity of chloropolysporin B to that of avoparcin complex, Avotan, as well as stronger *in vitro* activities than β-avoparcin and vancomycin against anaerobic Gram-positive enterobacteria such as *C. perfringens* may result in advantages of chloropolysporins as a feed additive.

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