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Mechanisms of Insecticidal Action of Deoxypodophyllotoxin (Anthricin). I.[1] Distribution of Deoxypodophyllotoxin in Tissues of the 5th Instar Larvae of Silkworm, *Bombyx mori* LINNE

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It has already been reported that among the constituents of the root of *Anthriscus sylvestris* HOFFM., deoxypodophyllotoxin (anthricin: I), anthriscinol methyl ether and (*Z*)-2-angeloyloxymethyl-2-butenoic acid show insecticidal action against several species of insects.

In order to clarify the mechanisms of insecticidal action of I, which showed the strongest action among the above chemicals, the distribution of I in the tissues of the 5th instar larvae of silkworm, *Bombyx mori*, fed on mulberry leaves treated with I was investigated. I was analyzed by high-pressure liquid chromatography. The amount of I in the blood increased rapidly after the administration and no decrease was seen even after 96 h. Compound (I) accumulated in the fat body in a larger amount for a longer time, as compared with the other tissues. It was also accumulated at the skin, the ventriculus and the Malpighian tubules in somewhat higher amounts than in other tissues. Degeneration of the skin and the fat body were observed in the intoxicated insects. The concentration of I in feces reached its peak at 48 h after administration.

Keywords——deoxypodophyllotoxin; *Anthriscus sylvestris*; silkworm, *Bombyx mori*; skin; fat body; distribution; delayed insecticidal action; high-pressure liquid chromatography

Previously, Kozawa *et al.*[2] reported that among the constituents of the root of *Anthriscus sylvestris* HOFFM., deoxypodophyllotoxin (anthricin: I, Fig. 1), which is a kind of lignan, as well as anthriscinol methyl ether, and (*Z*)-2-angeloyloxymethyl-2-butenoic acid, showed delayed insecticidal action against several species of insects. Among the consituents, I showed the most potent activity.

In the present study, in order to clarify the mechanism of insecticidal action of I, the distribution of I in the tissues of the 5th instar larvae of silkworm was investigated by means of high-pressure liquid chromatography (HPLC).

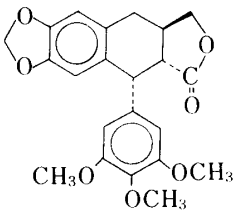

Fig. 1. Chemical Structure of Deoxypodophyllotoxin (I) (Anthricin)

Materials and Methods

Chemical——Deoxypodophyllotoxin[3] was used for the experiment.

Insect——The 5th instar larvae of silkworm, *Bombyx mori* L. (body weight: 1.7—2.2 g), were used. For each dosage group, 6 larvae were used.

Administration and Dose——The leaf-dipping method[4] was used. Acetone solution of I was prepared at

concentrations of 100, 500 and 1000 ppm. Fresh mulberry leaves were dipped into the solution and dried in a room. For the investigation of distribution of I in tissues, 1000 ppm was used; this dosage was about equal to the median lethal dose. Leaves treated with acetone were used as a control group. A treated leaf was kept in a Petri dish and the larvae were released on it.

Quantitative Analysis of Deoxypodophyllotoxin (I) in Blood, Tissues and Feces——The concentration of I in blood, tissues and feces were analyzed quantitatively by the following procedure of HPLC. Apparatus : high speed liquid chromatograph (Toyo Soda HLC-803 series UV-8).

Method: absolute calibration curve method. The calibration curve was determined by the least-squares method [Y, the peak height; x, concentration of I (blood, μg/ml; tissues and feces, μg/g); r, correlation coefficient]. Flow rate: 1.0 ml/min (blood, 5.0 ml/min); chart speed, 5 mm/min; ultraviolet (UV), 294 nm; range, 0.005—0.04; loop, 10 μl; sample, 15 μl; column, TSK LS-310, 30 cm × 4 mm i.d. (blood, 30 cm × 8 mm i.d.). The solvent system[5] was as follows: blood, $CHCl_3$: AcOEt : hexane = 6 : 4 : 4 (t_R, 2 min 48 s); tissues, $CHCl_3$: AcOEt : hexane = 6 : 4 : 6 (t_R, 4 min 48 s) (for analysis of the concentration in the small intestine and rectum, 6 : 4 : 8; t_R, 5 min 30 s); feces, hexane : AcOEt = 13 : 4 (t_R, 14 min 48 s). For the tissues, blood and feces of the control group, no peak was found at the position of retention time of I.

1) Blood: After administration of I, the abdominal legs of the larvae were cut off at the prescribed time, and the blood was collected and analyzed by HPLC. The amount of I was determined from a calibration curve by the least-squares method ($Y=0.248x+0.170$, $r=0.987$. Concentration range: 1—30 μg/ml).

2) Tissues: After administration of I, the larvae were anesthetized with CO_2 and dissected to obtain the skin, ventriculus, small intestine, colon, rectum, Malpighian tubules and fat body. The tissues were washed with 0.85% NaCl solution, and then homogenized in AcOEt. The concentration of I in the filtrate of the homogenate was analyzed by HPLC. The calibration curves were determined by the least-squares method [small intestine $Y=0.210x+0.235$ ($r=0.9991$), concentration range 8—48 μg/g; colon $Y=0.211x-0.636$ ($r=0.9914$), concentration range 8—72 μg/g; ventriculus $Y=0.205x-0.524$ ($r=0.9978$), concentration range 8—80 μg/g; rectum $Y=0.194x-0.589$ ($r=0.9809$), concentration range 8—56 μg/g; skin $Y=0.067x-0.162$ ($r=0.9988$), concentration range 24—360 μg/g; Malpighian tubules $Y=0.062x+0.074$ ($r=0.9923$), concentration range 24—240 μg/g; fat body $Y=0.050x+0.819$ ($r=1.0000$), concentration range 24—330 μg/g].

3) Feces: Excreted feces of the treated larvae were collected and weighed every 24 h. Then quantitative analysis of I in the feces was done. After being dried in a desiccator (P_2O_5) at a room temperature and weighed, the feces were homogenized and extracted with AcOEt for analysis of I. The calibration curve was determined by the least-squares method ($Y=0.071x+0.425$, $r=0.9981$, concentration range 8—240 μg/g).

Temperature——Every experiment was carried out at 23.5 ± 1.5 °C.

Results

I. Toxicity and Symptoms

General Findings——After administration of I to the larvae, general symptoms were observed. The delayed toxicity which had already been recognized in the previous experiment[2] was reconfirmed in the present experiment. Until 24 h after administration, no apparent change was found in the treated groups. However, after 24 h movement and the amount ingested decreased greatly in all treated groups. At 48 h all of the treated larvae were paralyzed and most of them were becoming immobilized.

Body Weight Change——After administration of I to the larvae, changes in the body weight were observed. The result is shown in Fig. 2. Until 24 h after administration, every group increased its body weight, though the rate of increase in the treated groups was apparently lower than that of the control group. However, the weight gain of the treated group was extremely suppressed after 24 h.

Amount of Feces——After ingestion of I by the larvae, the amount of feces excreted was measured in each 24 h period. The results are shown in Fig. 3. Until 24 h every administered group excreted a certain amount of feces, though it was considerably less than that of the control group. However, the amount was very greatly decreased from 24 to 48 h.

II. Distribution of Deoxypodophyllotoxin (I)

In blood and Feces——After administration of I to the larvae, the changes in the concentration of I in the blood were investigated. The results are shown in Fig. 4. The peaks of blood levels of administered groups were as follows: that of the 100 ppm group was found at

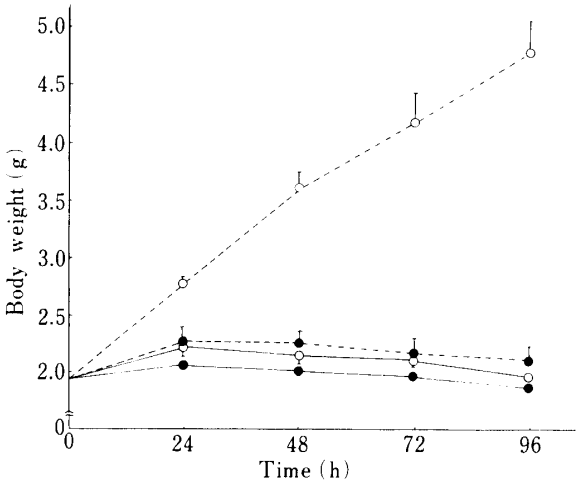

Fig. 2. Body Weight Change in the 5th Instar Larvae of Silkworm, *B. mori* after Administration of Deoxypodophyllotoxin (I)

Each value represents the mean ±S.D. ($n=3$). ---○---, control; ---●---, 100 ppm; —○—, 500 ppm; —●—, 1000 ppm.

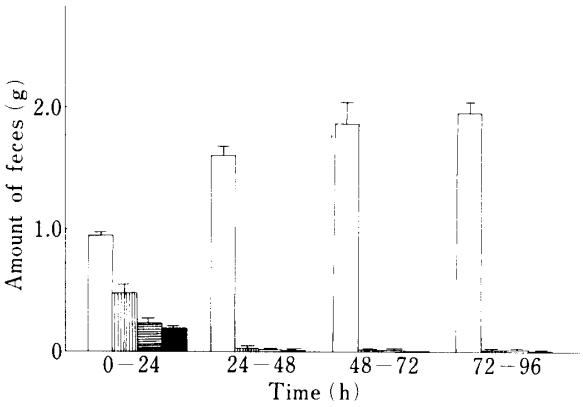

Fig. 3. Amount of Feces Excreted by the 5th Instar Larvae of Silkworm, *B. mori* after Administration of Deoxypodophyllotoxin (I)

Each value represents the mean ±S.D. ($n=3$). □, control; ▥, 1000 ppm; ▤, 500 ppm; ■, 100 ppm.

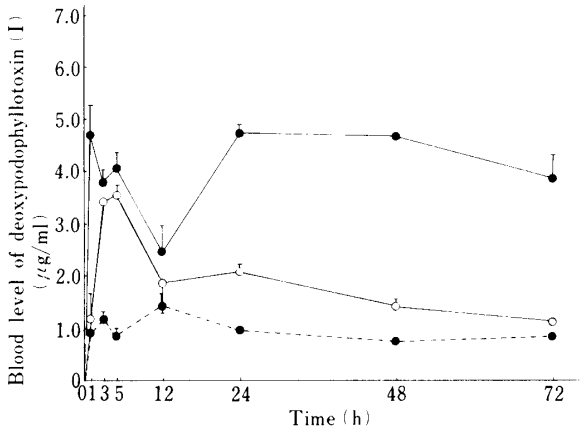

Fig. 4. Blood Level of Deoxypodophyllotoxin (I) in 5th Instar Larvae of Silkworm, *B. mori*

Each value represents the mean ±S.D. ($n=3$). ---●---, 100 ppm; —○—, 500 ppm; —●—, 1000 ppm.

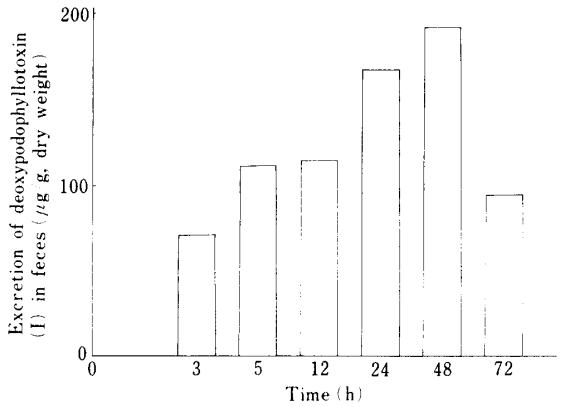

Fig. 5. Amount of Deoxypodophyllotoxin (I) Excreted in Feces of the 5th Instar Larvae of Silkworm, *B. mori*

Each value represents the average of 3 groups. Dose: 1000 ppm.

12 h after administration, that of the 500 ppm group at 5 h and that of the 1000 ppm group at 1 and 24 h (the blood level of the group given 1000 ppm peaked at 1 h, dropped sharply to the lowest value at 12 h, but approached the peak value again at 24 h). Thereafter the blood level of every group began to decrease slowly. However, the compound had not disappeared from the blood even at 72 h.

The analytical results for the feces are shown in Fig. 5. The amount reached a peak at 48 h after administration, and thereafter it began to decrease.

In Tissues——The results obtained from analysis of I in various tissues are summarized in Table I. The highest accumulation was observed in the fat body. Compound (I) was also accumulated in the skin, the ventriculus and the Malpighian tubules in somewhat larger amounts compared with the other tissues. The fat body and the skin of the treated larvae gradually degenerated after the ingestion of I (visual observation).

TABLE I. Distribution of Deoxypodophyllotoxin (I) in Tissues of the 5th Instar Larvae of Silkworm, *B. mori*

Tissue	Time after administration (h)						
	1	3	5	12	24	48	72
Skin	57.0	46.7	66.5	47.9	32.2	16.5	13.2
Fat body	205.2	168.0	291.8	92.6	89.3	28.0	15.8
Malpighian tubule	63.4	97.0	145.8	106.0	64.0	44.1	31.4
Ventriculus	98.3	39.9	68.9	64.9	36.5	14.5	5.7
Small intestine	12.6	24.2	40.4	26.3	14.3	10.6	5.1
Colon	30.4	28.1	76.5	26.4	35.5	20.4	10.3
Rectum	14.3	21.9	48.0	21.4	15.7	13.1	13.5

Values are the means of 30 silkworms.
Quantitative analysis: HPLC (Toyo Soda HLC-803A).
Dose: 1000 ppm.
Unit: μg/g.

Discussion

The present experiments demonstrated a high toxicity of deoxypodophyllotoxin (I) against silkworm larvae. It was confirmed that the symptoms caused by I developed slowly; namely, I is a delayed toxicant. Considering that most conventional insecticides are neurotoxicants which cause convulsive symptoms rapidly in insects, it seems likely that the mechanism of action of I is very different from that of the neurotoxicants.

As a preliminary to an investigation of the mechanism of action, the distribution of I in the silkworm tissues was examined in the present paper. In every treated larva, I remained in the blood even at 72 h after administration (Fig. 4). In the previous paper,[6] we reported that in silkworm larvae the concentration of racemomycin-D (a streptothricin antibiotic) in the blood decreased slowly. Thus, it seems likely that streptothricin antibiotics remain in the blood at rather high concentration when ingested by insects. The behavior of racemomycin-D described above is incompatible with that in mammals.[7-9] When racemomycin-D was administered to mice and rats, it disappeared rapidly from the blood and was not detectable at 4 h after administration. Such a difference is presumably caused by the different metabolic activities of insects and mammals. Further detailed investigation will be required. Furthermore, the cause of the blood level change of I in the 1000 ppm group at 12 h was not clarified.

The quantitative analytical results (Table I) demonstrated that I accumulated at the fat body in the highest concentration among the tissues, followed by the Malpighian tubules, the skin and the ventriculus. In visual examination of excised tissues after administration of I, degeneration of the skin and the fat body was clearly observed along with the development of the toxic symptoms. However, it is premature to conclude that the site of action of I is the fat body, because there is a possibility that the fat body degeneration is due to the deterioration of various physiological activities of insects (*e.g.*, caused by starvation).

It appears likely that the decrease of amount of feces excreted and the loss of body weight gain are associated with the paralytic action of I which leads to starvation. Therefore, the possibility of the direct inhibition of the excretory mechanism by I is not proven by the present experiments. However, it would be interesting to investigate in a further study[10] the histopathological changes in the tissues, especially the fat body, the skin, the Malpighian tubules and the ventriculus where I accumulated in large amounts. Such work is planned, together with the analysis of uric acid in the fat body and the skin, and xanthine

dehydrogenase activity.

In this study, I was detected only by HPLC and its metabolites were not examined. For studies of the metabolism, the synthesis of a labelled compound will be required.

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References and Notes

1) This work was presented at the 102nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 1982.
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10) The results of histopathological research on the tissues in which deoxypodophyllotoxin accumulated in higher amounts will be reported in the next paper.