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154. Kunihiro Nakajima : Structure-Activity Relationship of Colistins.

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1) Purified samples of polymyxin group antibiotics were shown to be remarkable antimicrobial agents as colistin against *E. coli*.

2) Removal of the C_9-fatty acid from the colistin molecule resulted in the decrease of the activity.

3) The side chain moiety itself showed no antimicrobial activity.

4) The cyclic peptide moiety showed an antimicrobial activity although it was less active than the parent antibiotic.

These results indicate that the "active" structure responsible for the antimicrobial activity is the "cyclic peptide moiety" in colistin molecule.

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Polymyxin is a generic name for a group of chemically related antibiotics isolated from various strains of *Bacillus polymyxa*, a soil bacteria. They were discovered in 1947 by three independent groups of investigators[1~3] and the recent increase of infections due to gram-negative bacilli has provoked interest in them. Five polymyxins have been described, named A, B, C, D and E,[4] all of which are basic, cyclic peptides and contained some L- and D-amino acids including L-α,γ-diaminobutyric acid and C_9-fatty acid, 6-methyloctanoic acid or isooctanoic acid. Nearly all species of gram-negative bacilli are highly sensitive to polymyxins with the notable exception of *Proteus*.[5]

Colistin was first reported by Koyama, *et al.*,[6] and its chemical structure was found to be the same as that of polymyxin E.[7,8] This antibiotic is not very toxic and is now used clinically. Circulin, isolated from *Bacillus circulans*,[9] also belongs chemically to polymyxin group antibiotics.[10]

During investigations on the mechanism of action of colistin, various kinds of polymyxins became available and thus it became possible to study the relationship between the structure of colistin and its antimicrobial activity. This paper shows that the cyclic peptide moiety of colistin is the essential structural unit of the molecule.

Materials and Methods

Organism——The polymyxin sensitive *Escherichia coli* strain B was used.

Medium and Conditions of Culture——The organism was grown at 37° in Simmons' medium[*2] (pH 7.2)[11,12] containing 0.3% glucose and shaken by a rotary shaker at 130 r.p.m. until the cells reached the logarithmic phase of growth.

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*2 Simmons' medium : This medium contains 2.5 g. $(NH_4)_2HPO_4$, 1.5 g. KH_2PO_4, 0.2 g. $MgSO_4 \cdot 7H_2O$, 5 g. NaCl and 3 g. sodium glutamate per liter of distilled water.

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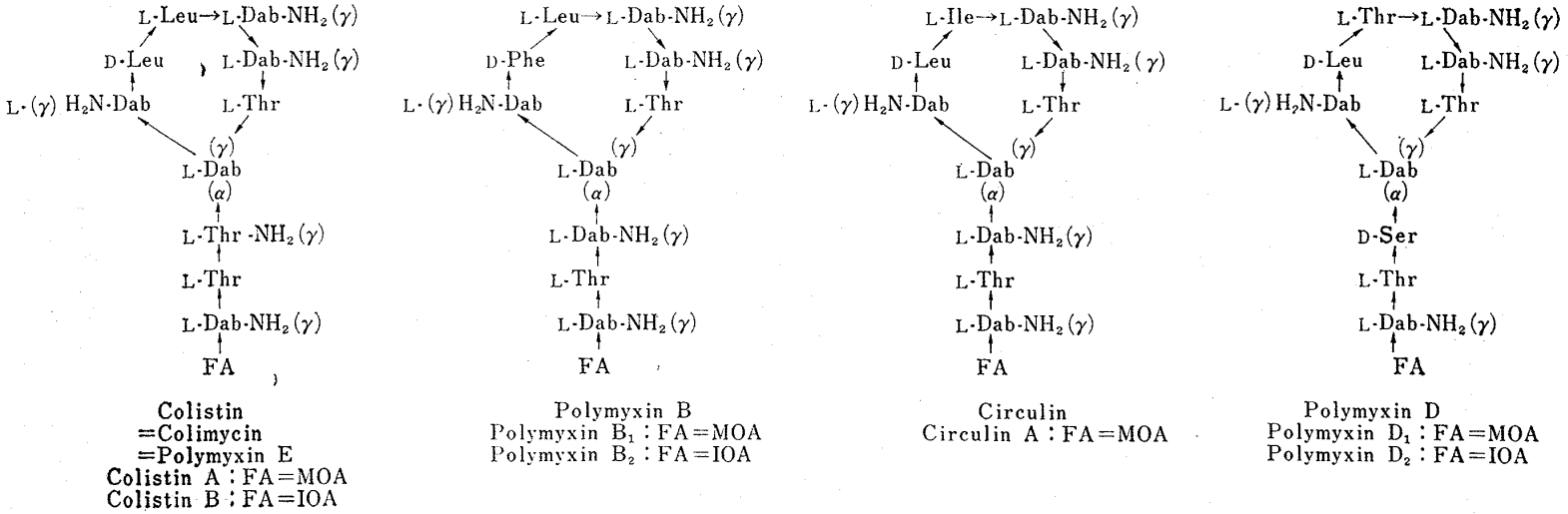

Fig. 1. Chemical Structures of Polymyxin Group Antibiotics

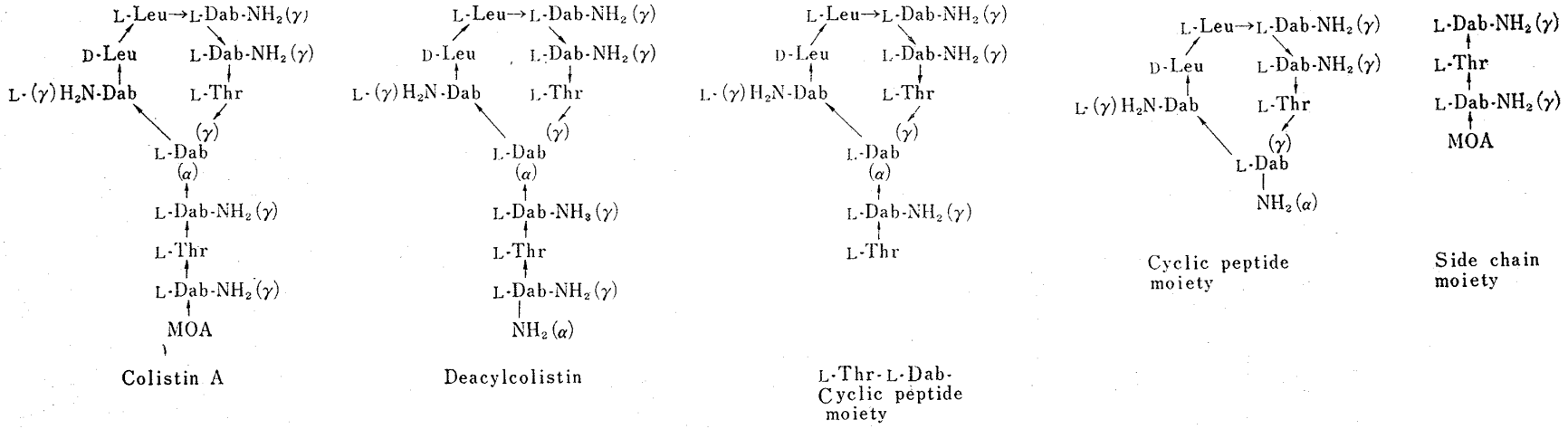

Fig. 1. Chemical Structures of Colistin A and Its Degradation Compounds

The following abbreviations are used : Leu=leucine, Thr=threonine, Phe=phenylalanine, Ser=serine, Ile=isoleucine, Dab=α,γ-diaminobutyric acid, MOA=6-methyloctanoic acid, IOA=isooctanoic acid, Colistin A=polymyxin E_1, Colistin B=polymyxin E_2

Polymyxins and the Degradation Compounds of Colistin——The chemical structures of the polymyxin group antibiotics used are listed in Fig. 1 and those of colistin and its degradation compounds in Fig. 2.

Samples of polymyxins and degradation compounds of colistin were kindly supplied by Prof. T. Suzuki and Dr. K. Hayashi (Institute for Protein Research, Osaka University). They are hydrochloride salt except colistin sulfate and polymyxin B sulfate which are products of Kayaku Antibiotics Research Co., Ltd., Tokyo and Taito Pfizer Co., Ltd., Tokyo, respectively. The polymyxins used were generally readily soluble in water. When assayed for antimicrobial activity, the samples of antibiotics were dissolved in distilled water and sterilized by passage through a Millipore filter (GS 0.22 μ).

Measurement of Antimicrobial Activity against *E. coli*——*E. coli* B at the logarithmic phase was harvested by centrifugation at 3000 r.p.m. for 15 minutes and resuspended in fresh glucose-Simmons' medium ($E_{660}=0.3$). Unless otherwise stated, the incubation mixture (3 ml.) consisted of 0.3 ml. of aqueous solution of antibiotic at the concentration shown for each experiment and 2.7 ml. of a suspension of *E. coli* After shaking in a water bath for 60 minutes at 37°, the incubation mixture was diluted with Simmons' medium and viable cells were counted on a plate (pH 7.2) containing 10 g. polypeptone, 2.5 g. NaCl and 12 g. of agar per liter by the conventional counting technique.

Observation of Release of 260 mμ Absorbing Materials from *E. coli*——To a 2.7 ml. portion of washed cell suspension ($E_{660}=0.3$) of *E. coli* in Simmons' medium was added 0.3 ml. of antibiotic solution in distilled water to give a volume of 3.0 ml. in a L-shaped culture tube and the suspension was rocked in a water bath at 37° for 60 minutes. Then it was chilled in an icewater bath to stop the reaction and centrifuged at 4° first for 15 minutes and then for 10 minutes at 5000 r.p.m. The absorbancy of the supernatant thus obtained was measured at 260 mμ with a Hitachi spectrophotometer (EPU-2).

Results

1. Comparison of Antimicrobial Activity against *E. coli*

E. coli B cells at the logarithmic phase of growth were treated with 10 μg. per ml. of polymyxin for 60 minutes at 37°. The antimicrobial activity against *E. coli* was expressed as surviving cells after treatment with antibiotics.

Table I. Effect of Polymyxin Group Antibiotics on the Viable Cells of *E. coli* B

Polymyxin group antibiotics	Incubation time (min.)	Conc. of antibiotic (μg./ml.)	Viable cells (per ml.)
Without antibiotic	0	0	4.0×10^8
	60	0	1.5×10^9
Polymyxin B[a)]	60	10	4.5×10^6
Polymyxin B_1	60	10	4.0×10^6
Polymyxin B_2	60	10	4.2×10^6
Polymyxin D_1	60	10	5.3×10^6
Polymyxin E_1 (Colistin A)	60	10	2.0×10^6
Polymyxin E_2 (Colistin B)	60	10	2.5×10^6
Colistin sulfate[b)]	60	10	2.2×10^6
Colistin A	60	10	1.1×10^6

E. coli cells in the logarithmic phase of growth were harvested and incubated in glucose-Simmons' medium containing 10 μg. per ml. of polymyxin with shaking for 60 minutes at 37°. The incubation mixture was then diluted with Simmons' medium and viable cells of the bacteria were counted on the agar plate by the conventional counting technique.
a)=a product of Taito Pfizer Co., Ltd., Tokyo.
b)=a product of Kayaku Antibiotics Research Co., Ltd., Tokyo.

As shown in Table I, the samples of polymyxin group antibiotics used in the experiment were found to be as effective in antibacterial activity as colistin against *E. coli*. This indicates that the nature of the amino acids in the cyclic peptide moiety of polymyxin does not affect the intensity of bactericidal action significantly. Table II shows the relationship between the structure and activity of the colistin molecule.

Table II. Antimicrobial Activity of Colistin A and Its Degradation Compounds against *E. coli*

Drug	Incubation time (min.)	Conc. of drug (μg./ml.)	Viable cells (per ml.)
Without drug	0	0	5.1×10^{8}
	60	0	1.9×10^{9}
Colistin A	60	3	7.2×10^{7}
	60	10	2.4×10^{6}
	60	30	8.1×10^{5}
	60	100	1.1×10^{4}
Deacylcolistin	60	3	1.2×10^{9}
	60	10	8.2×10^{7}
	60	30	7.8×10^{6}
	60	100	7.7×10^{5}
L-Thr-L-Dab-Cyclic peptide moiety	60	3	6.9×10^{8}
	60	10	7.6×10^{7}
	60	30	3.7×10^{6}
	60	100	6.8×10^{5}
Cyclic peptide moiety	60	3	3.4×10^{8}
	60	10	4.2×10^{7}
	60	30	5.8×10^{6}
	60	100	4.5×10^{5}
Side chain moiety	60	3	2.0×10^{9}
	60	10	1.8×10^{9}
	60	30	1.9×10^{9}
	60	100	1.6×10^{9}

E. coli in the logarithmic phase of growth was collected and incubated with shaking for 60 minutes at 37° in glucose-Simmons' medium containing various concentrations of antibiotic and then the viability of the bacteria was measured as described in Table I.

As illustrated in Table II, it is of interest that deacycolistin, which lacks the C_9-fatty acid moiety, was shown to be less active than colistin, the side chain moiety (containing the C_9-fatty acid) itself having no activity. Two kinds of compound with a cyclic peptide moiety also had an appreciable activity although they were less active than the parent antibiotic. From the evidences mentioned above, it can be concluded that the cyclic peptide moiety is essential structure responsible for the bactericidal activity against *E. coli*.

2. Release of Materials Absorbing at 260 mμ from Bacterial Cells

The addition of polymyxins to a washed cell suspension of the susceptible strain resulted in the release from the cells of materials with an absorption maximum at 260 mμ.[13~15]

As illustrated in Table III, samples of purified polymyxins at a concentration of 10 μg. per ml. caused a remarkable release of these materials from the cells. There was no significant difference in the release of UV-absorbing materials among different members of polymyxin group antibiotics. To identify the "active" structure of colistin with respect to the release of these materials, a comparison was made of purified colistin A or its degradation compounds. *E. coli* cells were incubated with

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Table III. Release by Polymyxin Group Antibiotics of Materials Absorbing at 260 mμ from Washed Cells of *E. coli* B

Polymyxin group antibiotics	UV-absorbing materials from *E. coli* cells (E_{260})	Polymyxin group antibiotics	UV-absorbing materials from *E. coli* cells (E_{260})
Colistin sulfate	0.530	Polymyxin B_2	0.512
Colistin A (Polymyxin E_1)	0.528	Polymyxin D_1	0.531
Colistin B (Polymyxin E_2)	0.508	Colistin A	0.541
Polymyxin B	0.478	Without antibiotic	0.025
Polymyxin B_1	0.502		

A portion of 2.7 ml. of washed cell suspensoin of *E. coli* (E_{660}=0.3) was shaken with 0.3 ml. of aqueous antibiotic solution (at a final concentration of 10 μg./ml.) for 60 minutes at 37°. The mixture was centrifuged twice at 4° for 15 and 10 minutes, respectively at 5000 r.p.m. The absorbancy of the resulting supernatant was measured at 260 mμ with a Hitachi spectrophotometer (EPU-2).

colistin A or its degradation compounds at a concentration of 100 μg./ml. at 37° for 60 minutes and the amount of UV-absorbing materials released into the medium was measured. As shown in Table IV, deacylcolistin caused a slight release of these materials, whereas incubation with the side chain moiety itself did not. Both of the

Table IV. Effects of Colistin A and Its Degradation Compounds on the Release of UV-absorbing Materials from Washed Cells of *E. coli* B

Colistin A and its degradation compounds	UV-absorbing materials from *E. coli* cells (E_{260})	Colistin A and its degradation compounds	UV-absorbing materials from *E. coli* cells (E_{260})
Colistin A	0.530	Cyclic peptide moiety	0.350
Deacylcolistin	0.175	Side chain moiety	0.010
L-Thr-L-Dab-Cyclic peptide moiety	0.401	Without antibiotic	0.015

The incubation mixture was rocked with colistin A or a degradation compound at a level of 100 μg./ml. at 37° for 60 minutes in a water bath.

compounds with the cyclic peptide moiety, however, resulted in an appreciable release of UV-absorbing materials although they were not as effective as intact colistin. These data coincided more or less with the results shown in Table II. However, the reason why "deacylolistin" caused only slight release of these materials even though it showed appreciable antimicrobial activity is still obscure, because the mode of action of colistin is now not to be clarified entirely.

Discussion

Colistin is mainly composed of two different structural units, *viz.*, the "cyclic peptide moiety" and the "side chain moiety." Polymyxin group antibiotcs differ chemically from each other in the nature of the amino acids components in the cyclic peptide moiety. The similarity in structure of these antibiotics is shown in Fig. 1. From experiments on the antimicrobial activity and the release of UV-absorbing materials, the following results were obtained; (1) the samples of polymyxins tested so far all showed as much antimicrobial activity as colistin against *E. coli*, (2) the side chain moiety itself showed no activity, (3) removal of the C_9-fatty acid from the colistin molecule led to some loss in activity, (4) the cyclic peptide moiety showed an appreciable activity although it was not as active as the parent antibiotic. Therefore,

it can be concluded from these results that the "active" structure of colistin is the cyclic peptide moiety. Although the exact function of the C_9-fatty acid is as yet unknown, it may play a role in the penetration of the colistin molecule into the cell through the cytoplasmic membrane which is rich in phospholipids.

In this paper the author systematically investigated the relationship between the structure and activity of the colistin molecule using purified samples of polymyxin group antibiotics and degradation compounds of colistin. These experiments showed that the structural requirement for the antibiotic activity of colistin is the "cyclic peptide moiety" in the molecule. The identification of the "active" structure will lead to the development of new and more effective chemotherapeutic agents, since it now seems possible[16,17] by a slight modification of the side chain moiety to prepare a wide variety of semisynthetic colistins with different biological and pharmacological characteristics.

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