ISOLATION OF GALANTINS I AND II, WATER-SOLUBLE BASIC PEPTIDES

STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. III

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Two water-soluble basic antibiotics named galantins I and II were isolated from a strain resembling *Bacillus pulvifaciens*. Both antibiotics are peptides containing glycine, alanine, ornithine, lysine and some unknown ninhydrin-positive components. An approximate empirical formula $C_{50\pm1}H_{98\pm2}O_{17}N_{16}$ is indicated for galantin I. These are active against some gram-positive, acid-fast and gram-negative bacteria.

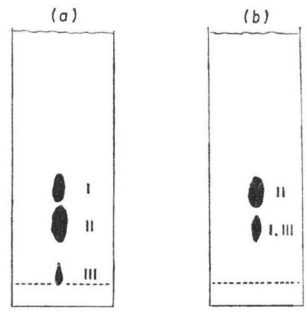
In the course of our screening program for new antibiotics from the genus *Bacillus*,[1] a strain numbered 52—33 which was isolated from a soil sample from New Guinea and preliminarily identified with *Bacillus pulvifaciens* was found to produce three or more antibiotic substances. The substances were isolated from the culture filtrate by adsorption on an IRC-50 (Na) column, elution with dilute hydrochloric acid followed by adsorption on activated carbon and elution with acidified aqueous acetone.

When the crude preparation was chramatographed on a carbon plate with 0.1 N hydrochloric acid-methanol (4:1), three active components named I, II and III were detected on a bioautogram against *B. subtilis* (Fig. 1). These components were separated from one another by chromatography on a carbon column. The components I and II thus obtained were named galantins I and II, respectively, by reason of their amino acid constituents. When these components were tested by paper chromatography with *n*-propanol-pyridine-acetic acid-water (15:10:3:12), reversed mobilities between I and II were observed. The components I and III were not separable on this system (Fig. 1).

Fig. 1. Illustrated mobilities of components I, II and III on thin layer and paper chromatograms.

a) Carbon plate: 0.1 N Hydrochloric acid-methanol (4:1)

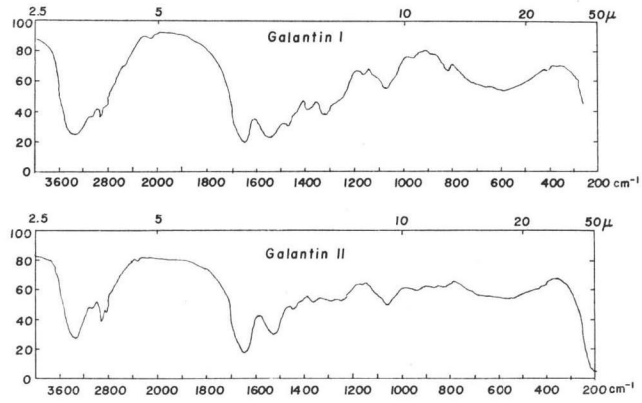
b) Toyo Roshi No. 51: *n*-Propanol-pyridine-acetic acid-water (15:10:3:12). Bioautograph on *Bacillus subtilis* assay plate



Galantin I (free base) is a colorless amorphous powder, m.p. 143~150°C, $[\alpha]_D^{23°}$ $+4.5\pm0.9°$ (*c* 0.510, H_2O), $[\alpha]_D^{23.5°}$ $-2.3\pm0.9°$ (*c* 0.477, 0.5 N HCl), soluble in water, methanol and ethanol, but insoluble in acetone, ethyl acetate, chloroform and ether. Its basic nature was indicated by paper electrophoresis. It is positive to ninhydrin, weakly positive

to PAULY (yellow) but negative to SAKAGUCHI reaction. No characteristic absorption was observed in the ultraviolet absorption spectrum measured in water. Typical bands of peptide were shown in the infrared absorption spectrum (Fig. 2).

Fig. 2. Infrared absorption spectra of galantins I and II (KBr).



From the elemental analyses and molecular weight determination of galantin I and its 2,4-dinitrophenyl derivative, an approximate empirical formula $C_{50\pm1}H_{98\pm2}O_{17}N_{16}$ was proposed for the free base. The dinitrophenyl derivative obtained by the usual way gave a single spot on a Silica gel GF plate with a solvent system [5% of dioxane was added to a mixture of chloroform-methanol (4:1)], providing an additional proof for homogeneity.

Galantin II was also prepared as colorless amorphous powder, m.p. 145~153°C, $[\alpha]_D^{24.5°}$ 0.00, $[\alpha]_{365}^{24.5°}+6.2\pm1.0$(*c* 0.472, 0.5 N HCl). Similar properties to galantin I were observed such as solubility, color reaction, uv and ir spectra (Fig. 2), and elemental analysis. Its 2, 4-dinitrophenyl derivative gave also a single spot on the TLC.

These antibiotics were hydrolyzed with constant-boiling hydrochloric acid and analyzed by an automatic amino acid analyzer. Glycine (0.87)*, alanine (0.82), ornithine (0.84) and trace amount of lysine were found with galantin I. Similarly, glycine (0.90), alanine (0.86), ornithine (0.59) and lysine (0.15) were observed with galantin II. This suggested that these antibiotic preparations were not homogeneous in regard to ornithine or lysine residue, in which both amino acids were considered to be replaceable to each other by cultural environment as often seen in peptide antibiotics.[2] In addition to these usual amino acids, four unidentified peaks, common in galantins I and II, were shown (Fig. 3). Their content ratio were seemed to be somewhat different between galantins I and II from the peak areas.

The component III shown in the TLC, was not obtained in a pure preparation and was found to show only weak antibiotic activity. Amino acid analysis of this prepararion revealed that the substance contained no alanine and ornithine, but glycine (1.79) and some unknown ninhydrin-positive components.

Galantins I and II were active against some gram-positive, gram-negative and acid-fast bacteria (Table 1), but not against yeast and fungi. It is noteworthy that these antibiotics showed remarkable preference even for strains of the same species. When galantin I was

* The analytical results were expressed in μmoles of amino acid found per mg of antibiotic.

Fig. 3. Amino acid analyses of the hydrolyzates of galantins I and II.

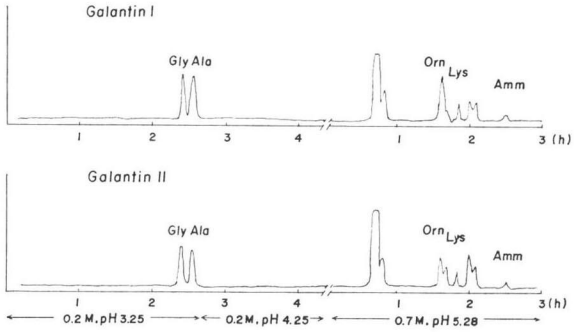


Table 1. Antimicrobial spectra of galantins I and II

Organism	MIC (μg/ml)	
	Galantin I	Galantin II
Bacillus subtilis PCI 219	1.56	3.13
Bacillus anthracis	25	>50
Staphylococcus aureus FDA 209P JC-1	0.78	3.13
Staphylococcus aureus Smith	12.5	>50
Diplococcus pneumoniae type I	>50	>50
Streptococcus pyogenes C-203	>50	>50
Mycobacterium tuberculosis $H_{37}Rv$	12.5	25
Escherichia coli	>50	>50
Klebsiella pneumoniae	6.25	12.5
Salmonella typhimurium	>50	>50
Pseudomonas aeruginosa Ps-24	>50	>50

Obtained by the usual agar dilution method

administered intraperitoneally three times (0, 4 and 8 hours after infection) to mice infected with *Klebsiella pneumoniae*, curative effect was observed (ED_{50}: 0.86 mg/kg×3), but not when administered subcutaneously. Lethal dose was shown to be below 50 mg/kg intraperitoneally.

Experimental

Fermentation

Spores of the strain 52—33 were inoculated into 130 ml of a medium of peptone 1.0%, meat extract 0.5% and sodium chloride 0.3%, pH 7.0 in a 500-ml shaking flask and shake-cultured at 27°C for 24 hours. The culture was then seeded to a medium consisting of glucose 1.0%, peptone 1.0%, meat extract 0.5% and sodium chloride 0.3%, which was shake-cultured for 5 days in the same manner.

Isolation and purification

Because of its viscous nature, the broth was acidified to pH 2.0 by hydrochloric acid and a nearly equal volume of methanol was added; this resulted in coagulation of viscous materials that were filtered off. Some 5 liters of the culture filtrate thus obtained were subjected to evaporation, adjusted to pH 7.0 by sodium hydroxide and passed through an IRC-50 (Na) column (250 ml). The adsorbed antibiotics on the column were eluted with 0.5 N hydrochloric acid. The fractions active to a *B. subtilis* assay plate were collected, neutralized to pH 7.0 by sodium hydroxide and adsorbed on 30 g of Darco G-60. The carbon was filtered, washed with water

and eluted with 50% aqueous acetone acidified to pH 2.0 by dilute hydrochloric acid. The eluate was then neutralized to pH 5.0 by an IR-4B (OH), concentrated and lyophilized, giving a colorless powder (1,300 mg).

The crude powder was then chromatographed on a carbon column (Wakō, Activated Charcoal, 70 g, 2.3×74 cm) with 500 ml of mixtures of 0.1 N hydrochloric acid and methanol, (4:1, 3:2 and 1:1), successively. Components I, II and III were found to be eluted in that order, when the fractions were tested with the TLC and PC. The fractions containing the components I, II and III, respectively, were separately processed by neutralization to pH 5.0 with an IR-4B (OH), evaporation and lyophilization, giving preparations of component I (410 mg), II (206 mg) and III (170 mg) as their hydrochlorides.

The hydrochloride of galantin I (referred to as component I in above) was dissolved in water, passed through a Dowex 1×8 (OH) column and lyophilized to give a colorless amorphous powder of the free base.

Anal. Found: C, 50.51; H, 8.56; N, 18.76
Calcd. for $C_{80}H_{98}O_{17}N_{16}$: C, 50.25; H, 8.21; N, 18.76, MW 1194

Similarly galantin II free base was obtained as a colorless amorphous powder.

Anal. Found: C, 50.32; H, 8.34; N, 18.76

2, 4-Dinitrophenylation

Galantin I free base (30 mg) and $NaHCO_3$ (30 mg) were dissolved in water (0.3 ml). A 0.6-ml portion of 5% 2, 4-dinitrofluorobenzene in ethanol was added and stirred for 2 hours at room temperature. The 2, 4-dinitrophenyl derivative that precipitated was filtered and washed with water, ethanol and ether, successively. The precipitate was then dissolved in a small amount of dimethylsulfoxide, reprecipitated by addition of water and washed with the above solvents to give a preparation, free from dinitrophenol, as a yellow powder (35 mg), m.p. 126~135°C.

Anal. Found: C, 46.44; H, 4.82; N, 17.70; MW 2184 (Osmometry in pyridine)
Calcd. for $C_{50}H_{92}O_{17}N_{16}(C_6H_3O_4N_2)_6$: C, 47.12; H, 5.02; N, 17.90; MW 2190.
Calcd. for $C_{49}H_{90}O_{17}N_{16}(C_6H_3O_4N_2)_6$: C, 46.88; H, 4.96; N, 18.01, MW 2176.

Similar procedure gave a 2, 4-dinitrophenyl derivative of galantin II as a yellow powder, m.p. 136~148°C.

Anal. Found: C, 45.22; H, 5.43; N, 16.36

References

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2) Shoji, J. & R. Sakazaki: A new peptide antibiotic complex S-520. II. Further characterization and degradative studies. J. Antibiotics 23: 432~436, 1970