Studies on the Promoting Agent Obtained from *Houttuyna cordata* (Dokudami) for the Production of the Antibiotic Substance by a Strain of Gram Positive, Spore Bearing Bacilli.

By

Kei-ichiro Hosisima and Hyoe Okabe.

(星島 啓一部)

(岡部 兵二)

(From the Bacteriological Department, Faculty of Medicine, Tohoku University, Sendai. Director: Prof. M. Kuroya.)

(Received for publication July 11, 1949)

Some substances to promote the production of antibiotics were reported already by many authors.

According to Knight¹⁾ the use of corn steep liquor in the media of *Penicillium chrysogenum* increased the production of Penicillin. Koffler and others²⁾ investigated on the promoting elements of corn steep liquor and reported that one element was the combination of a Fe-ion and a soluble phosphor salt.

It became clear that the phenyl-acetic acid is a cleavage product of penicillin G. So many authors investigated on the promoting action of phenyl-acetic acid and its derivatives for penicillin production.

Higuchi and others,³⁾ Jarvis and Johnson,⁴⁾ Moyer and Goghill,⁵⁾ Cook and Brown,⁶⁾ and Umezawa⁷⁾ reported on the action of phenyl-acetic acid and its derivatives (for instance p-phenyl ethylamine, p-hydroxy phenyl acetic acid, phenylacetoamide, nitrophenyl acetic acid, p-amino-phenyl acetic acid, p-oxyphenyl acetic acid and one derivative contained in corn steep liquor.)

Okunuki and others⁸⁾ had reported that some peptides had promoting activities on penicillin production.

Regarding the promoting substances for another antibiotic produced by actinomycetes, mould and bacteria, there are many descriptions to be mentioned.⁹⁾

One of us (H. Okabe)¹⁰⁾ had isolated a strain of Gram positive spore forming bacilli from some soils collected in Sendai, which possessed an antagonistic property.

For the production of the antibiotic the following was used as a basal medium.

Meat extract	10 g
Peptone	10 g
NaCl	5 g
KH ₂ PO ₄	1 g
Na ₂ HPO ₄ ·12H ₂ O	0.25 g
Agar	2.5 g
Aq. dest.	1000 cc

adjusted to pH 7.2.

When cultivated on the basal medium, the antibiotic titre of the culture filtrate against *Staphylococcus aureus* ("Terashima" strain) was only 1:10.

It was shown, however, that when "Dokudami" (*Houttuynia cordata*) extract, prepared as described below, was added at the rate of 2 cc per liter of the basal media, antibiotic titre increased to 1:640 (in highest titre) after one day incubation at 30°C.

With regards to the details of this antibiotic itself another paper will be written by one of us elsewhere.¹⁰ The present paper is concerned with the nature of the active substance contained in the extract and with the chemical isolation and purification of this substance.

Isolation and Purification of the Substance.

200 g. of dried pieces of Dokudami plant containing the stems and leaves were added with 5000 cc. of tap water and heated at 100°C, 5-6 hours and filtered. The extract thus obtained was concentrated in vacuo at 40°C to 1/10 of original volume. The resulting precipitates which had no activity, were removed by centrifugation and discarded. The supernatant was proved to be active as described above.

By adding to this supernatant methanol, ethanol, acetone, and ammonium sulfate at various concentrations, it was not possible to separate clearly the active substance. It was not possible also to extract the active agent with petroleum-ether, benzin, ligroin, xylene and toluen.

In the course of purification, however, it was found that the active agent was thoroughly extractable by ether and ethylacetate and that the substance was always accompanied by sterol reaction. (Liebermann's test).

The purification of the substance, therefore, was performed as follows:

The dried pieces of "Dokudami" plant (including the stems and leaves) were extracted with ether, and the extracts were dried with sodium sulfat anhydride and evaporated. The oily residue was saponified with 30% potassium hydroxide in alcohol by boiling for 3 hours and the resulting solution was diluted with water and extracted with ether. After desiccation of the extract the ether was evaporated. The resulting dark green masses were recrystallised by boiling absolute alcohol 12 times, and once

80% alcohol and the alcoholic solution of the substance was diluted with water and extracted with ether. The ether solution was dried and evaporated in vacuo and the residue was recrystallised by boiling in absolute alcohol 7 times, until pure substance was obtained.

From 2600 g of dried "Dokudami" were obtained ca. 1 g of pure substance.

TABLE I.
The Purification of the Active Substance.

Chemical and Physical Properties of the Active Substance.

The pure substance thus obtained were crystals in the form of a colorless plate, and melted at 132°–133°C without decomposition.

The alcoholic solution of the substance was precipitated by digitonin dissolved in alcohol and the small needles crystallized out. (Digitonin test).

When its chloroform solution was mixed with acetic anhydride and conc. sulphuric acid, the solution became immediately yellow and showed a ting of fluorescence and later turned to yellowish green. (Liebermann's test).

When to the chloroform solution of the substance was added the solution of trichloroacetic acid, then the solution became faint red. (Rosenheim's test).

Further, when to the chloroform solution was added conc. sulphuric acid and shaken well, the chloroform layer became red and soon turned to green, and later discoloured; the acid layer presented greenish fluorescence. (Salkowski's test).

From the chemical and physical properties mentioned above, this substance seems to be a certain sort of "sterol," especially of phytosterol. From the point of view of the melting point this substance resembled most closely a certain sort of "Sitosterol."

The Promoting Action of the Pure Substance for the Production of Antibiotic.

When the substance was added to the basal media in the concentration of 0.0001%, and a strain of the soil bacteria isolated by one of us, was cultivated in this media, the production of antibiotic titre reached its maximum after incubation for 24 hours at 30°C. The culture filtrate thus obtained completely checked the growth of *Staphylococcus aureus* ("Terashima" strain) in dilution of 1:160.

As indicated in Table II it was proved that there was a optimal concentration of this substance to obtain a highest titre of the culture filtrates, and higher or lower concentrations of the substance brought about a decreasing of the titre.

No biological differences—as such the degrees of the growth, those of the sporulation and the characteristics of colonies of the bacilli cultivated in the culture media with or without addition of the substance were confirmed.

The Promoting Action of Another Few Kinds of Sterols and of Phenantren Compounds for the Production of Antibiotic.

As shown above already, this substance seems to be a certain sort of sterol, so another few kinds of sterol and of phenantrene compounds were examined on their activities.

TABLE II.

The Activity of the Pure Substance Obtained from the Ether
Extracts of "Dokudami."

Concentration of the substance to be added to basal media	Dilution titre of the culture filtrate for the inhibition of the growth of <i>Sta.</i> <i>aureus</i> (Terashima strain)		
	incubated for (days)		
	1	2	3
0.01%	lower than 1:10	lower than 1:10	lower than 1:10
0.005%	1:10	1:10	lower than 1:10
0.001%	1:40	1:40	1:10
0.0005%	1:80	1:80	1:40
0.0001%	1:160	1:80	1:80
0.00005%	1:80	1:80	1:40
0.00001%	1:40	1:10	lower than 1:10
without substance	lower than 1:10	lower than 1:10	lower than 1:10

As shown in Table III, it was elucidated that phenantrene, cholesterol, follicular hormone ($C_{18}H_{22}O_2$), and testicular hormone had an almost same activity, while a sample of phytosterol (mp. $102^\circ C$), Sodium desoxycholate, Sodium acetylcholate and Sodium xylocholate had no such activity.

As it was proved that cholesterol was active and easily obtainable, the following media were used usually in the latter part of experiment.

Meat extract	10 g
Pepton	10 g
NaCl	5 g
KH_2PO_4	1 g
$Na_2HPO_4 \cdot 12H_2O$	0.25 g
1% Cholesterol (alcoholic solution)	10 cc
Aq. dest.	1000 cc

SUMMARY.

1) A certain sort of sterol was isolated from *Houttuyna cordata* (Dokudami), a Japanese plant drug, which had the ability to promote the production of the antibiotic substance produced by a certain strain of Gram positive, spore forming bacilli.

TABLE III.

The Activities of Phenantrene and Sterol Compounds to Promote the Production of the Antibiotic Substance.

Substances to be added	Optimal concentration of the substance to be added to basal media for the maximal production of antibiotic	Dilution titre of the culture filtrate for the inhibition of the growth of <i>Sta. aureus</i> (Terashima strain)
This substance	0.0001%	1:160
Phenantrene	0.001%	1:80
Cholesterol	0.001%	1:320
Follicular hormone	0.0005%	1:80
Testicular hormone	0.0005%	1:320
A sample of phytostrol (mp. 102°C)	0.01%—0.00001%	lower than 1:10
Sodium desoxycholate	ditto	ditto
Sodium acetylcholate	ditto	ditto
Sodium xylocholate	ditto	ditto
Control (without substance)		lower than 1:10

2) It was proved, that the sterol isolated from the plant closely resembled sitosterol from the chemical and physical points of view.

3) There was a optimal concentration of this sterol to promote the production of the antibiotic.

4) It was shown that Phenantrene, cholesterol and other similar compounds had almost similar activities.

We wish to express our thanks to Prof. M. Kuroya for his guidance and his kind advice.

We also express our thanks to the staff of the Institute of Tuberculosis and Leprosy, of the Pharmacological Department and of the Department of the Organic Chemistry, Tohoku University who kindly gave us phenantrene and sterol compounds.

References.

- 1) Knight, *Science*, 1945, **102**, 617.
- 2) Koffler, Knight, Frazier and Bunis, *J. Bact.*, 1946, **51**, 385.
- 3) Higuchi, Jarvis Peterson and Johnson *J. Amer. Chem. Soc.*, 1946, **68**, 1669.
- 4) Jarvis and Johnson, *ibid.*, 1947, **69**, 301.
- 5) Moyer and Goghil, *J. Bact.*, 1947, **53**, 329.

- 6) Cook and Brown, Nature, 1947, **159**, 367.
 - 7) Umezawa, J. Penicillin, 1947, **1**, 363.
 - 8) Okunuki, Yakushiji, Hayashi and Tayama, J. Penicillin, 1947, **1**, 237.
 - 9) Waksman, "Microbial antagonisms and Antibiotic Substances", Common Wealth Fund, New York, 1947.
 - 10) Okabe, Tohoku J. Exp. Med., 1950 255, 1949.
-