

## INHIBITORY ACTIVITY OF AN IMIDAZOLE COMPOUND ON ECDYSIOSYNTHETIC ORGANS IN MEALWORMS UNDER *IN VIVO* AND *IN VITRO* CONDITIONS

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### Abstract

KK-42, an imidazole derivative was tested on adult females of *Tenebrio molitor* L. (*Insecta: Coleoptera*). The compound was applied topically (1, 5, 10 µg/insect) on 0- or 2-day old adult females or added to the culture medium (1 and 10 µM) of abdominal sternites explanted from newly ecdysed pupae or ovaries removed from 2- and 4-day old adult females. Ecdysteroid measurements using an enzyme-immunoassay demonstrated that this compound applied on newly emerged adult females reduced the hormonal amounts in ovaries. However, when applied later, i.e. on 2-day old females corresponding to the beginning of the vitellogenesis, it had no significant effect on the amount of ovarian ecdysteroids with the lowest dose. Finally, the compound was also tested on the *in vitro* production of ecdysteroids. The amount of ecdysteroid released into the culture medium by ovaries or integumental explants were significantly reduced by KK-42.

**Key words:** mealworms, ovary, integument, ecdysteroids, KK-42, insect growth regulator.

### Résumé

KK-42, un dérivé de l'imidazole a été testé sur les femelles adultes de *Tenebrio molitor* L. (*Insecta: Coleoptera*). Le composé a été administré par application topique (1,5 et 10 µg /insecte) sur des femelles adultes âgées de 0 ou 2 jours, ou additionné au milieu de culture (1 et 10 µM) de sternites abdominaux explantés de nymphes nouvellement exuviées ou d'ovaires prélevés de femelles adultes âgées de 2 ou 4 jours. La quantification des ecdystéroïdes par une méthode enzymo-immunologique montre que ce composé, appliqué sur des femelles adultes nouvellement exuviées, réduit le taux hormonal dans les ovaires. Cependant, quand il est appliqué plus tard, c'est-à-dire sur des femelles adultes âgées de 2 jours correspondant au début de la vitellogenèse, il n'a pas d'effet significatif sur le taux ovarien d'ecdystéroïdes avec la dose la plus faible. Finalement, le composé a été également testé sur la production d'ecdystéroïdes *in vitro*. Les taux d'ecdystéroïdes libérés dans le milieu de culture par les ovaires ou les explants tégumentaires sont significativement réduits par le KK-42.

**Mots clés:** vers de farine, ovaire, tégument, ecdystéroïdes, KK-42, régulateurs de croissance.

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### ملخص

لقد تم اختبار مادة KK-42 الذي هو مشتق إيميدازول على بالغات إناث *Tenebrio molitor* (حشرات غمدية الأجنحة). أضيف هذا المركب موضوعيا إلى الإناث عند العمر 0 يوم أو 2 يوم بتركيز 1, 5, 10 ميكروغرام/حشرة، أو أضيف إلى وسط الزرع بتركيز 1 و 10 ميكرومول إلى المنطقة الصدرية البطنية المتحصل عليها من الإنسلاخ الجديد للعداري أو من المبايض المنزوعة من البالغات ذات الأعمار 2 و 4 أيام. إن معايرة هرمون الإنسلاخ بالطريقة الإنزيمية المناعية أوضحت بأن هذا المركب المضاف إلى الإناث البالغات المنسلقات حديثا قد انخفض تركيزه في المبايض، في حين أنه عندما أضيف إلى الإناث ذات عمر 2 يوم و هي المدة التي تشير إلى بداية تشكيل المح، فإنه لم يكن له تأثير معنوي على كمية هرمون الإنسلاخ المبيضي سجل أخفض مستوى له. أخيرا، أختبر المركب كذلك خارج الجسم لغرض دراسة هرمون الإنسلاخ المفرز داخل وسط الزرع بواسطة المبايض أو الجلد انخفض معنويا تحت تأثير مركب KK-42. **الكلمات المفتاحية:** حشرات غمدية الأجنحة، المبايض، هرمون الإنسلاخ، KK-42، ضابط النمو.

Several developmental and physiological processes are under the control of the steroid moulting hormone (ecdysteroids) such as molting, growth, reproduction and gametogenesis [1]. This developmental hormone is considered as potential specific target sites for pest control [2]. Agrochemical research has resulted in the discovery of several chemically novel insecticides that mimic or inhibit the action of ecdysteroids [3, 4]. KK-42, an imidazole compound, was found to interfere with the normal growth and development in several insect species [5-7]. The normal development of *Tenebrio molitor* L. (*Insecta: Coleoptera*) ovaries, the vitellogenesis process [8-14] and the ovarian ecdysteroid production have been reported [15]. These data provide an experimental basis for investigations on KK-42 or more potent insect growth regulators for controlling pest species.

In addition to secreting the eggshell, ovarian follicle cells are the site of ecdysteroid synthesis [16]. The ovarian ecdysteroids play multiple and fundamental roles such as reinitiation of meiosis, induction of choriogenesis and determination of ovarian protein pattern [1]. Therefore, we first evaluated the activity of KK-42, topically applied on adult females of mealworms on the ecdysteroid amounts of ovaries. Since both pupal integument [17] and developing ovaries of mealworms were found to synthesize ecdysteroids *in vitro* [15], the compound was also tested on the hormonal release into the culture medium by such organs.

## MATERIAL AND METHODS

### Experimental animals

*T. molitor* pupae from a stock colony were sexed and kept separately until adult ecdysis. Adults were collected 0-4h following emergence and reared on wheat flour at 27°C and 80% r.h. in almost continuous darkness.

### Culture of ovaries and integumental explants

Explants were cultured following the method previously described by Soltani et al. [13]. Four abdominal sternites from newly ecdysed pupae and ovaries from 2- or 4-day old adult females were dissected. Each explant was excised, cleaned of extraneous tissue, rinsed three times in a saline solution and then incubated for 5 days at 26°C in 0.5 ml of Landureau's medium [18].

### Insecticidal treatments

KK-42 was kindly provided by D.R. Kwano (Kyushu University, Japan). Treatments were made either *in vivo* or *in vitro*. In the case of *in vivo* treatment, the compound was dissolved in acetone (2 µl per female) and topically applied at three doses (1.5 and 10 µg/female) to 0- or 2-day old females. In the case of *in vitro* treatment, KK-42 was added to the culture medium in 5 µl ethanol per well to give a final concentration of 1 or 10 µM. In controls, the culture medium contained 5µl ethanol per culture.

### Enzymo-immunoassay of ecdysteroids

At appropriate times culture media of ovaries were collected and subjected to ecdysteroid extraction. The samples were extracted with methanol by sonication. After centrifugation at 5,000 g for 10 min, the supernatants were taken and evaporated. The extracts were suitably resuspended in phosphate buffer (0.1 M, pH 7.4) and each individual sample was analyzed in duplicate by an enzyme immunoassay (EIA) as previously described [19] using peroxidase as an enzymatic tracer and tetramethyl benzidine as a color reagent. The ecdysteroid antiserum (L2 polyclonal antibody) used was 7 times more sensitive to ecdysone than to 20-hydroxyecdysone. Data are expressed as pg ecdysone equivalents/culture or /mg ovaries.

### Statistical analysis

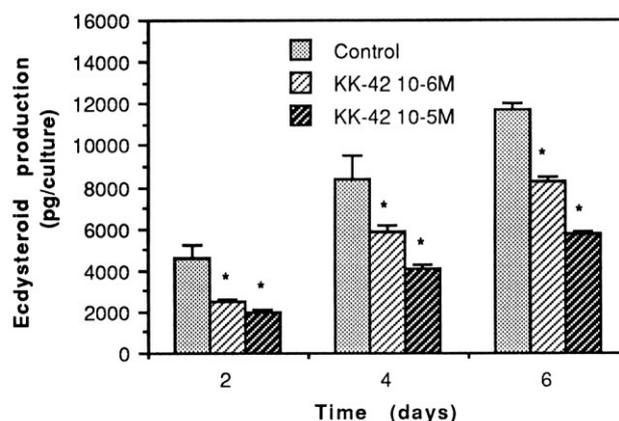
Results are reported as means ± standard deviation (s) of measurements established on individuals samples. The age and the number of animals tested per series are given with the results. The comparison of mean values between control and treated series was made by the Student's *t*-test at 5% level.

## RESULTS

### Ecdysteroid production by integumental explants *in vitro*.

Abdominal sternites from newly ecdysed pupae were cultured without addition of exogenous hormone and the amounts of ecdysteroids released into the culture medium

were determined by EIA at various times of incubation. As illustrated in figure 1, results showed that the amounts of ecdysteroids released into the culture medium by sternal explants increased in all series during the incubation period. Moreover, the two tested doses of KK-42 caused a significant reduction in the ecdysteroid production as compared to control series.



**Figure 1:** Effect of KK-42 on ecdysteroid amounts in culture medium at various times during incubation of sternal integumental explants from newly ecdysed pupae of *Tenebrio molitor*. Each value is the mean ± s of 6-8 cultures measured in duplicate by EIA.

### Ecdysteroid production by ovaries *in vivo*

The ecdysteroids produced by ovaries of *T. molitor* under normal conditions were reported in a previous study [15]. The amount of ecdysteroids produced *in vivo* by ovaries during the sexual maturation peaked at day 4 declined thereafter at the start of egg laying. Moreover, ovaries incubated *in vitro* released significant amount of ecdysteroids into the culture medium. This hormonal release of ecdysteroids varied according to the age of explantation. Thus, the maximum value was recorded for ovaries explanted from 4-day old females.

Age (days)	Control	KK-42 1µg	KK-42 5µg	KK-42 10µg
0	11.9±2.3			
2	29.1±0.4	19.9±2.3	15.2±3.3	11.9±0.8
4	51.0±4.0	23.8±0.4	21.6±2.3	15.0±2.1

**Table 1:** Effect of KK-42 on the amount of ecdysteroids (pg ecdysone equivalents/mg) in the ovaries of *Tenebrio molitor* adult females treated topically at adult ecdysis (m±s, n = 6-10 females).

Age (days)	Control	KK-42 1µg	KK-42 5µg	KK-42 10µg
2	29.1±0.4			
4	51.0±4.0	48.6±2.9	40.0±8.0	39.1±8.0

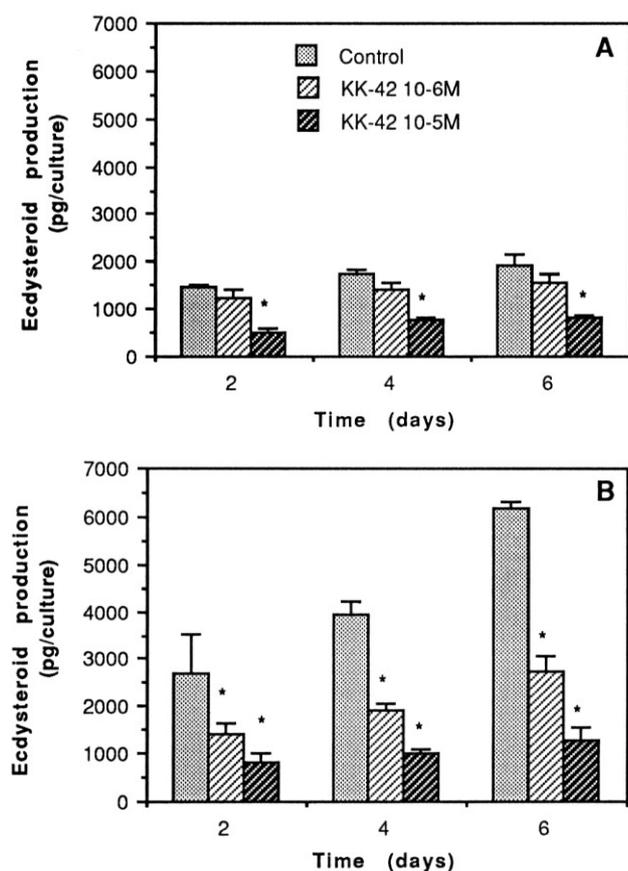
**Table 2:** Effect of KK-42 on the amount of ecdysteroids (pg ecdysone equivalents/mg) in the ovaries of *Tenebrio molitor* adult females treated topically at day 2 after adult ecdysis (m±s, n=8-10 females).

KK-42 was first assayed on *in vivo* production of ecdysteroids by developing ovaries (Tables 1, 2). Results showed that KK-42 applied topically on newly ecdysed

females caused a significant reduction in the *in vivo* production of ecdysteroids by ovaries respectively at day 2 with the 5 ( $t = 7.14$ ;  $Df = 14$ ;  $p < 0.0001$ ) and 10 ( $t = 57.85$ ,  $Df = 14$ ,  $p < 0.0001$ )  $\mu\text{g}$  doses, and at day 4 with the three doses (1  $\mu\text{g}$ :  $t = 21.32$ ,  $Df = 14$ ,  $p < 0.0004$ ; 5  $\mu\text{g}$ :  $t = 16$ ,  $Df = 14$ ;  $p < 0.0001$ ; 10  $\mu\text{g}$ :  $t = 20.26$ ,  $Df = 14$ ,  $p < 0.0001$ ), compared to controls of the same age (Table 1). As shown in table 2, the compound applied on 2-day old females, i.e. at the beginning of the vitellogenesis, had no significant effect on the ovarian amount of ecdysteroids only with 1  $\mu\text{g}$  dose ( $t = 1.42$ ,  $Df = 16$ ,  $p = 0.1718$ ).

### Ecdysteroid production by ovaries *in vitro*

The imidazole compound was then tested on the *in vitro* production of ecdysteroids by ovaries explanted from 2- and 4-day old adult females, corresponding respectively to follicles at the beginning and the end of vitellogenesis.



**Figure 2:** Effect of KK-42 on ecdysteroid amounts in culture medium at various times during incubation of ovaries explanted from 2-(A) and 4-day (B) old adult females of *Tenebrio molitor*. Each value is the mean  $\pm$  s of 4-5 cultures measured in duplicate by EIA.

Figure 2A showed that the compound at the highest concentration decreased significantly the amount of ecdysteroids released into the culture media by ovaries explanted from 2-day old females at day 2 ( $t = 23.26$ ,  $Df = 7$ ,  $p < 0.0001$ ), 4 ( $t = 17.60$ ,  $Df = 7$ ,  $p < 0.0001$ ) and 6 ( $t = 8.96$ ,  $Df = 7$ ,  $p = 0.0002$ ) during the incubation period,

respectively. In the case of the *in vitro* production of ecdysteroids by ovaries explanted from 4-day old females, the compound at the two tested doses reduced significantly ( $p < 0.05$ ) this release of ecdysteroids at day 2 (1  $\mu\text{M}$ :  $t = 3.13$ ,  $Df = 7$ ,  $p = 0.0162$ ; 10  $\mu\text{M}$ :  $t = 5.21$ ,  $Df = 7$ ,  $p = 0.0139$ ), 4 (1  $\mu\text{M}$ :  $t = 12.75$ ,  $Df = 7$ ,  $p = 0.0001$ ; 10  $\mu\text{M}$ :  $t = 18.98$ ,  $Df = 7$ ,  $p < 0.0001$ ) and 6 (1  $\mu\text{M}$ :  $t = 22.08$ ,  $Df = 7$ ,  $p < 0.0001$ ; 10  $\mu\text{M}$ :  $t = 33.29$ ,  $Df = 7$ ,  $p = 0.0001$ ), respectively (Fig. 2B).

### DISCUSSION AND CONCLUSION

In various insect species, besides prothoracic glands and ovaries, they are alternative sites of moulting hormone production [17]. It is frequently admitted that prothoracic gland are the sole ecdysteroid source in immature stages, i.e. when ecdysteroids are involved in moult control, whereas ovaries represent the only source during late pupal and adult stages, i.e. during reproduction. In mealworms, both pupal integument [17] and developing ovaries are able to release ecdysteroids *in vitro* [15]. This ecdysteroid release by pupal integumental explants from mealworms was significantly reduced by KK-42 added to the culture medium.

Mealworms presents 12 telotrophic meroistic ovarioles per ovary. Each ovariole includes a germarium, where germ cells proliferate and follicle are formed, and a vitellarium, where follicles undergo accumulation of yolk proteins and choriogenesis [20]. Egg-laying begins at day 4 following adult ecdysis in mealworms [12]. As in other insect species studies [21], ovaries of *T. molitor* were found to produce ecdysteroids and their changes are correlated with reproduction [15]. Indeed, the rate of ecdysteroids produced *in vivo* or *in vitro* by mealworm ovaries, changed during oocyte maturation in a characteristic way and reached a maximum at day 4 after emergence, when animals started to deposit eggs [15].

The effect observed in ovarian ecdysteroid production in mealworms varies according to the moment of application during the oocyte maturation. The ovarian amount of ecdysteroids determined by EIA in adult females of mealworms were affected particularly when the compound was applied *in vivo* at the adult emergence. When topical treatment was made later, i.e. on 2-day old females corresponding to the beginning of the vitellogenesis, it had no significant effect on the amount of ovarian ecdysteroids only with the lowest dose.

Concerning the *in vitro* hormonal production, the amounts of ecdysteroids released into the culture medium by developing ovaries explanted from 2- or 4-day old females were significantly reduced by KK-42. Similarly, the imidazole compound was also found to reduce the ovarian amount of ecdysteroids when applied topically before the starting of vitellogenesis process in *Bombyx mori* L. (*Insecta: Lepidoptera*) pupae [6]. This inhibitory action of KK-42 on ecdysteroid production in prothoracic glands and ovaries was evidenced under *in vivo* and *in vitro* conditions in several insect species [22, 23].

Conclusively, the inhibitory activity of the compound on ecdysteroid production by integumental explants or

ovaries of mealworms confirm its primary mode of action reported on several insects species. The effects of KK-42 on ecdysteroid production *in vivo* were more marked when applied on newly emerged adults comparatively to treatment on 2-day old females corresponding to the beginning of the vitellogenesis. Moreover, this action on ecdysteroid production *in vitro* seemed rapid since a reduction in hormonal release into culture medium was recorded within 2 days of incubation undepending to the age of explantation.

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