# ANTAGONISTS OF ANTIFUNGAL SUBSTANCE POLYOXIN

MITSUAKI MITANI and YUKIO INOUE

Research Division, Kaken Chemical Company Ltd., Tokyo, Japan

(Received for publication June 25, 1968)

Polyoxin inhibited the growth of a number of fungi with *Pellicularia sasakii* and *Rhizoctonia solani* being the most sensitive. The growth of *P. sasakii* in shake culture was inhibited completely at all growth stages by the antibiotic. *P. sasakii* which had been treated with polyoxin resumed growing when transferred to fresh CZAPEK-DOX medium. The inhibitory action of polyoxin was prevented by the addition of certain nitrogen compounds to the media. Dipeptides were the most effective, while a number of amino acids and bases had no effect. Although the ultraviolet absorption spectrum of polyoxin was modified by the addition of many nitrogen compounds, there was no relationship between the shift in the ultraviolet spectrum and the antagonistic effect.

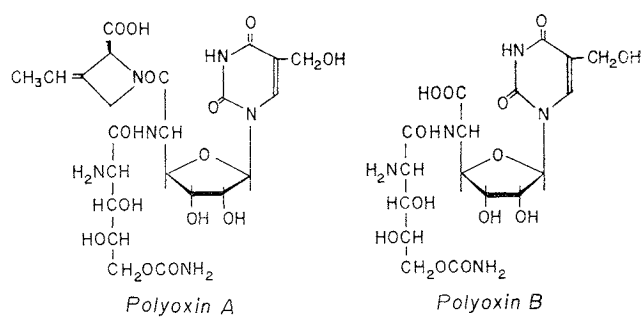
An antifungal substance, polyoxin, was isolated from *Streptomyces cacaoi* var. *asoensis* by SUZUKI *et al.*[1] in 1964. The antibiotic could be separated into several components which were divided into three groups[2,3] on the basis the chromophore present; 5-hydroxymethyl uracil, polyoxins A, B, C and G (described as ABG mixture); uracil-5-carboxylic acid, polyoxins D, E, F and I (described as DEF mixture); and thymine, polyoxin H. The proposed structures of polyoxins A and B are shown in Fig. 1[4]. Polyoxins C and I are not antifungal. Though the mode of action of polyoxin is unknown, it was observed that when nitrogen-containing substances such as peptone, yeast extract, or milk casein were added to the polyoxin assay agar plate, the antifungal activity was completely prevented.

The present study was conducted to explore the effect of nitrogen compounds on the inhibitory action of polyoxin.

## Materials and Methods

*Pellicularia sasakii*, *Rhizoctonia solani*, *Piricularia oryzae*, *Penicillium oxalicum* IAM 7221, *Penicillium notatum*, *Botrytis cinerea* IAM 5127, *Alternaria kikuchiana* A-14, *Cladosporium fulvum* C-65, *Fusarium oxysporum* F-3, *Aspergillus niger* ATCC 9642 and 6275, *Asp. flavus* ATCC 9643, *Asp. chevalieri* NI 5550, *Streptomyces griseus* WAKSMAN *et* HENRICI, *S. fradiae* IMRU 3535,

Fig. 1. The structure of polyoxins A and B



*S. lavendulae* IMRU 3440, *Escherichia coli* NIHJ, *Bacillus subtilis* ATCC 6633 and *Saccharomyces pastrianus* NI 7271 were obtained from the Research Division of Kaken Chemical Company.

To determine the antimicrobial spectra, fungi were grown on Petri plates containing CZAPEK-DOX agar medium and the advancing colony edge was cut with cork borer. The cut discs were placed in the center of new CZAPEK-DOX agar plates, which contained various concentrations of the antibiotic, and incubated at 28°C. The fungal growth was measured as the colony diameter. For shake culture experiments, a small amount of fungal inoculum obtained from CZAPEK-DOX agar slants was placed in a 300 ml Erlenmeyer flask containing 40 ml of CZAPEK-DOX medium and incubated on a rotary shaker. After 2~5 days of incubation the culture broth was blended for 30 sec. in a Waring blendor. Portions of blended cells were transferred into 300 ml flasks containing 40 ml of CZAPEK-DOX medium and incubated. The fungal growth rate were determined by dry weight of mycelium.

To determine the antagonism of nitrogen compounds on polyoxin activity, various nitrogen sources such as peptides, amino acids, and bases were added to CZAPEK-DOX agar plates containing various concentrations of polyoxin. The antagonistic activities of the nitrogen compounds were calculated from the diameters of 2~4 days old colonies. A control colony was grown on CZAPEK-DOX agar plate containing the nitrogen compounds. The polyoxin concentration used completely inhibited the growth of *Pellicularia sasakii*.

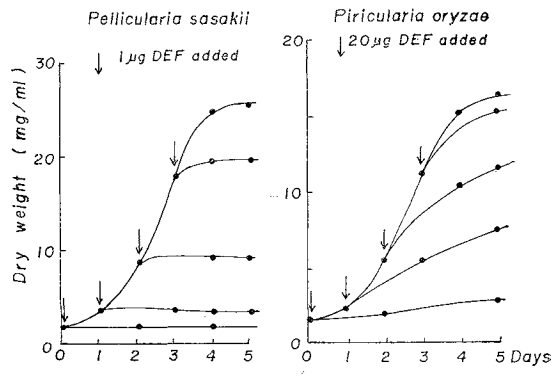
The absorption spectra are reported as difference spectra. The ultraviolet absorption was measured in cuvettes containing 50 mM Tris buffer, pH 7.0, 13 μg/ml polyoxin, various concentrations of peptides, and water to a final volume of 3 ml. Controls were run in which polyoxin or the peptides were omitted. A Beckmann DB-G spectrophotometer wes used for spectrophotometric studies.

Polyoxins ABG and DEF of 80 % purity were used throughout this study, of the difficulty of obtaining single pure entities. In a few experiments single components were used.

## Results and Discussion

The ABG mixture, at 10 μg/ml, completely inhibited the growth of *Pellicularia sasakii*, *Rhizoctonia solani* and *Penicillium oxalicum*, and at 25 μg/ml, inhibited *Alternaria kikuchiana*. The DEF mixture, at 1 μg/ml inhibited the growth of *Pellic. sasakii* and *R. solani*, and at 50 μg/ml inhibited *P. oxalicum* and *Alt. kikuchiana*. Fifty μg/ml or higher concentrations of polyoxin were required for 50 % growth inhibition of the other fungi. The antibiotic did not inhibit the growth of *Penicillium notatum* and *Asp. chevalieri* at any concentration tested. The ABG mixture caused the lysis of old mycelium of *Botrytis cinerea* at 50 μg/ml. Streptomycetes and bacteria were not inhibited by the antibiotic in shake culture, while growth of *Saccharomyces pastricum* in the presence of the antibiotic was stimulated. Results from shake culture experiments were similar to ones on CZAPEK-DOX agar plates. We were unable to determine the

Fig. 2. Polyoxin inhibition on the growth of *Pellicularia sasakii* and *Piricularia oryzae*



antifungal spectrum for *Piricularia oryzae* since growth of this fungus was very slow under our experimental conditions. Although *Piricularia oryzae* and *Pellicularia sasakii* are completely inhibited by low concentrations of polyoxin *in vitro*, a hundred-fold greater concentration of polyoxin is necessary to inhibit *Piricularia* in field tests[2]. Experiments were carried out to determine the influence of polyoxin when added at various growth phases in shake culture (Fig. 2). The growth of *Pellicularia* was completely inhibited by the antibiotic at all growth phases, but without lysis. The growth of *Piricularia*, on the other hand, was only completely inhibited when the antibiotic was added during the lag growth phase, while growth rate was decreased by the addition of the antibiotic at other time. Therefore, because of the difference in the sensitivity of *Pellicularia* and *Piricularia* in shake culture to the antibiotic, one would expect such phenomenon in the field. If these fungi were exposed to the antibiotic and then transferred to fresh medium which did not contain the antibiotic, fungal growth occurred again. Both fungi produced a brown pigment in the supernatant fluid after the addition of the antibiotic. Therefore, the antibiotic is not fungicidal, but is fungistatic. Similar results were obtained with the ABG mixture.

Table 1. The influence of nitrogen compounds on the polyoxin inhibitory action

| Nitrogen compounds | Percent polyoxin inhibition | | | | |
|---|---|---|---|---|---|
| | 1.0% | 0.5% | 0.1% | 0.05% | 0.01% |
| Peptone* | 0 | 10 | 60 | 95 | — |
| Yeast extract* | — | 36 | 64 | 95 | 100 |
| Casein* (Hammarstein) | — | 31 | 70 | 95 | 100 |
| | 20 mM | 10 mM | 2 mM | 1 mM | |
| Glycine** | 100 | 100 | 100 | 100 | |
| Glycylglycine** | 32 | 57 | 88 | 100 | |
| Glycylglycylglycine** | 72 | 95 | 100 | 100 | |
| Glycylglycylglycylglycine** | 53 | 66 | 72 | 95 | |

* *Rhizoctonia solani* was used as the test organism and 10 μg/ml DEF mixture was used.
** *Pellicularia sasakii* was used as the test organism and 13 μg/ml DEF mixture was used.

Table 2. Antagonistic action of nitrogen compounds on the antifungal activity of polyoxin

| Nitrogen compounds (mM) | Percent polyoxin inhibition |
|---|---|
| None | 100 |
| Glycylglycine 10 | 30 |
| Glycyl L-alanine 10 | 0 |
| Glycyl DL-valine 10 | 0 |
| Glycylglycylglycine 10 | 95 |
| Glycylglycylglycylglycine 10 | 55 |
| DL-Alanyl DL-alanine 10 | 100 |
| DL-Alanyl glycine 10 | 0 |
| DL-Alanyl DL-methionine 10 | 64 |
| DL-Alanyl DL-valine 10 | 100 |
| DL-Leucyl DL-leucine 10 | 95 |
| γ-Aminobutyrate 100 | 100 |
| L-Asparagine 100 | 100 |
| L-Arginine 10 | 72 |
| L-Cysteine 100 | 47 |
| L-Glutamic acid 100 | 100 |
| D-Glutamic acid 100 | 100 |
| Glycine 200 | 100 |
| L-Glutamine 100 | 95 |
| DL-α-Alanine 100 | 100 |
| DL-β-Alanine 100 | 100 |
| L-Isoleucine 100 | 95 |
| DL-Methionine 100 | 86 |
| DL-Valine 50 | 95 |
| L-Phenylalanine 100 | 100 |
| L-Lysine* 5 | 79 |
| L-Ornithine* 10 | 92 |
| Ascorbic acid 10 | 100 |
| Adenine 10 | 100 |
| Uracil 10 | 100 |
| Xanthine 10 | 95 |
| Thymine 10 | 100 |
| Cytosine 10 | 100 |

DEF mixture : 13 μg/ml. * Polyoxin D : 1 μg/ml.

Fig. 3-1. Modifications of the glycylglycine ultraviolet absorption spectra by polyoxin

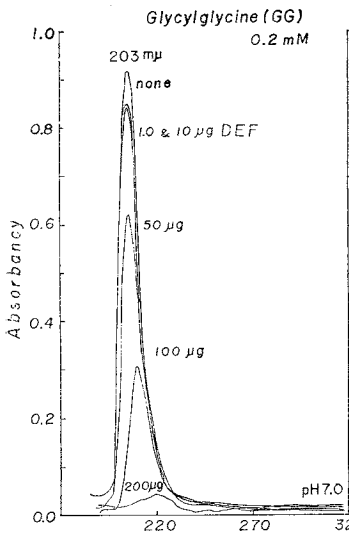



Fig. 3-2. Modifications of the polyoxin ultraviolet absorption spectra by glycylglycine

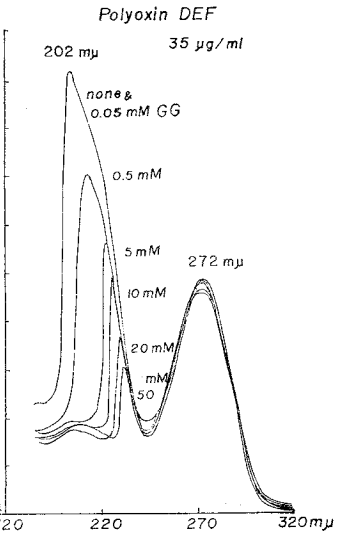
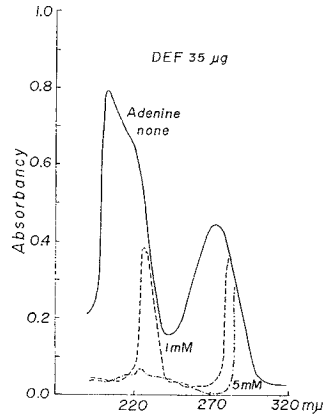



Fig. 4. Modifications of the polyoxin ultraviolet absorption spectra by adenine




Previous results indicated that *Pellicularia sasakii* and *R. solani* were the most sensitive of the fungi that were tested for the growth inhibiting effect of polyoxin. *Pellicularia sasakii* was used for the remaining experiments. Although growth of *Pellicularia sasakii* was inhibited by low concentration of polyoxin, the inhibitory action of the antibiotic was prevented by the addition of peptone, yeast extract, or milk casein (Table 1). Although glycylglycine, glycylglycylglycine and glycylglycylglycylglycine antagonized polyoxin inhibition, the extent of antagonism was not related to the length of the peptide chain. Glycine was not antagonistic to polyoxin inhibition. Among ten dipeptides used, DL-alanyl DL-alanine and DL-alanyl DL-valine did not show any antagonistic action, while DL-leucyl DL-leucine and glycylglycylglycine were slightly antagonistic (Table 2). Both purified polyoxins D and B yielded similar results. Among various amino acids and bases, L-cysteine yielded 50% decrease in polyoxin inhibition, DL-methionine, L-arginine, and L-lysine showed slight activity. However, in general much higher concentrations of the free amino acids and bases were necessary to cause the antagonistic action as compared to the dipeptides. Exceptions were L-arginine, L-lysine, L-ornithine and xanthine. We were unable to obtain accurate data on the effect of hydrogen ion concentration on the polyoxin inhibitory action; polyoxin, however, was completely denatured at pH 9.0 in the cold room after 18 hours. Purified polyoxins did not antagonize each other but showed an additive effect. Sometimes fungi when grown on CZAPEK-DOX agar plate containing polyoxin showed irregular growth. In such cases, we could not measure the colony diameters. No differences in fungal growth and antibiotic activity were observed with sucrose or glucose as carbohydrate sources.

Polyoxin possesses an absorption spectrum in the ultraviolet range[3]. The absorption spectrum of the antibiotic was altered by the addition of various peptides and nucleic acids or bases (Figs. 3 and 4). Likewise glycylglycine has a peak at 203 mμ

which is altered by the addition of polyoxin (Fig. 3 left). Even though the absorption maximum for polyoxin decreased and was shifted to a longer wave length by the addition of adenine (Fig. 4), antifungal activity was not affected. Other bases produced similar results as adenine. Therefore, the modification of the polyoxin absorption spectrum in the ultraviolet range does not appear to be related to the antagonistic action by various nitrogen compounds on the antifungal activities under these conditions.

Acknowledgement

We thank Dr. S. SUZUKI of the Antibiotic Laboratory of the Institute of Physical and Chemical Research for his generous supply of polyoxin.

## References

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