A NEW ANTIBIOTIC, GATAVALIN

I. ISOLATION AND CHARACTERIZATION

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(Received for pulbication December 28, 1971)

Gatavalin, a new peptide antibiotic, was isolated from cells of *Bacillus polymyxa* var. *colistinus* KOYAMA. The antimicrobial spectrum of this antibiotic is characterized by activity against gram-positive bacteria, mycobacteria, yeasts and molds. This antibiotic was named gatavalin because it contains glutamic acid, aspartic acid, threonine, alanine and valine. The isolation, purification, physico-chemical and biological properties of gatavalin are described, and the relationship with other *colistinus* peptides, colistin and jolipeptin, is discussed.

Colistin, a peptide antibiotic, active against *Escherichia coli* is produced by *Bacillus polymyxa* var. *colistinus* KOYAMA[1]. In the commercial production of colistin the culture broth is heated at acid pH to increase colistin yields and to facilitate filtration. The filtrate thus prepared usually exhibits considerable antibacterial activity against gram-positive bacteria, in contrast with negligible activity shown by non-heated culture filtrates or colistin itself. This led to the concept of the existence of another antibiotic which is effective against gram-positive bacteria and which is not released from the poducing organism without special treatment. When cells of the *colistinus* strain were extracted with an organic solvent such as butanol-water, 2 kinds of peptide antibiotics other than known colistin members were isolated; the one was jolipeptin[2] active against both gram-positive and gram-negative bacteria and the other active against only gram-positive bacteria. The chemical and antimicrobial properties of this latter antibiotic are distinct from those of previously described antibiotics and it was named gatavalin because of its amino acid composition. This paper deals with isolation, purification and properties of this new peptide antibiotic, gatavalin.

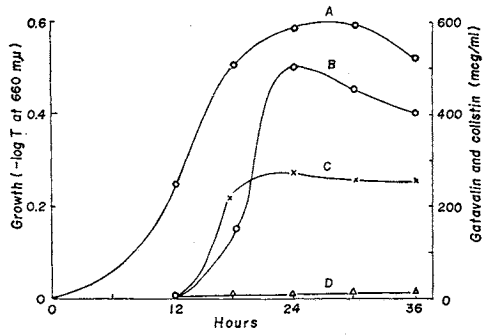
Fermentation Studies

Two liters of a 24-hour culture of *B. polymyxa* var. *colistinus* KOYAMA grown at 30°C in reciprocal shaker was used to inoculate a 500-liter fermenter containing 200 liters of medium and incubation was continued at 30°C for 24 hours with adequate aeration. The medium contained 10 g starch, 5 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 3 g $CaCO_3$, 0.5 g NaCl, 150 mcg $MnSO_4$ and 10 mcg biotin per liter; pH 7.0. Cell growth and gatavalin production are illustrated in Fig. 1. Gatavalin, which was scarcely detected in the supernatant of cultured broth throughout the fermentation

process, was observed when the broth acidified to pH 3.0 was heated at 80°C for 10 minutes. This shows that gatavalin biosynthesized within cells and scarcely diffused into the culture filtrate, unless the cultured broth containing cell mass is heated at acid pH region. Maximum yields of approximately 500 mcg gatavalin and 300 mcg colistin per ml were observed coincident with maximum cell growth. Determination of antibacterial activities of both antibiotics was made by agar diffusion pulp disc method, using *Staphylococcus aureus* FDA 209P as test organism for gatavalian and *Escherichia coli* NIHJ for colistin.

Fig. 1. Growth curve and gatavalin production.

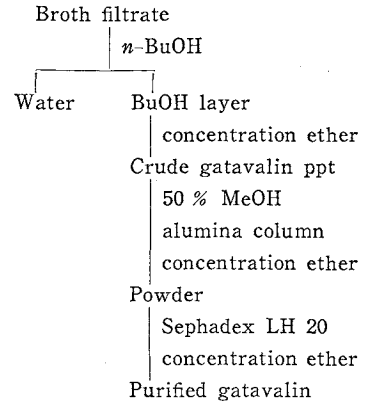
A : cell growth
B : gatavalin activity after acid-heating (80°C, 10 min., pH 3.0)
C : colistin activity
D : gatavalin activity in the supernatant of broth



Isolation of Gatavalin

Cultured broth was acidified at pH 3.0 with 4 N HCl and heated at 80°C for 10 minutes, then filtered. The 80°C, 10-minute heating at pH 3.0 exhaustively deprived cells of this organism of gatavalin but of no jolipeptin. After cooling to 20°C, the filtrate was further acidified to pH 1.0 with 4 N HCl, to which 100 liters of *n*-butanol was added with stirring for one hour to extract gatavalin. The butanol layer was concentrated to 10 liters *in vacuo*, and gatavalin was precipitated with 3 volumes of ethylether. The precipitated and dried powder (crude gatavalin), 100 g, was dissolved in 5 liters of 50 volume (percent) methanol, adjusted to pH 4.0 with N HCl, and passed through alumina column which was (30×500 mm) packed with 200 g of active alumina. The effluent was concentrated *in vacuo* to 3 liters and extracted with 3 liters of *n*-butanol with acidified to pH 1.0 for the extraction of gatavalin. The butanol layer containing gatavalin was concentrated *in vacuo* to a half volume, and gatavalin was precipitated by the addition of ethylether. After drying, *ca.* 20 g of white powder of gatavalin was obtained, which was entirely free from colistin. For further purification, the above white powder was dissolved in 50 % methanol and passed through a Sephadex LH 20 column. The passed solution was concentrated *in vacuo*, from which gatavalin was precipitated with ethylether, separated and dried. This purified, non-crystalline gatavalin preparation was used for studies of physico-chemical properties. The isolation and purification of gatavalin are outlined in Fig. 2.

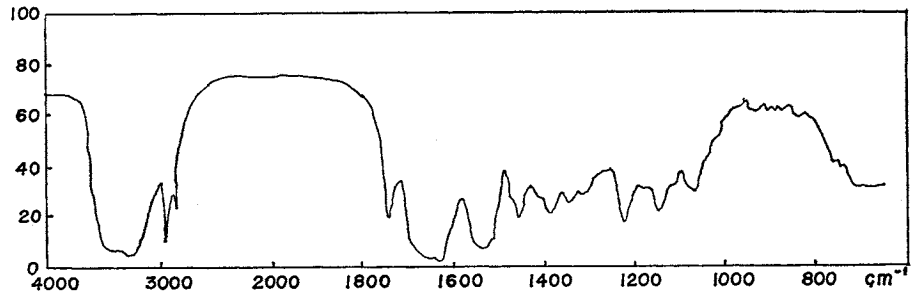
Fig. 2. Isolation of gatavalin.



Properties of Gatavalin

Gatavalin is a neutral substance and melts at 245~248°C. It shows no UV absorption at 260 mμ and 280 mμ. $[\alpha]_D^{23}$ +22.4° (50 % methanol). Gatavalin is soluble in dimethyl sulfoxide, acetic acid, ethyleneglycol, methanol, and ethanol, and slightly soluble in water, and insoluble in ethylether, butyl acetate, benzene, and acetone. Infrared absorption spectrum measured in KBr disc is shown in Fig. 3. Elemental analysis is as follows: C 51.19 %, H 8.19 %, N 14.16 %. Color reactions: positive to biuret and TBHCl (*tert.*-butyl hypochlorite)[3], negative to ninhydrin, FEHLING, and MOLISCH.

Fig. 3. IR spectrum of gatavalin

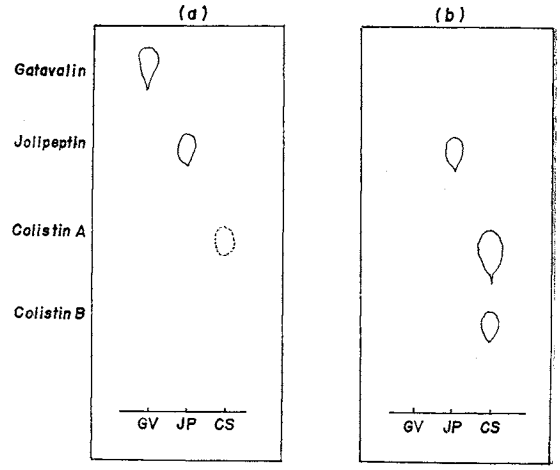


Gatavalin was hydrolyzed with 6 N HCl at 105°C for 24 hours and glutamic acid, aspartic acid, alanine, threonine and valine were detected in the hydrolysate by thin-layer chromatography. For the investigation of molar ratio of amino acid composition, gatavalin was hydrolyzed in a sealed tube under the same conditions as above, and the filtrate was evaporated to dryness *in vacuo*, dissolved in 1 ml of citrate buffer (pH 2.2), and then applied to automatic amino acid analyzer, Yanagimoto LC-2 type. The molar ratio of amino acids in gatavalin, colistin and jolipeptin are indicated in Table 1. Gatavalin dialyses through cellophane and a presumptive molecular weight is *ca.* 2,000 as estimated

Table 1. Amino acids composition of gatavalin, jolipeptin and colistin

	Molar ratio		
	Gatavalin	Jolipeptin[2]	Colistin[4]
Glutamic acid	1.08	1.19	0
Alanine	1.90	1.73	0
Valine	3.02	1.85	0
Aspartic acid	1.00	0	0
Threonine	2.98	0	0.93
α,γ-Diamino butyric acid	0	2.01	3.15
Leucine	0	0	1.0
Glycine	0	1.0	0
Serine	0	2.18	0

Fig. 4. Bioautographs of gatavalin, jolipeptin and colistin.
(a) : *Staphylococcus aureus* FDA 209P
(b) : *Escherichia coli* NIHJ



GV : Gatavalin JP : Jolipeptin CS : Colistin

Table 2. Antimicrobial spectrum of gatavalin

Organism	Minimal inhibitory concentration (mcg/ml)	Organism	Minimal inhibitory concentration (mcg/ml)
Bacillus subtilis	1.25	*Achromobacter liquidum*	100
Bacillus cereus	2.5	*Flavobacterium sulfureum*	100
Staphylococcus aureus	0.625	*Serratia marcescens*	100
Staphylococcus aureus (Mac-R*)	0.625	*Xanthomonas oryzae*	100
Sarcina lutea	0.625	*Candida krusei*	10
Brevibacterium ammoniagenes	1.25	*Candida pseudotropicalis*	0.625
Micrococcus lysodeikticus	0.312	*Saccharomyces cerevisiae*	5
Mycobacterium 607	0.625	*Aspergillus oryzae*	5
Escherichia coli	100	*Aspergillus niger*	1.25
Pseudomonas aeruginosa	100	*Penicillium expansum*	1.25
Proteus mirabilis	100	*Absidia butterri*	2.5
Proteus rettgeri	100	*Fusarium* sp.	1.25
Aerobacter aerogenes	100		

* (Mac-R): Resistant to macrolide antibiotics.

by gel filtration using Sephadex G-25. As other components such as fatty acid were not found in acid hydrolyzate of gatavalin, this antibiotic is assumed as a simple peptide composed of 10 amino acids. The investigation of its chemical structure is now in progress.

The antimicrobial spectrum of gatavalin is shown in Table 2. This antibiotic is effective not only against gram-positive bacteria but also mycobacteria, yeast and molds, whereas no activitiy is observed on gram-negative bacteria. The antimicrobial activity is very stable in acid *milieu*, and even 30-minute heating at 100°C and pH 3.0 causes no reduction in activity. In contrast, it is quite unstable in alkaline *milieu*, *e.g.* no activity is detectable after the incubation at room temperature for 3 hours at pH 10. When each of gatavalin and colistin was applied on a paper strip and developed by a solvent system of *n*-butanol-acetic acid-water (3:1:1), bioautography of the paper chromatogram of gatavalin on a *S. aureus* FDA 209P plate gave an inhibitory spot of Rf 0.9 (Fig. 4a), and no inhibition zone on a *E. coli* plate. In contrast, colistin was detectable at Rf on a *E. coli* plate but not clearly on a *S. aureus* plate. The Rf of jolipeptin when developed by the same solvent system was 0.67. The LD_{50} of intraperitoneally administered gatavalin is 22.5 mg/kg.

Discussion

The antimicrobial spectrum and physico-chemical properties of gatavalin are distinct from not only colistin as well as jolipeptin but also from other known peptide antibiotics, thus it is considered to be a new peptide antibiotic. The activitiy of gatavalin against mycobacteria, fungi and gram-positive bacteria may be regarded as antipodal to the anti-*coli* activity of colistin whereas jolipeptin effective on both gram-positive and gram-negative bacteria is assumed to be situated intermediately in this respect. Thus, the *colistinus* strain is of particular interest because it produces 3 peptide antibiotics which can attack almost all of aerobic microbes by their co-operative actions. On the other hand, these 3 *colistinus*-peptide antibiotics have some chemical properties in common. They are all dialyzable, and, although the exact molecular weight of jolipeptine and gatavalin are not known as yet, both peptides are presumed to be near colistin in molecular weight. On this basis of comparable molecular weights, these *colistinus* antibiotics

are composed of 10 moles of amino acids. Furthermore, except for glutamic acid, the component amino acids are confined in three groups, *viz.* pyruvate, serine and aspartate series. Accordingly, these *colistinus* peptide may be regarded to be in a homologous group, though different from each other in antimicrobial spectrum and amino acid composition. Extending this concept of homology, one may speculate that the presence of L-α,γ-diaminobutyric acid in colistin and jolipeptin molecules confers in these compounds their inhibiting capacities on gram-negative bacteria while the glutamic acid in jolipeptin and gatavalin confers activity against gram-positive bacteria. Further comparative studies with gatavalin, colistin and jolipeptin are in progress.

Acknowledgements

The authors wish to express their thanks to Dr. H. SATO in our laboratory for amino acid analyses, and also to Mr. S. UECHI and Mr. M. YAHATA for technical assistance.

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