Karalicin, a New Biologically Active Compound from *Pseudomonas fluorescens/putida*

II. Biological Properties

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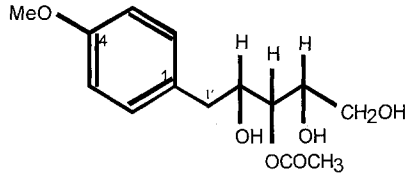
The biological activities of karalicin, a new product from the *Pseudomonas fluorescens/putida* strain SS-3 (CCM 4430) are described.

It shows a weak, but specific and irreversible, antiviral activity on Herpes simplex viruses. It also presents some inhibitory activity on different species of yeasts.

We have recently devised a new, simple and rapid method, (unpublished data), for screening a great number of microbial colonies for cytotoxic and antiviral activity, which has led to the detection of some microbial strains with interesting antiviral properties[1]. Among these, was a strain of *Pseudomonas fluorescens/putida* SS-3, which produced the biologically active compound karalicin.

The taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and structure elucidation of karalicin have been reported in a separate paper[2]. In this paper we describe the biological properties of this compound, which has shown an inhibitory effect on the multiplication of herpes viruses *in vitro*.

Fig. 1. Structure of karalicin.



Materials and Methods

Cell and Viruses

The heteroploid cell line, Vero, and euploid embryonic cells, Flow 2002, were used. The cells were either grown in DULBECCO's modified Eagle medium or MEM supplemented with non-essential aminoacids. All were purchased from ICN-Flow (Costa Mesa, CA.).

Vaccinia virus (VV), Herpes simplex virus type 1 (HSV1), Herpes simplex type 2 (HSV2) and Poliovirus Sabin type 1 (1S) were obtained from NIH (Rockville, Maryland).

Cytotoxicity Assays

For all the experiments, karalicin was dissolved in DMSO (BDH) at the concentration of 10 mg/ml and it was diluted at least 100 fold in test medium before use.

The cytotoxicity of the biologically active extract was evaluated by the inhibition of [DL-4,5-^{3}H]leucine incorporation into cellular monolayers. Either Vero or Flow 2002 cells grown in DMEM with 2% FCS on 35 mm petri dishes, were incubated with different doses of the test compound at 37°C for 3 hours (in triplicate). Then, 1 μCi/ml of [^{3}H]leucine (40 Ci/mmol, Amersham) was added to the plates and left for a further hour. The cells were washed three times with fresh HANK's solution, treated twice with 2 ml/plate of cold 5% TCA, lysed with 1 ml of 0.1 N NaOH and the radioactivity was

counted in a liquid scintillation β-counter. The minimal toxic dose 50% (MTD_{50}) was the concentration of the compound which inhibited the radioisotope incorporation into the cells by 50%.

[DL-4,5-^{3}H]leucine, [5-^{3}H]uridine (47 Ci/mmol) and [methyl-^{3}H]thymidine (85 Ci/mmol) were used as indicators of cell macromolecular biosynthesis, both in virus-infected and uninfected cells. Vero cell monolayers were infected at room temperature for 1 hour. Then the cells were washed and fresh DMEM with 2% FCS, which contained different doses of karalicin, was added. Mock infected cells and cell monolayers without karalicin were used as controls. After 1 hour of incubation with the substance, 1 μCi/ml of each radioisotope precursor was added to a series of plates (in triplicate) and left for a further hour. Subsequently the cells were washed and radioactivity was detected, as indicated above.

Antiviral Activity Assay

Antiviral activity of the compound was evaluated as either inhibition of the viral cytopathic effect or as a plaque reduction test.

Viral cytopathic reduction assays were performed in 48 well plates where the cells were infected with a multiplicity of infections (MOI) of 0.001 infectious viral particles per cell. Cytopathic effect was scored after 36~48 hour at 37°C under a light microscope.

The viral plaque reduction test was performed in 35 mm petri dishes containing a confluent cell monolayer. About 200 infectious viruses per plate were incubated for 1 hour at room temperature. Then the cells were covered with a purified agar solidified nutrient medium. After 2~3 days the plaques were stained with 0.01% neutral red and counted macroscopically. The ID_{50} was defined as the dose of the substance that inhibited either viral plaque formation or viral cytopathic effect by 50%.

Irreversibility of Antiviral Activity and the Action of Karalicin on the Viral Cycle

For the experiments on the reversibility of the antiviral activity of karalicin, Vero cells were infected with HSV1 at a MOI of 0.1. Karalicin was added at time 0 after infection in a concentration of 0.032 μg/ml. The compound was left in the culture for different times (from 0 to 10 hours) then it was removed and the cells, covered with fresh medium, were incubated for a further 24 hours. The viruses produced, were titrated as plaque forming units on Vero cells.

To study the action of karalicin on the viral cycle, the drug (0.032 μg/ml) was added to HSV1 infected cells at different times after infection. Karalicin was added into the plates (in triplicate) from 0 to 10 hours after infection. After 24 hours of incubation in a CO_2 incubator at 37°C, the cells were collected and the virus was titrated as plaque forming units.

Antibiotic Activity Assay

For antibacterial and antifungal assays the following strains were used: *Staphylococcus aureus* ATCC25932, *Enterococcus faecalis* ATCC27989, *Escherichia coli* ATCC25922, *Candida albicans* CDCB385 and Y0109, *C. kefyr* Y0601, *C. tropicalis* CBS94. *Pseudomonas aeruginosa* Ca1 was from our Institute's collection and was identified according to conventional procedures[3].

Antibacterial and antifungal activity was studied by a dilution method in multiwell plates with the use of Muller-Hinton broth (for bacteria)[4] and Sabouraud broth (for fungi) according to standard conditions[5].

Results

The toxicity of karalicin was studied on both Vero and Flow 2002 cells as the incorporation of [^{3}H]-leucine. The MTD_{50} is reported in Table 1. MTD_{50} was 0.064 μg/ml on Vero cells and 0.128 μg/ml on Flow 2002 cells.

The ID_{50} of karalicin was obtained for different viruses and is reported in Table 2. The ID_{50} was 0.004 μg/ml for HSV1 0.008 for HSV2 and a little higher for VV and 1S.

Fig. 2 shows the incorporation of [^{3}H]-leucine, [^{3}H]-uridine, [^{3}H]-thymidine in Vero cells, which were either virus-infected or uninfected.

Under these experimental conditions, no effect was observed on thymidine uptake in both viral-infected or uninfected cells. Leucine uptake was slightly inhibited in both types of cells, but in infected cells it was reduced more than in uninfected ones. Only at 0.064 μg/ml karalicin reduced leucine incorporation by about 50%. Uridine uptake decreased in non-infected cells from 0.064 μg/ml onwards, whilst it was even enhanced in HSV1 infected cells at drug concentrations from 0.016

Table 1. Cytotoxicity of karalicin on both Vero and Flow 2002 cells.

Cell line	Protein biosynthesis MTD50 (μg/ml)
Vero	0.064
Flow 2002	0.128

Table 2. Virus inhibition by karalicin.

Viral strain	ID_{50} μg/ml
HSV1	0.004
HSV2	0.008
VV	0.016
1S	0.016

Fig. 2. Macromolecular precursor uptake by karalicin treated cells.

The symbols represent: □ ^{3}H-thymidine, △ ^{3}H-uridine, ○ ^{3}H-leucine.

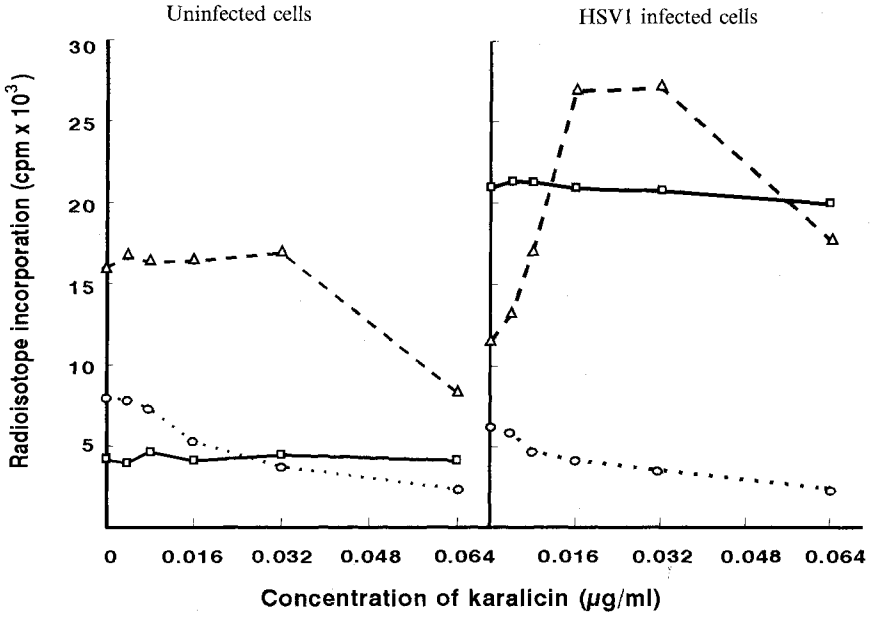
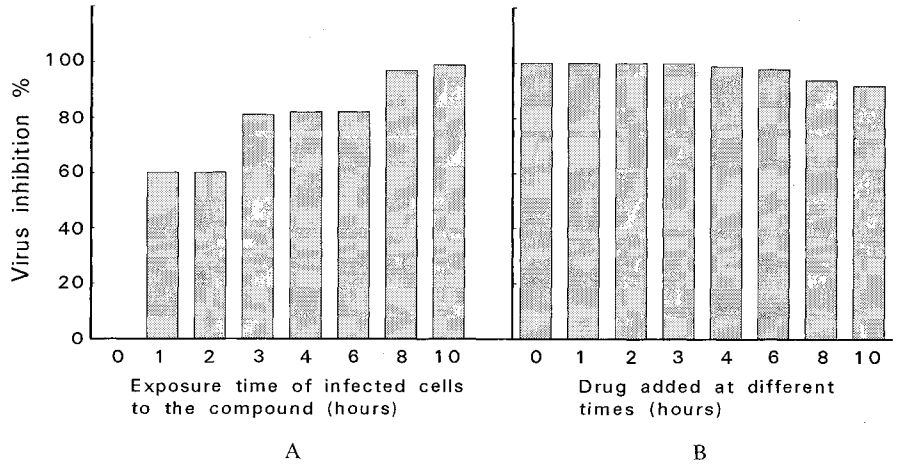


Fig. 3. HSV1 inhibition in Vero cell cultures.

A: After exposure of the infected cells to the compound karalicin for different times, B: After addition of karalicin at different times after infection.



to 0.045 μg/ml.

A contact time of as low as 1 hour for karalicin with HSV1 infected cells resulted in an inhibition of more than 50% of viral yield (Fig. 3a). 8 hours of contact between the drug and the infected cells were sufficient to block viral replication by more than 99%. These findings suggest that the action of karalicin on viral multiplication is not reversed by removal of the drug from the culture.

It resulted, as shown in Fig. 3b, that the virus was still inhibited by karalicin by more than 90% even when the drug was added 10 hours after infection; at this time

Table 3. Antimicrobial activity of the compound karalicin.

Microbial strains	MIC (μg/ml)
Staphylococcus aureus ATCC 25923	> 400
Escherichia coli ATCC 25922	> 400
Enterococcus faecalis ATCC 27989	> 400
Pseudomonas aeruginosa Cal	> 400
Candida albicans CDC B385	12
C. albicans Y0109	25
C. kefyr Y0601	0.75
C. tropicalis CBS 94	100

most steps of the viral cycle are almost completed and only virus morphogenesis is still ongoing.

Table 3 shows the minimum inhibiting concentrations (MIC) of karalicin against various microorganisms. Only a modest antifungal effect was observed, in particular on *Candida albicans* with a MIC of 12 μg/ml and on *C. kefyr* with a MIC of 0.75 μg/ml; no effect on bacteria was observed.

Discussion

Pseudomonas fluorescens has been found to produce several bioactive compounds. Among them pyoluteorin[6], pyrrolnitrin[7], 2,4-diacetylphloroglucinol[8], hydrogen cyanide[9] and pyoverdine siderophore[9] were described.

In this paper a new biologically active substance, named karalicin, has been described as being produced by an environmental strain belonging to this species.

Karalicin shows interesting antiviral properties, although its potency is not very high. It mainly affects Herpes viruses, whilst polio virus and vaccinia virus appear to be less sensitive. Anti-HSV activity was irreversible: in fact, a few hours of contact between karalicin and infected cells during viral multiplication blocked viral growth, even though the substance was removed from the culture medium. Furthermore, the addition of karalicin to the cells up to 10 hours after infection still resulted in more than 90% viral inhibition. Taken together, these findings suggest that karalicin specifically affects some step in viral multiplication, which is irreversibly impaired. RNA was found to accumulate in virus-infected cells, but not in uninfected ones; this fact is not surprising, since other antibiotics which affect proteins synthesis, such as cycloheximide, gave similar results in our hands on HSV1 (data not shown) and by other authors on different viruses[10,11]. In addition, considering that DNA synthesis was unaffected by karalicin at non-toxic doses and that leucine uptake seemed the activity mainly affected by it, especially in HSV1 infected cells, we suggest that some step in the protein synthesis regulation or in viral morphogenesis are the most likely to be altered.

Acknowledgments

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References

1) LAMPIS, G.; D. DEIDDA, C. MAULLU, M. A. MADEDDU, R. POMPEI, F. D. MONACHE & G. SATTA: Sattabacins and sattazolins: new biologically active compounds with antiviral properties extracted from a *Bacillus* sp. J. Antibiotics 48: 707～712, 1995
2) LAMPIS, G.; D. DEIDDA, C. MAULLU, S. PETRUZZELLI, R. POMPEI, F. D. MONACHE & G. SATTA: Karalicin, a new biologically active compound from *Pseudomonas fluorescens/putida*. I. Production, isolation, physico-chemical properties and structure elucidation. J. Antibiotics 49: 260～262, 1996
3) GERALD, L. G.: *Pseudomonas*. *Ed.*, E. H. LENNETTE *et al.*, 4th edn., pp. 526～541, American Society for Microbiology, Washington, D.C., 1985
4) AMSTERDAM, D.: Susceptibility testing of antibiotics in liquid media. *In* Antibiotics in Laboratory Medicine. *Ed.*, LORIAN V., 3rd edn., pp. 53～105, Williams and Wilkins, Baltimore, 1991
5) MCGINNIS, M. R. & M. G. RINALDI: Antifungal drugs: mechanism of action, drug resistance, susceptibility testing, and assay of activity in biological fluids. *In* Antibiotics in Laboratory Medicine. *Ed.*, LORIAN V., 3rd edn., pp. 198～257, Williams and Wilkins, Baltimore, 1991
6) HOWELL, C. R. & R. D. STIPANOVIC: Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology 70: 712～715, 1980
7) HOWELL, C. R.: Control of *Rhizoctonia solari* in cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69: 480～482, 1979
8) NOWAK-THOMPSON, B.; S. J. GOULD, J. KRAUS & J. E. LOPER: Production of 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Can. J. Microbiol. 40: 1064～1066, 1994
9) KRAUS, J. & J. E. LOPER: Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of Pythium damping-off of cucumber. Phytopathology 82: 264～271, 1992
10) MOSS, B. & R. FILLER: Irreversible effects of cycloheximide during the early period of vaccinia virus replication. J. Virol. 5: 99～108, 1970
11) WERTZ, G. W. & M. LEVINE: RNA synthesis by vesicular stomatitis virus and a small plaque mutant. Effects of cycloheximide. J. Virol. 12: 253～264, 1973