Anti-Juvenile Hormone Activity of 1-Citronellyl-5-phenylimidazole in the 3rd Instar Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae)

Shoji ASANO¹

*Laboratories of Agricultural Chemical Research, Tokushima Research Institute, Otsuka Pharmaceutical
Co., Ltd., 463-10 Kagasuno, Kawauchi, Tokushima 771-01, Japan*

Eiichi KUWANO² and Morifusa ETO

*Department of Agricultural Chemistry, Kyushu University,
Fukuoka 812, Japan*

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Some biological effects of anti-juvenile hormone active compound, 1-citronellyl-5-phenylimidazole (KK-22), on the 3rd instar silkworm, *Bombyx mori* L., were investigated by feeding treatment with artificial diet for successive days of the 3rd instar period. Drastic precocious metamorphosis was induced in response to doses of KK-22: 100% at 50-200 ppm, 95% at 25 ppm, and 45% at 12.5 ppm in the diet. Some 3rd instar larvae ingesting diet containing 50 to 200 ppm of KK-22 were metamorphosed to pupae within the same instar stage; they spun to make cocoons and pupate, but failed in adult emergence. The larval development of the treated 3rd instar silkworms was prolonged for 1 to 5 days, accompanied by a gain of body weight exceeding the controls. The induction of precocious metamorphosis seems to correlate with prolongation of the larval developmental period in the 3rd instar.

INTRODUCTION

Juvenile hormone (JH) active compounds (juvenoids) show similar biological effects on a variety of insect species as natural hormones and some of them have already been introduced into practical use for pest control as insect growth regulators (STAAL, 1975, 1982). However, the successful application of juvenoids has some restrictions. The drawbacks that are inherent in the use of juvenoid for insect control should be greatly reduced or absent when anti-JH compounds are used (STAAL, 1982). These latter compounds are thus considered more useful for controlling pest insects of field crops because the induction of precocious metamorphosis by anti-JH action can be expected to curtail larval damage. The discovery of insect anti-JHs in plants by BOWERS et al. (1976) stimulated the chemical approach in the field of anti-JH research

¹ Present address: Department of Technical Research, Earth Chemical Co., Ltd., 3218-12 Sakoshi, Ako, Hyogo 678-01, Japan

² Correspondence to: Dr. Eiichi KUWANO, Department of Agricultural Chemistry, Kyushu University, Fukuoka 812, Japan

and several anti-JHs have been discovered, although practical chemicals for pest control have not yet been developed. Recently KUWANO et al. (1983) synthesized a new class of terpenoid imidazoles with potent anti-JH activity against the silkworm, one of the lepidopterous insects.

The present experiment was made to elucidate some biological effects of 1-citronellyl-5-phenylimidazole (KK-22) as a representative of the terpenoid imidazoles on the 3rd instar silkworms by incorporating it into artificial diet.

MATERIALS AND METHODS

Third instar larvae of a hybrid strain (Shunrei $\times$ Shogetsu) of the silkworm, *Bombyx mori* L., were used as test insects and reared on an artificial diet of Silkmate-1-S (Nippon Nosanko, Co., Ltd.) under 25°C laboratory conditions.

1-Citronellyl-5-phenylimidazole (K-22) was dissolved in acetone to make different concentrations and these were mixed into an artificial silkworm diet in powder form, Silkmate-1-M. After evaporating acetone from the mixed diet powder under room temperature, 2% agar solution was added to solidify. The control diet was prepared by a similar method except that KK-22 was not included. Both diets, with and without the test compound, were given successively to 3rd instar silkworms within 24 hr after ecdysis through 3rd instar stage. The 3rd instar larvae which did not develop to next instar in 5 days were transferred onto fresh diet without the test chemical until spinning time.

Containers used for the test silkworms were one-ounce plastic cups, and 120 ml and 500 ml polyethylene cups for the 3rd, 4th, and 5th instar larvae, respectively. Each cup was shared by 5 larvae and each dose level had 4 replicates. Total larval body weight of the 5 specimens in each cup was measured daily during the 3rd instar period and the standard deviation of mean weight was corrected by multiplying the variance calculated by 5.

RESULTS

Induction of precocious metamorphosis

Precocious silkworms were induced by feeding diet containing KK-22 to the 3rd instar larvae. The results are presented in Table 1. The hybrid strain of silkworm used in the present experiment usually ecdyses 4 times in the larval stage, these are the so-called 4-molter silkworms. Their final larval stage is the 5th instar, followed with wandering, spinning a cocoon and pupating in it. As shown in Table 1 some 3rd instar larvae fed on diet with 12.5 to 400 ppm of KK-22 spun in the 4th instar (3-molter silkworms) and some spun in the 3rd instar (2-molter silkworms) at 50 to 200 ppm. In the latter case the 3rd instar larvae ingesting the treated diet were thus committed to pupae within the same instar without any further ecdysis. Some of them were able to spin cocoons and pupate in them but all failed in adult emergence.

Percent induction of precocious silkworms occurred at dose-responses: 100% at 50–200 ppm in the diet, 95% at 25 ppm, and 45% at 12.5 ppm; exception 17% occurred at the highest dose of 400 ppm. Percent induction of precocious 2-molter silkworms at 200, 100, and 50 ppm was 60, 55, and 15%, respectively. No precocious 2-molter silkworms were induced at 400 ppm and the induction rate of precocious 3-molter

Table 1. Induction of precocious silkworms on dietary application of KK-22 in the 3rd instar stage

Conc. (ppm) in diet	No. of larvae used	No. of molters ^a			% precocious induction
		A	B	C	
400	19	0	3	16	17
200	20	12	8	0	100
100	20	11	9	0	100
50	20	3	17	0	100
25	20	0	19	1	95
12.5	21	0	9	12	45
6.25	20	0	0	20	0
3.13	20	0	0	20	0
Controls	20	0	0	20	0

^a A: 2-molter silkworm (precocious), B: 3-molter silkworm (precocious), and C: 4-molter silkworms (normal).

Table 2. Average time of ecdysis and spinning of silkworms fed on diet containing KK-22 in the 3rd instar

Conc. (ppm) in diet	No. of larvae used	Ecdysis time in days to		Spinning time in days of molters		
		4th instar	5th instar	A	B	C
400	19	9.8**	14.8**	—	17.5	21.3**
200	20	10.0**	—	10.8**	18.0	—
100	20	9.7**	—	10.5**	15.8*	—
50	20	8.9**	—	11.0**	15.2**	—
25	21	8.2**	12.0	—	15.4**	—
12.5	20	6.6**	10.7	—	15.1**	17.4
6.25	20	5.2	10.8	—	—	18.8
3.13	20	5.1	10.4	—	—	18.4
Controls	20	5.2	10.4	—	—	17.1

* and ** Significant at $P=0.01$ and $P=0.001$ between treatments and controls.

A, B, and C: as defined in Table 1.

worms was only 17%. It seems that a high dose of K-22 has different effects from lower doses on the 3rd instar larvae, although no dead larvae were observed at any test dose levels, even at the highest dose of 400 ppm.

Effect on the larval developmental period

The average time of ecdysis to the 4th or 5th instar and the time of spinning of test silkworms are shown in Table 2 from the beginning of 3rd instar, and in Figs. 1 and 2 as relationships between the individual frequency and elapsed days. The time of ecdysis to the 4th instar was delayed 1 to 5 days as compared with that of controls in response to diet concentrations of KK-22. Higher doses caused even longer delay. Almost all 4th instar larvae at 200 to 25 ppm entered into precocious metamorphosis without ecdysis to the subsequent 5th instar, although some ecdysed to the 5th instar at the highest dose of 400 ppm and doses below 6.25 ppm, where there was no difference

Fig. 1. Time of the 3rd ecdysis (empty bars) and the 4th ecdysis (solid bars) in silkworms fed on KK-22 during 3rd instar stage.

in the time of ecdysis to the 5th instar between treated worms and controls. The time of ecdysis to the 4th instar when treated at 25 to 200 ppm was delayed 5 days which corresponds to the time of ecdysis to the 5th instar in controls.

The time of spinning varied among different molters. Average spinning time of 2-molter silkworms was around 11 days, which was about 6 days earlier than the 4-molters in controls. However, 3-molter silkworms spun one day earlier than 4th molter one in controls, although there was no significant difference at $P=0.01$ at 200 and 400 ppm. In 4-molter treated silkworms spinning time was similar to that of controls, except for the 400 ppm. Spinning time of different molters is compared in Fig. 2. Roughly speaking, the spinning time of precocious 2-and 3-molter treated silkworms corresponded to the ecdysing time to the 5th instar and the spinning time of normal 4-molter silkworms in controls, respectively.

Effect on the growth rate of 3rd instar silkworms

The body weight of 3rd instar silkworms fed on diet containing different concentrations of KK-22 was daily compared with that of controls (Fig. 3). In controls the

Fig. 2. Time of spinning of 2-molters (dotted bars), 3-molters (solid bars), and 4-molters (empty bars) derived from silkworms fed on KK-22 during 3rd instar stage.

Table 3. Body weight of 3rd molted silkworms fed on diet containing KK-22

Conc. (ppm) in diet	No. of specimens	Average weight (mg) of 3rd molted larvae*
400	19	193±68 a
200	8	285±35 c
100	9	347±45 bc
50	17	339±22 bc
25	20	311±37 c
12.5	21	242±90 c
6.25	20	175±12 a
3.13	20	173±23 a
Controls	20	171±44 a

* Different letters indicate significant difference at $P=0.01$.

Fig. 3. Growth curve of 3rd instar silkworms fed on diet containing KK-22.

larval weight increased regularly for the first 3 days and reached a plateau when larvae went into the molting stage without feeding. On the other hand, the treated 3rd instar silkworms had a feeding period 1 to 5 days longer than controls. The degree of prolongation varied depending on dose levels in the diet. Although higher doses caused greater prolongation in the 3rd instar period, the weight gain for the first several days at doses of 200 and 400 ppm was less than controls but eventually became larger. In other doses, 12.5 to 100 ppm, there was no difference in weight for the first 2 days between treated worms and controls. However, after 3 days the larval weight at the above doses became significantly greater than controls.

To compare the degree of weight gain during the 3rd instar period between treatments and controls the weight of molting 3rd instar larvae just before ecdysis to the 4th instar was measured and the average values at each dose level are summarized in Table 3. The weight of molting larvae at doses of 12.5 to 400 ppm was significantly higher than controls, showing the highest around 100 ppm, where a weight gain twice that of controls was observed. Since two higher doses, 200 and 400 ppm, showed less weight increase than the 100 ppm, an appropriate dose for growth increasing effects may be around 100 ppm and this may also be the most effective dose level for anti-JH activity without adverse effects.

Average weight of the 3rd instar larvae at the beginning of the test varied a little among test plots although this difference was not significant at $P=0.01$. To make a comparison of daily weight graphically the relative weight values against those of the starting day were calculated for each plot and are shown in Fig. 3. In controls the larval weight increased in a direct line for the first 3 days but then reached a plateau because of the approaching molting stage and lack of feeding. On the other hand, the larval growth rate at high doses of 400 and 200 ppm was suppressed in the early 3rd instar stage but later recovered. The larval growth curves at less than 100 ppm were similar to that of controls in the first 2 days and then were higher depending on the prolongation of the 3rd instar feeding period. When the 3rd instar larvae were transferred onto fresh diet without the test chemical on the 5th day, the growth rate was enhanced and increased at a higher rate, especially those worms previously treated at 400 ppm.

DISCUSSION

The chemical induction of precocious metamorphosis in silkworms has been demonstrated by dietary application of kojic acid (MURAKOSHI, 1972), its related compounds (MURAKOSHI and ICHIMOTO, 1972), abietic acid derivatives (MURAKOSHI et al., 1975), and quinolone alkaloids (MURAKOSHI et al., 1977). However, no compound administered by topical application has yet been reported as causing precocious induction, perhaps because of the hydrophilic characters of such compounds.

In a previous paper the topical application of KK-22 to 0–8 hr old 3rd instar silkworms induced up to 100% precocious metamorphosis when used at more than 2.5 μg per individual, where all precocious silkworms were 3-molters, that is, they prematured in the 4th instar (KUWANO et al., 1983). In the present experiment the dietary application of the same compound to 3rd instar larvae induced not only some 3-molter precocious silkworms but also 2-molter ones, which prematured within the 3rd instar without any further ecdysis. The different results between the two experiments might be due to the application method of single or successive supply. Doses inducing the precocious 2-molter silkworms were restricted to a range of 50 to 200 ppm in the diet and percent induction did not exceed 60%. All doses of KK-22, except the highest 400 ppm, caused the induction response of precocious silkworms; at 400 ppm the 3rd instar larval growth was highly suppressed, contrary to other doses (Fig. 3). However, this suppressed growth rate in early 3rd instar at higher doses of 400 and 200 ppm was recovered after transference onto fresh diet without the test compound. These facts suggested that KK-22 may also have some antifeeding effect on silkworms in addition to its anti-JH action. In the preliminary anti-feeding assay with mulberry leaf disks dipped in 0.1% acetone solution there was no significant difference in feeding rate for 3 hr. Therefore, the observed antifeeding effects may be caused by some physiological occurrence after ingestion of the chemical.

As MURAKOSHI et al. (e.g., 1975) described for the prolongation of 4th instar larval period in precocious 4-molter silkworms with dietary application of kojic acid and other compounds, effective doses of KK-22 induced precocious silkworm prolongation a feeding period in 3rd instar which is about 1 to 5 days longer than controls.

There seems to be some relationship between the degree of prolongation in the 3rd instar stage and the precocious induction rate. Prolongation of 3 to 5 days induced

the precocious 2-molter silkworms. Prolongation for 5 days in the 3rd instar corresponded to the ecdysing time to the 4th instar in controls. Although the ecdysing time to the 4th or 5th instar varied with dose levels of the test chemical, the larval maturing time based on spinning was similar among the same molter silkworms regardless of dose (Table 2 and Fig. 2). The spinning time of treated precocious 2- and 3-molter silkworms corresponded respectively to the ecdysing time to the 5th instar and one day prior to the spinning time of normal 4-molter silkworms in controls. The larval developmental period was shortened by the anti-JH action resulting in smaller matured larvae followed by miniature pupae. Although no emergence was observed in pupae from 2-molter silkworms, emerged adults from pupae of 3-molter silkworms were about 7/10 normal size, and all could mate and laid viable eggs.

STAAL et al. (1981) classified five main and ten possible mechanisms of action of JH antagonists. We think these mechanisms can also be briefly classified into three categories: 1) interference with the neural and humoral regulation of corpora allata, 2) interference with JH biosynthesis in corpora allata, and 3) interference with JH transport and JH receptor.

KUWANO et al. (1983) reported that the anti-JH action of KK-22 can be reversed by simultaneous application of an active juvenoid, methoprene. HAMMOCK and MUMBY (1978) examined the effects of some chemicals on the NADPH-dependent conversion of tritiated methyl farnesoate to JH III (methyl 10, 11-epoxy-farnesoate). Among them, *o*-bromophenoxymethylimidazoles showed the highest inhibition of methylfarnesoate epoxidation. These facts suggest that the present test compound, KK-22, might have epoxidation inhibition to juvenile hormones in corpora allata of insects and might be classified among the 2nd of the above suspected mechanisms for JH antagonists.

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