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Antiviral, Antimicrobial, and Cytotoxic Properties of Peptavirins A and B Produced by *Apiocrea* sp. 14T

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Two peptaibol antibiotics, peptavirins A and B, which exhibited strong inhibitory effect against *Tobacco mosaic virus* (TMV) infection, were isolated from steam-cooked rice culture of *Apiocrea* sp. 14T. The peptavirins were identified as new derivatives of chrysospermins, which are 19-mer and have been reported to be produced in a fungal isolate. The physicochemical properties of the peptavirins were mostly identical with chrysospermins A through D except for the UV absorption spectrum. The peptavirins inhibited the growths of the Gram-positive bacteria tested, including the plant pathogenic bacterium, *Corynebacterium lilium*, and the fungus, *Aspergillus niger*. Peptavirin A was somewhat cytotoxic to cancer cell lines, especially K562 (leukemia) and UACC 62 (melanoma), whereas peptavirin B only exhibited slight cytotoxicity.

Keywords : antimicrobial activity, *Apiocrea* sp., cytotoxicity, peptavirins, peptaibol antibiotics.

Peptaibols are a type of peptide with an N-terminal acylated amino acid residue and C-terminal amino alcohol, containing many α,α-dialkylated amino acids, α-aminoisobutyric acid (Aib), and isovaline (Iva). Peptaibol antibiotics, which have been isolated mostly from *Sepedonium* (teleomorph: *Apiocrea*) spp., *Trichoderma* spp., and *Boletus* sp., are well known as transmembrane channel-forming agents active against fungi and Gram-positive bacteria. They have also exhibited various kinds of biological activities, either hemolytic or inhibitory to platelet aggregation (Bruckner et al., 1984; Chikanish et al., 1997), neuroleptic (Ritzau et al., 1997), antiamoebin (Snook et al., 1998), antibacterial, and antifungal activities (Berg et al., 1996; Bodo et al., 1985; Dornberger et al., 1995; Grafe et al., 1995).

During recent screening work for anti-phytoviral materials, it was found that the culture of *Apiocrea* sp. 14T showed strong inhibitory activity against the *Tobacco mosaic virus* (TMV) infection in tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc NN). The main antiviral components produced by the fungus were isolated and purified using various chromatographies. In total, four peptaibols with 19-mer amino acid residues were produced in a steamed rice culture of the fungus. Two of them were identified as chrysospermins B and D that are known compounds produced by *Apiocrea Chrysosperma* (Dornberger et al., 1995); their anti-phytoviral, antimicrobial, and cytotoxic activities were earlier reported by the authors (Kim et al., 2000). The other two are new chrysospermin derivatives designated as peptavirins A and B (Yun et al., 2000), which had strong anti-phytoviral activity against TMV. The molecular structures of peptavirins A and B were identified (Fig. 1), along with their molecular weights of 1909 and 1886, and the molecular formulae of $C_{92}H_{144}N_{22}O_{22}$ and $C_{90}H_{144}N_{21}O_{23}$, respectively. The amino acid residues differed at positions 3 and 5 (peptavirin A), and the C-terminal amino alcohol was substituted with phenylalaninol instead of tryptophanol (peptavirin B).

The present study examined the physico-chemical, antibiotic, and cytotoxic properties of these new chrysospermin derivatives, peptavirins A and B. Also, their antiviral activity against TMV infection was reexamined based on the concentration of the materials

Materials and Methods

Fermentation and purification of the peptavirins. The peptavirins were produced in steam-cooked rice culture of the fungus (*Apiocrea* sp. 14 T) that had been incubated at 27°C for 3 weeks. The rice-cultured material was extracted with ethanol, concentrated *in vacuo*, and partitioned between H_2O and butanol (BuOH). The BuOH phase was concentrated and subjected to flash chromatography on a silica gel column (Silica gel 60, Merck, 70/230 mesh) with $CHCl_3$-methanol (MeOH) (100:0 →

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Chrysospermin B	AcPhe Aib **Ser** Aib **Aib** Leu Gln Gly Aib Aib Ala Ala Aib Pro Iva Aib Aib Gln **Trpol**
Chrysospermin D	AcPhe Aib **Ser** Aib **Iva** Leu Gln Gly Aib Aib Ala Ala Aib Pro Iva Aib Aib Gln **Trpol**
Peptavirin A	AcPhe Aib **Ala** Aib **Iva** Leu Gln Gly Aib Aib Ala Ala Aib Pro Iva Aib Aib Gln **Trpol**
Peptavirin B	AcPhe Aib **Ser** Aib **Iva** Leu Gln Gly Aib Aib Ala Ala Aib Pro Iva Aib Aib Gln **Pheol**

Fig. 1. Antimicrobial peptaibols produced by *Apiocrea* sp. 14T. Bold-faced letters indicate the residues that differ between the peptaibols (AcPhe: N-acetyl phenylalanine, Aib: α-aminoisobutyric acid, Iva: isovaline, Trpol: tryptophanol, Pheol: phenylalaninol).

30:1→10:1→8:1→6:1→4:1→2:1→1:1, v/v). The crude antiviral fractions were combined and subjected to flash chromatography on an ODS (C18, Waters, 70/230 mesh) column with H_2O-MeOH (100:0→90:10→70:30→50:50→30:70→10:90, v/v). The active H_2O-MeOH (10:90) fraction was concentrated and further purified by reverse-phase HPLC (ODS, Capcell Pak; 62% MeCN; flow rate of 3.0 ml/min; UV detection at 220 nm).

Physico-chemical characters of the peptavirinsB The two purified compounds were subjected to instrumental analyses to examine their physico-chemical properties. UV-visible spectrum was measured with a Shimazu UV-260 spectrophotometer. Melting points were measured with a Fisher Johns melting point apparatus. Their solubility and *Rf* values on TLC were examined by usual methods.

Inhibitory effect of the peptavirins on TMV Infection. TMV-infected plant sap in 0.02 M phosphate buffer (pH 7.3) was prepared, which was a 100-fold dilution of dried leaf debris. Each purified compound was dissolved in a small volume of ethanol to make a 200-, 20-, and 2-μg/ml concentrations (containing 5% ethanol). These solutions were mixed with equal volumes of TMV sap. These mixtures were inoculated on the half leaves of the local lesion host plants, *Nicotiana tabacum* cv. Xanthi-nc, and the other half leaves were inoculated with the control infected plant sap (TMV inoculum in 5% ethanol) as above in the screening experiment. The inoculated plants were placed at 25-27°C in a greenhouse, and the number of lesions formed on a treated half leaf was measured and compared with that on the other control half leaf 3-4 days after inoculation.

Antimicrobial activity of KGT 141 and KGT 142 against bacteria and fungi. Antimicrobial activity was determined by paper disc method using Mueller-Hinton agar and PDA for bacteria and fungi, respectively, using paper discs 8 mm in diameter. The paper discs were soaked in solution of each compound (30 μg per paper disc), and were placed on the media seeded with bacteria or fungi. One to two days later, clear zones around the paper discs were examined and measured in size.

Cytotoxicity of KGT 141 and KGT 142 on Tumor Cells. Cytotoxicity of the purified compounds against human cancer cell lines was tested by SRB method (Yeo et al., 1994), examining the growth of cells in RPMI 1640 media with 10% fetal calf serum (FCS). The media contained each compound (dissolved in DMSO to give the final concentration of 0.1% DMSO) in wells of tissue culture microplates, incubated at 37°C in a 5% CO_2 incubator. The results were expressed as 50% effective dosage (ED50) values (concentrations of the compounds responsible for inhibiting cell growth by 50%).

Results

Physico-chemical characters of the peptavirins. The purified peptavirins by reverse-phase HPLC were in the form of white powder, and readily soluble in dimethyl sulfoxide (DMSO), MeOH, and ethanol (EtOH), but were found to be insoluble in water (Table 1). The UV spectrum showed maxima at 214, 224, and 282 nm for peptavirin A, and 200, 219, and 280 nm for peptavirin B in MeOH. Their *Rf* values were 0.38 and 0.42 for peptavirins A and B, respectively. Melting point for both compounds was 250°C. These physicochemical properties were identical with those of chrysospermins A, B, C, and D, except for the UV absorption peaks.

Antiviral activity against TMV. The peptavirins also showed strong inhibitory activity against TMV infection (Fig. 2) when they were applied to the upper surface of tobacco (*N. tabacum* cv. Xanthi-nc NN) leaves in a mixture with TMV. Both compounds inhibited TMV almost completely at 100 μg/ml and 70-80% at 10 μg/ml. The antiviral efficacies of the peptavirins were similar with that

Table 1. Some physico-chemical properties of peptavirins A and B

Character	Peptavirin A ($C_{92}H_{144}N_{22}O_{22}$)	Peptavirin B ($C_{90}H_{144}N_{21}O_{23}$)
Appearance	White powder	White powder
MP(°C)	250	250
UV λmzx (MeOH, nm)	214, 224, 282	200, 219, 280
Rf value on TLC[a]	0.38	0.42
Solubility	soluble in DMSO, MeOH insoluble in H_2O	soluble in DMSO, MeOH insoluble in H_2O

[a] Silica gel (ODS) TLC:MeOH-H_2O (90:10).

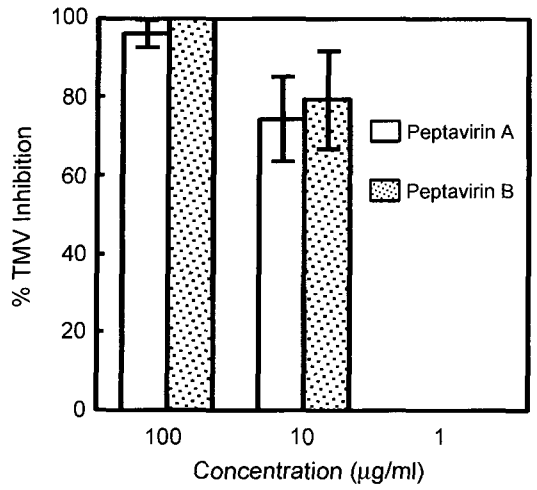

Fig. 2. Bioassay of TMV infection using a local lesion host, *Nicotiana tabacum* cv. Xanthi nc. The left half of the leaf was only inoculated with TMV-infected plant sap, whereas, the right half was inoculated with the mixture of the culture extract and TMV-infected plant sap.

of chrysospermin D, and higher than that of chrysospermin B (Kim et al., 2000).

Antimicrobial activity. The antimicrobial activities of the peptavirins were measured by inhibition zones around the paper discs soaked in each compound (Table 2). Both peptavirins inhibited Gram-positive bacteria, particularly the plant pathogenic bacterium, *Corynebacterium lilium*. However, they showed no definite inhibitory effect on the Gram-negative bacterium *Escherichia coli* in most of the fungi tested, except for *Aspergillus niger* where both peptavirins were active, and *Saccharomyces cerevisiae* where only peptavirin A was active.

Table 2. Antifungal and antibacterial activity of peptavirins produced by *Apiocrea* sp. 14T

Microorganism	Diameter of inhibition zone (mm)[a]	
	Peptavirin A	Peptavirin B
Bacillus subtilis IAM 1069	12	12
Staphylococcus aureus R-209	11	11
Streptococcus sp.	10	11
Corynebacterium lilium	20	19
Escherichia coli AB 1157	0	0
Candida albicans IAM 4905	0	0
Aspergillus niger ATCC 9642	11	10
Saccharomyces cerevisiae IFO 1008	9	0
Magnaporthe grisea IFO 5994	0	0
Colletotrichum lagenarium IFO 7513	0	0
Fusarium solani	0	0
Mucor ramannianus IAM 6218	0	9
Alternaria mali IFO 8594	0	0

[a] Paper disc (8 mm in diameter) was treated with 50 μg of each purified compound, and then placed on microorganism-seeded media (Mueller Hinton agar for bacteria and PDA for fungi).

Table 3. Cytotoxicity of antibiotics produced by *Apiocrea* sp. 14T to human cancer cell lines

Cell line	GI50 (μg/ml)[a]		
	Peptavirin A	Peptavirin B	Adriamycin
PC-3 (Prostate)	3.96	>6	1.00
A549 (Lung)	5.73	>6	0.95
UACC62 (Melanoma)	3.29	>6	0.28
SW620 (Colon)	ND[b]	>6	0.58
K562 (Leukemia)	3.47	>6	0.91

[a] The value of GI50 (50% growth inhibition) was measured by SRB methods (Skehan et al., 1990).
[b] Not determined.

Cytotoxic activity. As regards the cytotoxic activities with human cancer cell lines, variations in the efficiencies were noted between the peptaibols produced by *Apiocrea* sp. 14T. Peptavirin A showed somewhat significant anti-tumor activities, especially against melanoma (UACC62) and leukemia (K562), although it was much less cytotoxic than adriamycin (Table 3). However, peptavirin B exerted no cytotoxic effects at the given concentration of 6 μg/ml.

Discussion

Apiocrea sp. 14T, which was studied previously (Kim et al, 2000; Yun et al., 2000), produced a total of four peptaibols. Although the antibiotic production was not compared quantitatively between the solid (steam-cooked rice) and liquid (potato dextrose broth) cultures, peptavirins A and B were hardly detectable in the liquid culture. Also, chrysospermins B and D were produced more in the solid medium (Kim et al., 2000). It is generally accepted that the production of certain materials by fungi can be affected by the constituents of the media. For example, the addition of glutamic acid to the culture medium of *Trichoderma longibrachiatum* increases the production of the acidic peptide that contains glutamic acid at position 19 of a 20-mer peptaibol instead of glutamine (Leclerc et al., 1998).

Both peptavirins showed an anti-phytoviral activity as strong as chrysospermins B and D. The antiviral efficacy of the four peptaibols produced by *Apiocrea* sp. 14T did not seem to differ much from each other, thereby suggesting that physicochemical modifications related to the substitution of one or two amino residues or C-terminus amino alcohol may cause remarkable changes in the biological activity. However, their usability may be limited because they are not systemic, plus they are unsuitable for genetic

engineering as they contain non-standard amino acids and an acylated N terminus and amino alcohol-C terminus.

The antimicrobial properties were similar between the peptavirins and chrysospermins B and D as well as between peptavirins A and B, having strong antimicrobial activity against Gram-positive bacteria. Peptavirin B, which inhibited efficiently the growth of Gram-positive bacteria, showed weak or no anti-tumor activity. However, as the stronger cytotoxicity to cancer cells may imply more toxic effect to normal cells, peptavirin B can be used as safe antibiotics for the control of human and animal bacterial diseases as well as *Corynebacterium* diseases in plants.

As with the anti-phytoviral activity, the antimicrobial activity did not appear to be changed much by differences in the molecular structures. As established in a previous study, antimicrobial peptaibols, including tylopeptins (Lee et al., 1999) and chrysospermins (Donberger et al., 1995), are mostly active against Gram-positive bacteria and mollicutes (Beven et al., 1998), yet not against Gram-negative bacteria. Thiopeptide antibiotics also exhibit activity against Gram-positive bacteria (Yeo et al, 1994). The difference in the antibacterial activity between Gram-positive and negative bacteria has not yet been examined. However, this can be explained in part by differences in the cell wall compositions. Gram-negative bacteria have much more complex cell walls than Gram-positive walls, plus mollicutes have no cell wall. Gram-negative walls are composed of a lipoproteneous outer membrane containing outer lipopolysacharides, a thin peptidoglycan layer, and periplasmic space, which may serve as a structural barrier outside of the plasma membrane.

This study and the previous one suggest that the cytotoxic efficiency is in order of chrysospermin D, peptavirin A, chrysospermin B, and peptavirin B. Peptaibols exert their physiological activities by forming pores (ion channels), thereby leading to membrane permeabilization (Balaram et al., 1992; Boheim et al., 1983; Duval et al., 1998; Grigoriev et al., 1995; Menestrina et al., 1986; Sansom, 1993). Consequently, their physiological activities are related to pore size and their life times (stability). The 20-mer peptaibol, trichocellin B-II with Glu-18 (charged) stabilizes larger pores than trichocellin A-II with Glu-18 (neutral) (Wada et al., 1997). A reduction in the channel lifetime has been observed with the longbrachins, which include Ala-2 in their 20-residue long-sequences compared with alamethicin, which has Pro-2 instead (Cosette et al., 1999). The current results of cytotoxic effects on cancer cells also suggest that structural modifications resulted in changes in the cytotoxic efficacies. The first and second cytotoxic peptaibols, chrysospermin D and peptavirin B, have a common Iva-5, whereas the third cytotoxic peptaibol has Aib-5. When examining the cytotoxicity in relation to their amino acid residues, Iva-5 seems to contribute more to the physiological activity than Aib-5. It has also been previously shown that the replacement of Iva at position 15 of the polypeptide chain by Aib results in a decrease in the channel effective radius and a respective decrease in channel conductance (Termovsky et al., 1997). Peptavirin B, which includes phenylalaninol at position 19 instead of tryptophanol as in peptavirin A and chrysospermins B and D, exhibited almost no cytotoxicity. This coincides with the previous report that C-terminal tryptophanol is more favorable to the ion channel forming activity than phenylalaninol (Duval et al., 1998). The most cytotoxic peptaibol was found to be chrysospermin D, which only differs in the C-terminal amino-alcohol from the least cytotoxic peptavirin B.

References

Balaram, P., Krishana, K., Sukumar, M., Mellor, I. R. and Sansom, M. S. 1992. The properties of ion channels formed by zervamicins. *Eur. Biophys. J.* 21:117-128.

Berg, A., Ritzau, M., Ihn, W., Schlegel, B., Fleck, W. F., Heinze, S. and Gräfe, U. 1996. Isolation and structure of bergofungin, a new antifungal peptaibol from *Emericellopsis donezkii* HKI 0059. *J. Antibiot.* 49:817-820

Bodo, B., Rebuffat, S., el Hajji, M. and Davoust, D. 1985. Structure of trichozianine A IIIc, an antifungal peptide from *Trichoderma harzianum*. *J. Am. Chem. Soc.* 107:6011-6017.

Beven, L., Duval, D., Rebuffat, S., Riddell, F. G., Bodo, B. and Wroblewski, H. 1998. Membrane permeabilisation and antimycoplasmic activity of the 18-residue peptaibols, trichorzins PA. *Biochim. Biophys. Acta.* 1372:78-90.

Boheim, G., Hanke, W. and Jung, G. 1983. Alamethicin pore formation: voltage dependent flip-flop of α-helix dipoles. *Biophys. Struct. Mech.* 9:181-191.

Brükner, H., Graf, H. and Bokel, M. 1984. Paracelsin; characterization by NMR spectroscopy and circular dichroism, and hemolytic properties of a peptaibol antibiotic from the cellulolytically active mold *Trichoderma reesei. Part B. Experientia* 40:1189-1197.

Chikanishi, T., Hasumi, K., Harada, T., Kawasaki, N. and Endo, A. 1997. Clonostachin, a novel peptaibol that inhibits platelet aggregation. *J. Antibiot.* 50:105-110

Cosette, P., Rebuffat, S., Bodo, B. and Molle, G. 1999. The ion-channel activity of longibrachins LGA I and LGB II: effects of Pro-2/Ala and Gln-18/Glu substitutions on the alamethicin voltage-gated membrane channels. *Biochim. Biophys. Acta* 1461:113-122.

Dornberger, K., Ihn, W., Ritzau, M., Gräfe, U., Schlegel, B., Fleck, W. F. and Metzger, J. W. 1995. Chrysospermins, new peptaibol antibiotics from *Apiocrea chrysosperma* AP 101. *J. Antibiot.* 48:977-989.

Duval, D., Cosette, P., Rebuffat, S., Duclohier, H., Bodo, B. and Molle, G. 1998. Alamethicin-like behaviour of new 18-residue peptaibols, trichorzins PA. Role of the C-terminal amino-

alcohol in the ion channel forming activity. *Biochim. Biophys. Acta.* 1369:309-319.

Duval, D., Riddell, F. G., Rebuffat, S., Platzer, N. and Bodo, B. 1998. Ionophoric activity of the antibiotic peptaibol trichorzin PA VI: a 23Na- and 35Cl-NMR study. *Biochim. Biophys. Acta* 1372:370-378

Gräfe, U., Ihn, W., Ritzau, M., Schade, W., Stengel, C., Schlegel, B., Fleck, W. F., Künkel, W., Hätel, A. and Gutsche, W. 1995. Helioferins, novel antifungal lipopeptides from *Mycogone rosea*. Screening, isolation, structures and biological properties. *J. Antibiot.* 48:126-133.

Grigoriev, P. A., Schlegel, R., Dornberger, K. and Gräfe, U. 1995. Formation of membrane channels by chrysospermins, new peptaibol antibiotics. *Biochim. Biophys. Acta* 1237:1-5.

Kim, Y. H., Yeo, W.-H., Kim, Y.-S., Chae, S.-Y. and Kim, K.-S. 2000. Antiviral activity of antibiotic peptaibols, chrysospermins B and D, produced by *Apiocrea* sp. 14T against TMV infection. *J. Microbiol. Biotechnol.* 10:522-528.

Leclerc, G., Rebuffat, S., Goulard, C. and Bodo, B. 1998. Directed biosynthesis of peptaibol antibiotics in two *Trichoderma* strains. I. Fermentation and isolation. *J. Antibiot.* 51: 170-177.

Lee, S.-J., Yun, B.-S., Cho, D.-H. and Yoo, I.-D. 1999. Tylopeptins A and B, new antibiotic peptides from Tyneofelleus. *J. Antibiot.* 52:998-1006.

Menestrina, G., Voges, K. P., Jung, G. and Boheim, G. 1986. Voltage-dependent channel formation by rods of helical polypeptides. *J. Membrane Biol.* 93:111-132.

Ritzau, M., Heinze, S., Dornberger, K., Berg, A., Fleck, W., Schlegel, B., Härtl, and Gräfe, U. 1997. Ampullosporin, a new peptaibol-type antibiotic from *Sepedonium ampullosporum* HKI-0053 with neuroleptic activity in mice. *J. Antibiot.* 50: 722-728.

Sansom, M. S. 1993. Alamethicin and related peptaibols - model ion channels. *Eur. Biophys. J.* 22:105-124.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S. and Boyd, M. R. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 82:1107-1112.

Snook, C. F., Woolley, G. A., Oliva, G., Pattabhi, V., Wood, S. F., Blundell, T. L. and Wallace, B. A. 1998. The structure and function of antiamoebin I, a proline-rich membrane-active polypeptide. *Structure* 6:783-792

Termovsky, V. I., Grigoriev, P. A., Berestovsky, G. N., Schlegel, R., Dornberger, K. and Grafe, U. 1997. Effective diameters of ion channels formed by homologs of the antibiotic chrysospermin. *Membr. Cell Biol.* 11:497-505.

Wada, S., Iida, A., Asami, K., Tachikawa, E. and Fujita, T. 1997. Role of the Gln/Glu residues of trichocellins A-II/B-II in ion-channel formation in lipid membranes and catecholamine secretion from chromaffin cells. *Biochim. Biophys. Acta* 1325: 209-214.

Yeo, W.-H., Kim, S.-K., Kim, S.-S., Yu, S.-H. and Park, E. K. 1994. Taxonomy and fermentation of *Kitasatosporia kimorexae* producing new thiopeptide antibiotics, kimorexins. *J. Microbiol. Biotechnol.* 4:354-359.

Yun, B.-S.,Yoo, I.-D., Kim, Y. H., Kim, Y.-S., Lee, S.-J., Kim, K.-S. and Yeo, W.-H. 2000. Peptavirins A and B, two new antiviral peptaibols against TMV infection. *Tetrahedron Letters* 41: 1429-1431.