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Effect of xanthurenic acid on P-450-dependent biotransformation by molting glands in vitro

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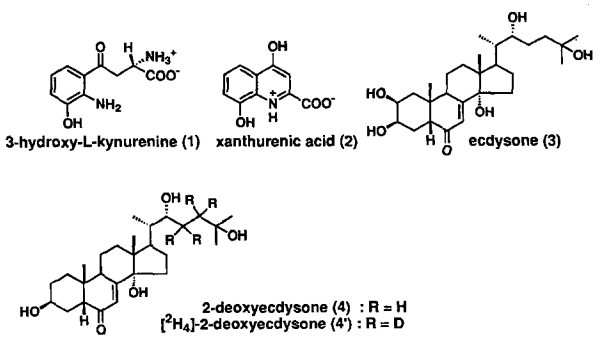
Abstract. Incubation of molting glands from the crayfish *Procambarus clarkii* (Y-organ) and the silkworm *Bombyx mori* (prothoracic gland) with 23,24-[$^2H_4$]-2-deoxyecdysone resulted in the production of deutero-ecdysone; this biotransformation was inhibited in the presence of xanthurenic acid. When the experiments were performed under an $^{18}O_2$ atmosphere, the ^{18}O atom was introduced into ecdysone, as confirmed by mass spectrometry. We therefore suggest that xanthurenic acid inhibits P-450-dependent hydroxylation of 2-deoxyecdysone. However, deutero-2-deoxyecdysone was not converted to 3-dehydroecdysone when using Y-organs in vitro, although it is a major product. We therefore conclude that the biosynthetic pathway of ecdysteroids in *P. clarkii* branches at an early step.

Key words. Xanthurenic acid; ecdysone; 23,24-[$^2H_4$]-2-deoxyecdysone; 3-dehydroecdysone; $^{18}O_2$-incorporation; P-450 monooxygenase; *Procambarus clarkii*; *Bombyx mori.*

It is accepted nowadays that the Y-organs in crustaceans have the ability to synthesize different ecdysteroids as the prothoracic glands (PG) do in insects[1]. A considerable number of studies revealed that the sequence of ecdysteroidogenesis in the molting gland was rather species-specific in both crustaceans[1] and insects[2]. The hydroxylation process of PG products is known to be mediated by cytochrome P-450 monooxygenases[3] which are localized in mitochondria and microsomes.

We previously reported[4] that 3-hydroxy-L-kynurenine (**1**) exhibited molt-inhibiting hormone (MIH) activity in vivo in the crayfish (*Procambarus clarkii*), and its metabolite, xanthurenic acid (**2**), acted directly as an ecdysone (**3**) biosynthesis inhibitor (EBI) in vitro. We also suggested[5] that the EBI effect was caused by the interaction between cytochrome P-450s and xanthurenic acid, although experiments were performed using a model P-450 induced by phenobarbital in rats. The presence of P-450 in the Y-organ and the step at which inhibition takes place, however, remained to be clarified.



Scheme.

In order to understand the mode of action of xanthurenic acid, we focused our investigation on C-2 hydroxylation of 2-deoxyecdysone (**4**), a proximal precursor, using molting glands in vitro (Y-organs from *P. clarkii* and PGs from *Bombyx mori*). The comparative experiments were performed with 23,24-[$^2H_4$]-2-deoxyecdysone (**4'**) under an $^{18}O_2$ atmosphere[6] in the presence or absence of xanthurenic acid as EBI.

Materials and methods

Animals. The 5th instar larvae of *B. mori* (day 7) were purchased and reared on an artificial diet at 26–28 °C under a 16L:8D photoperiod for 4 days. A pair of PGs was dissected from *B. mori* in Ringer's solution under a microscope. American crayfish, *P. clarkii*, were also purchased and male animals of 70–80 mm length were used. They were maintained at room temperature (18–22 °C) for 4 days after the removal of their eyestalks to stimulate the Y-organs[4]. The bilateral Y-organs were dissected in medium 199 under a microscope and used in the biotransformation experiments.

Chemicals. The commercial culture media were somewhat modified before use in the incubation of the PGs (a) and the Y-organs (b), respectively: (a) Tween 80 (0.002%) was added to Grace's insect medium (supplemented with lactalbumin hydrolysate, yeastolate, L-glutamate and methionine, without insect hemolymph, pH 6.5, GIBCO, USA); (b) Medium 199 containing Hank's salt (GIBCO) was modified according to Keller's method[7], and prior to use the pH was adjusted to 7.5 with NaOH. Oxygen-18 gas was purchased from MSD ISOTOPES, Canada (97.6%) or CEA, France (98.0%). All other reagents unless otherwise noted in the

text were obtained from Nacalai Tesque, Japan. [$^2H_4$]-2-Deoxyecdysone (23, 23, 24, 24-[$^2H_4$]-3β, 14α, 22R, 25-tetrahydroxy-5β-cholest-7-en-6-one) was synthesized according to a published method[8].

Biotransformation of [$^2H_4$]-2-deoxyecdysone. One of the bilateral molting glands was used for the experiment and the contralateral gland for the control. Each batch of glands (10 PGs or 3 Y-organs) was incubated at 25 °C for 18 h in 1 ml of the specified medium (a or b) in the presence of [$^2H_4$]-2-deoxyecdysone dissolved in 5 µl of ethanol (90 µg for PGs and 45 µg for Y-organ), while only ethanol (5 µl) was added to the medium for each control experiment.

Incorporation of molecular oxygen during the biotransformation process was examined in the presence or absence of heavy molecular oxygen ($^{18}O_2$)[6]; gas composition was adjusted to the proportion of $^{18}O_2$:N_2 = 1:4. The nitrogen gas was purified by passing through Fieser's solution to eliminate contamination by atmospheric oxygen. Hydrogen sulfide generated by Fieser's solution was removed by a saturated lead acetate solution[9]. Each incubation medium was degassed by sonication under vacuum for 10 s, then saturated with helium gas at 4 °C.

Inhibition of the biotransformation was examined in the presence or absence of xanthurenic acid. The concentration of xanthurenic acid was 10 mM when using PGs[5] and 100 µM for Y-organs. For comparison of the inhibitory effects, specific pharmacological inhibitors for cytochrome P-450s[3], such as piperonyl butoxide (100 µM) and SKF-525A (100 µM) were used with PGs.

Analysis. After incubation, the reaction mixture was diluted with water (10 ml) and applied onto a Sep-Pak C18 column (Waters) equilibrated with water. The ecdysteroids were eluted with methanol (5 ml), which was later removed under a stream of nitrogen gas. The residue was fractionated by HPLC (Shimadzu: LC-6A pump, SPD-6AV detector) at room temperature using a reverse phase column [LiChrospher 100, RP-18, 5 µm, 4 × 250 mm (Merck)] with a CH_3OH-H_2O gradient system (CH_3OH was 55% for the initial 30 min, increased to 100% for the following 10 min, and kept at 100% for an additional 10 min). Retention times were: 19.0 min (ecdysone), and 40.7 min (2-deoxyecdysone). After removal of the solvent, each fraction was analyzed by HPLC at 40 °C for quantitation using a photodiode-array detector [1090M (Hewlett Packard); Cosmosil $_5$C18, 4.6 × 250 mm; solvent for ecdysone: CH_3OH-CH_3CN-H_2O (3:1:6), for 2-deoxyecdysone: CH_3OH-H_2O (6:4)]. 3-Dehydroecdysone was also analyzed with a CH_3OH-H_2O gradient system (CH_3OH was increased from 40% to 50% for the initial 10 min, kept at 50% for 10 min, then increased to 100% over 20 min). Retention times were: 15.5 min (ecdysone), 16.7 min (3-dehydroecdysone), and 28.0 min (2-deoxyecdysone). Mass spectra were recorded on a JEOL JMX-HX110 spectrometer using FAB (fast-atom bombardment) positive ionization with a glycerol matrix.

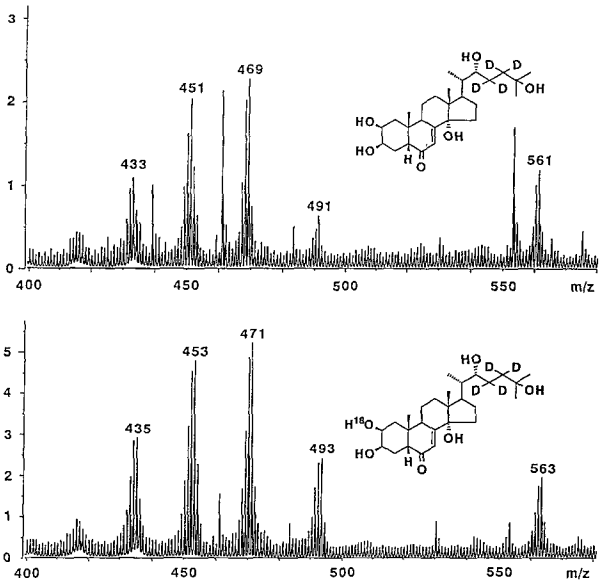


Figure 1. FAB mass spectra of deuterated ecdysones transformed from [$^2H_4$]-2-deoxyecdysone.
Above. The protonated molecular ion containing four deuterium atoms, produced under $^{16}O_2$, can be seen at m/z 469. Fragment ions at m/z 451 and m/z 433 due to sequential losses of water are also observed. The pseudomolecular ions $[M + Na]^+$ and $[M + \text{glycerol} + H]^+$ are present at m/z 491 and m/z 561, respectively. In addition to these major peaks, presence of the isotopes attributable to the incorporation of d_0-d_4 was indicated (see text). The residual intense peaks come from the glycerol matrix.
Below. Using $^{18}O_2$, all important peaks are shifted by two mass units higher.

Results

1) Biotransformation. In the presence of [$^2H_4$]-2-deoxyecdysone, the production of ecdysone was significantly increased in both molting glands (PGs and Y-organs). Using PGs, the production of ecdysone showed an increase of more than 200 times (18012 ± 7351 ng/10 PGs, n = 4) when compared with the controls (84 ± 7.8 ng/10 PGs, n = 4). Using Y-organs, ecdysone production increased more than 1000 times (1076 ± 384 ng/3 Y-organs, n = 3) even when ecdysone in the control was present only in trace amounts and undetectable under our experimental conditions (< 0.5 ng). The biotransformation was confirmed using mass spectrometry: the $[M + H]^+$ ion corresponding to [$^2H_4$]-ecdysone was observed at m/z 469 (fig. 1, above). The yield of the transformation was about 20% (calcd. from the starting material).

The biotransformation of the putative precursor ([$^2H_4$]-2-deoxyecdysone) was reexamined using the Y-organs from three groups of animals (n = 12–16) of different sizes and sexes [60–80 mm length, female; 80–100 mm, female; and 80–100 mm, male]. In all experiments, deutero-2-deoxyecdysone was converted to ecdysone

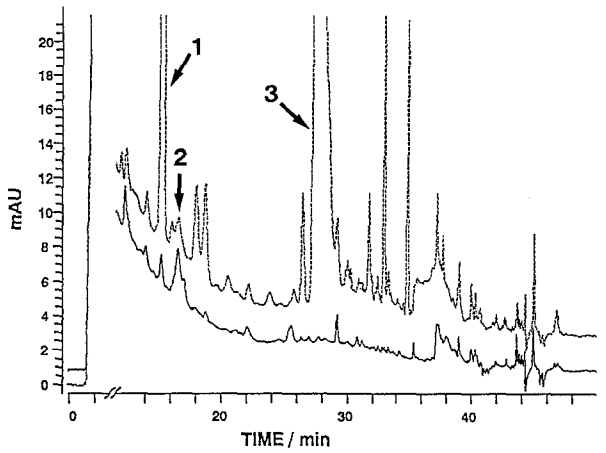


Figure 2. RP-HPLC profiles of ecdysteroids. Ecdysteroids, produced by incubation of the Y-organs in the presence (broken line) or absence (solid line) of [$^2H_4$]-2-deoxyecdysone, are indicated by arrows. **1**: ecdysone (15.5 min); **2**: 3-dehydroecdysone (16.7 min); **3**: 2-deoxyecdysone (28.0 min). The details are given in the text.

but not into 3-dehydroecdysone; the increase in ecdysone production respective to the controls was 9.4, 42 and 54 times, resepctively. Figure 2 shows a representative HPLC profile of ecdysteroids of the Y-organ, detected by photodiode-array detector (245 nm is the maximum of enone absorption in 50% aq. CH_3OH). Ecdysone (8–37 ng/Y-organ) and 3-dehydroecdysone (19–38 ng/Y-organ) were both detected in the controls.

2) Incorporation of $^{18}O_2$. The incorporation of molecular oxygen into ecdysone was also demonstrated using MS analysis. The mass spectrum exhibited an additional $[M+H]^+$ ion peak at m/z 471 (fig. 1, below), which indicated the incorporation of an ^{18}O atom into ecdysone, in both PGs and Y-organs. Under EI and FAB ionization modes, the mass spectra of our synthetic [$^2H_4$]-2-deoxyecdysone revealed that the relative abundance of $d_0:d_1:d_2:d_3:d_4$, was almost 1:2:6:10:10. The distribution of the isotopes was attributable to the contamination by H_2 of the $^2H_2$ gas used for catalytic reduction of the synthetic intermediate with an acetylenic function. A similar isotope distribution was observed with a model compound having a long-$(CH_2)_2$- sidechain. The ratio of ^{16}O-ecdysone ($[M+H]^+$, m/z 469) to ^{18}O-ecdysone (m/z 471) was estimated to be 12:88 (fig. 3). We suspect that the production of ^{16}O-ecdysone was mainly due to contamination by $^{16}O_2$.

3) Effect of xanthurenic acid. Addition of xanthurenic acid reduced the biotransformation as shown in the table.

4) Effect of pharmaceutical P-450 inhibitors on PG. The results obtained with piperonyl butoxide and SKF-525A, the P-450 inhibitors, are also given in the table. The inhibition was much more marked with piperonyl butoxide than with SKF-525A, although their exact mode of action remains to be clarified.

Comparison of ecdysone production under different experimental conditions.

Experimental condition	Relative production (organ vs. control) PG	Y-organ
Control (no addition)	1.00	1.00
+[$^2H_4$]-2-deoxyecdysone	210 ± 71.00	> 1000
Control (+[$^2H_4$]-2-deoxyecdysone)	1.00	1.00
+xanthurenic acid	0.47 ± 0.19	0.45 ± 0.37
+piperonyl butoxide	0.20 ± 0.11	
+SKF-525A	0.68 ± 0.63	

Discussion

Although the amounts of substrate added (a putative precursor) were much higher than physiological concentrations in the insect or the crayfish, we demonstrated that 2-deoxyecdysone was transformed into ecdysone in both Y-organs and PGs, at least in vitro. This step is generally considered to be the last step of ecdysteroidogenesis in insects[10]. Our results suggest that 2-deoxyecdysone is also an immediate precursor of ecdysone in the crayfish. On the other hand, 2-deoxyecdysone was not converted into 3-dehydroecdysone, which is a major product of the Y-organs in vivo and in vitro. 3-Dehydroecdysone was however metabolized into 20-hydroxyecdysone via ecdysone after release from Y-organs in vivo[11]. In addition, our experiments revealed that 3-oxidase was not involved in ecdysteroidogenesis by the Y-organs in vitro, and that C-2 hydroxylase in the Y-organs required at least 3-hydroxyl group as a substrate in *P. clarkii*. Recently, Böcking et al. [12] reported that Y-organs could not convert 3-dehydroecdysone to ecdysone and vice versa in *Orconectes limosus*, and that using two distant putative precursors (5β-ketodiol and 5β-diketol), 3β-reductase would work at an early stage of the biosynthesis. Our results are in agreement with those findings; the biosynthetic pathways of ecdysteroids in *P. clarkii* may also branch at an early stage. We have demonstrated that molecular oxygen is incorporated into ecdysone in both Y-organs and PGs. These results provide evidence that C-2 hydroxylation is mediated by a monooxygenase. According to a previous report on *Locusta migratoria*[13], this hydroxylation step is efficiently inhibited by piperonyl butoxide (I_{50} = 10 μM) but at a less efficient rate by SKF-525A, in agreement with our results with PGs from *B. mori*. Consequently, the transformation, which is inhibited by xanthurenic acid, is mediated by a cytochrome P-450 monooxygenase. It appears that xanthurenic acid regulates the hydroxylation step by binding to the active site of the P-450-monooxygenase as previously reported[5].

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