TITLE:

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AUTHOR(S):

Saito, Junki; Kimura, Ryota; Kaieda, Yuya; Nishida, Ritsuo; Ono, Hajime

CITATION:

Saito, Junki ...[et al]. Characterization of candidate intermediates in the Black Box of the ecdysone biosynthetic pathway in *Drosophila melanogaster*: Evaluation of molting activities on ecdysteroid-defective larvae. *Journal of Insect Physiology* 2016, 93-94: 94-104

ISSUE DATE:

2016-10-01

URL:

<http://hdl.handle.net/2433/231026>

RIGHT:

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**Characterization of candidate intermediates in the Black Box of the
ecdysone biosynthetic pathway in *Drosophila melanogaster*: evaluation
of molting activities on ecdysteroid-defective larvae**

Junki Saito¹, Ryota Kimura¹, Yuya Kaieda, Ritsuo Nishida, Hajime Ono*

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto
606-8502, Japan

*Corresponding author. Phone: +81-75-753-6310, Fax: + 81-75-753-6312.

E-mail address: onoono@kais.kyoto-u.ac.jp (H. Ono)

Postal address: Division of Applied Life Sciences, Graduate School of Agriculture,
Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto, 606-8502, Japan

¹ These authors contributed equally to this work.

23 **ABSTRACT**

24

25 The biosynthetic pathway of the insect steroid hormone ecdysone remains the “Black
26 Box” wherein the characteristic ecdysteroid skeleton is built. 7-Dehydrocholesterol (7dC)
27 is the precursor of uncharacterized intermediates in the Black Box. The oxidation step at
28 C-3 has been hypothesized during conversion from 7dC to 3-oxo-2,22,25-
29 trideoxyecdysone, yet 3-dehydroecdysone is undetectable in some insect species.
30 Therefore, we first confirmed that the oxidation at C-3 occurs in the fruitfly, *Drosophila*
31 *melanogaster* using deuterium-labelled cholesterol. We next investigated the molting
32 activities of candidate intermediates, including oxidative products of 7dC, by feeding-
33 rescue experiments for *Drosophila* larvae in which an expression level of a biosynthetic
34 enzyme was knocked down by the RNAi technique. We found that the administration of
35 cholesta-4,7-dien-3-one (3-oxo- $\Delta^{4,7}$ C) could overcome the molting arrest of ecdysteroid-
36 defective larvae in which the expression level of *neverland* was reduced. However,
37 feeding 3-oxo- $\Delta^{4,7}$ C to larvae in which the expression levels of *shroud* and *cyp6t3* were
38 reduced inhibited molting at the first instar stage, suggesting that this steroid could be
39 converted into an ecdysteroid-antagonist in loss of function studies of these biosynthetic
40 enzymes. Administration of the highly conjugated cholesta-4,6,8(14)-trien-3-one,
41 oxidized from 3-oxo- $\Delta^{4,7}$ C, did not trigger molting of ecdysteroid-defective larvae. These
42 results suggest that an oxidative product derived from 7dC is converted into ecdysteroids
43 without the formation of this stable conjugated compound. We further found that the
44 14 α -hydroxyl moiety of Δ^4 -steroids is required to overcome the molting arrest of larvae
45 in loss of function studies of *Neverland*, *Shroud*, *CYP6T3* or *Spookier*, suggesting that

46 oxidation at C-14 is indispensable for conversion of these Δ^4 -steroids into ecdysteroids
47 via 5β -reduction.
48
49
50
51 **Keywords:** Ecdysteroidogenesis; Ecdysone; Black Box; 7-Dehydrocholesterol; Cholesta-
52 4,7-dien-3-one (3-oxo- $\Delta^{4,7}$ C); *Drosophila melanogaster*
53
54 **Abbreviations:** 7dC, 7-dehydrocholesterol; 3-oxo-7dC, cholesta-5,7-dien-3-one; 3-oxo-
55 $\Delta^{4,7}$ C, cholesta-4,7-dien-3-one; 3-oxo- $\Delta^{4,6,8(14)}$ C, cholesta-4,6,8(14)-trien-3-one; 3-oxo-
56 5β - Δ^7 C, 5β -cholest-7-en-3-one; $\Delta^{4,7}$ C-3,6-dione, cholesta-4,7-diene-3,6-dione; Δ^4 -
57 diketol, 14α -hydroxy-cholesta-4,7-diene-3,6-dione; Δ^4 -ketodiol, 3β , 14α -dihydroxy-
58 cholesta-4,7-dien-6-one; ketol, 3β -hydroxy- 5β -cholest-7-en-6-one; diketol, 14α -hydroxy-
59 5β -cholest-7-ene-3,6-dione, 3-oxo-2,22,25-trideoxyecdysone; ketodiol, 3β , 14α -
60 dihydroxy- 5β -cholest-7-en-6-one, 2,22,25-trideoxyecdysone; 3DE, 3-dehydroecdysone;
61 E, ecdysone; 20E, 20-hydroxyecdysone; nvd, neverland; sro, shroud; spo, spook; spok,
62 spookier; PG, prothoracic gland
63

64 1. Introduction

65

66 Steroid hormones regulate many aspects of developmental and physiological
67 processes in higher organisms. Ecdysozoan animals are good models to study endocrine
68 control of developmental process, because their developmental transitions, including
69 molting, metamorphosis and diapause, are primarily regulated by their steroid hormones
70 (Niwa and Niwa, 2014; Wollam and Antebi, 2011). The steroid hormones of insects and
71 nematodes are ecdysteroids and dafachronic acids (DAs), respectively, both of which are
72 biosynthesized from dietary cholesterol (Gilbert and Warren, 2005; Iga and Kataoka,
73 2012; Motola et al., 2006). In insects, ecdysone (E) is biosynthesized in the prothoracic
74 glands and then secreted into the haemolymph during postembryonic development. The
75 released E is then hydroxylated to the principal molting hormone, 20-hydroxyecdysone
76 (20E), in peripheral tissues such as fat body. This conversion from cholesterol involves
77 the formation of the characteristic ecdysteroid skeleton, the structure of which includes a
78 *cis* junction of rings A and B, a 7-en-6-one chromophore, and a *trans* junction of rings C
79 and D with a 14 α -hydroxyl moiety. This synthetic process has been characterized as
80 involving a critical rate-limiting step or steps, the so-called “Black-Box”, during the more
81 than 50 years since the determination of chemical structure of E (Lafont et al., 2012;
82 Warren et al., 2009). In contrast, the biosynthetic pathway of the bile acid-like steroid
83 hormones, DAs, in the nematode, *Caenorhabditis elegans* has been elucidated.

84 The first step in the ecdysteroid biosynthetic pathway is the conversion of
85 cholesterol into 7-dehydrocholesterol (7dC), catalyzed by a Rieske oxygenase, Neverland
86 (Nvd) (Yoshiyama-Yanagawa et al., 2011; Yoshiyama et al., 2006). The analogous

process has also been reported in the DAs biosynthesis of *C. elegans*, in which the conversion of cholesterol into 7dC is catalyzed by a homolog of Nvd, i.e. DAF-36 (Rottiers et al., 2006; Wollam et al., 2011). While the biosynthetic pathway from 7dC to DAs has been unveiled in the nematode, the uncharacterized steps from 7dC to 14 α -hydroxy-5 β -cholest-7-ene-3,6-dione (the diketol) which help to build the ecdysteroid skeleton, have been called the Black Box in insects. During DAs biosynthesis, oxidation at C-3 in the early step, from lathosterol to lathosterone, is catalyzed by a short chain dehydrogenase, DHS-16, in *C. elegans* (Wollam et al., 2012). It should be noted that the homologous enzymes, Shroud (Sro) and Non-molting glossy (Nm-g), likely function in a reaction step of the Black Box in the fruitfly *Drosophila melanogaster* and the silkworm *Bombyx mori*, respectively (Niwa et al., 2010). In addition, Spook (Spo) and its paralog Spookier (Spok) have been thought to be the rate-limiting enzyme in the Black Box (Namiki et al., 2005; Ono et al., 2006; Rewitz et al., 2009). Furthermore, CYP6T3 has been identified as an enzyme which likely plays a role in the Black Box in *D. melanogaster* (Ou et al., 2011).

The experiments using radiolabeled cholesterols have shown that the 3 α -H of cholesterol is eliminated during E biosynthesis in the locust, *Schistocerca gregaria*, suggesting the involvement of 3-oxo-steroids in the Black Box (Davies et al., 1981). These results are consistent with the hypothesis that 3-dehydrogenation of 7dC is a first reaction in the Black Box (Gilbert et al., 2002). While the hypothesized initial product, cholesta-5,7-dien-3-one (3-oxo-7dC), from 7dC by the oxidation at C-3 is very unstable, a protected substrate of 3-oxo-7dC as a photosensitive ketal has been successfully converted into ecdysteroid conjugates and precursors of E after deprotection by

110 irradiation with long-wave UV-light in *D. melanogaster* and the tobacco hornworm
111 *Manduca sexta*, respectively (Warren et al., 2009). This result strongly supports the
112 possibility of the unstable 3-oxo-7dC as the first product of 7dC in the Black Box.

113 The 4 β -H of cholesterol is eliminated during the biosynthesis of E in the blow-
114 fly *Calliphora erythrocephala* and *S. gregaria* (Davies et al., 1981; Lockley et al., 1975),
115 suggesting the presence of a 3-oxo- Δ^4 intermediate in the Black Box. An ecdysteroid-like
116 steroid, 14 α -hydroxy-cholesta-4,7-diene-3,6-dione (Δ^4 -diketol), is the possible
117 intermediate, because this compound is converted into ecdysteroids in crustacean Y-
118 organs and showed molting activity in ecdysteroid-defective *Drosophila* larvae (Blais et
119 al., 1996; Ono et al., 2012). If the Δ^4 -diketol is an intermediate in ecdysteroid
120 biosynthesis, the 5 β -reduction which leads to the diketol is the final step of the Black
121 Box. However, there is no direct evidence in this reduction reaction to generate the A/B
122 *cis* ring junction. The diketol is converted into 3-dehydroecdysone (3DE) or E with the
123 additional reduction step at C-3 by successive hydroxylations (Bocking et al., 1993; Dolle
124 et al., 1991). The sequential hydroxylation reactions are catalyzed by three cytochrome
125 P450 monooxygenases, i.e. Phantom, Disembodied and Shadow (Chavez et al., 2000;
126 Niwa et al., 2004; Warren et al., 2002; Warren et al., 2004). While E is secreted from the
127 PG in many insects, 3DE or both of 3DE and E are released from the PG in several other
128 insect species (Kiriishi et al., 1990). Following secretion of 3DE or/and E from the PG
129 into haemolymph, subsequent reduction of 3DE to E occurs which then is rapidly
130 converted into 20E by the final hydroxylation step catalyzed by the cytochrome P450
131 monooxygenase, Shade (Petryk et al., 2003).

Although the involvement of 3-oxo-steroids has been postulated as mentioned above, no 3-oxo-steroid biosynthesized in the early steps has been detected in the PG of insects. Furthermore, exclusive secretion of E from the PG was observed in *B. mori* and the flesh fly *Sarcophaga peregrina* (Kiriishi et al., 1990), which leaves open the possibility that E is biosynthesized without oxidation at the 3-position. In this study, we first confirmed that the oxidation of dietary cholesterol at C-3 takes place in *D. melanogaster*, using deuterium-labeled cholesterol. Because the oxidative product of 7dC has been considered as an intermediate in the Black Box, we investigated if 3-oxo-steroids prepared from 7dC and their analogs can overcome developmental arrest of ecdysteroids-defective larvae in which the expression of ecdysteroid biosynthetic enzymes is knocked down in the PG. We further focused on the requirement of the 14 α -hydroxyl moiety of Δ^4 -steroids to trigger molting of the ecdysteroids-defective larvae.

2. Materials and methods

2.1. *Drosophila* strains

UAS-nvd-IR; *UAS-nvd-IR/Tm6B*, *Tb* was generated from *UAS-nvd-IR* strains (Yoshiyama et al., 2006). *UAS-sro-IR*; *UAS-sro-IR/Tm3*, *Ser*, *GFP* was generated from *UAS-sro-IR* strains (#50112 and #16388) obtained from the VDRC Stock Center. *UAS-Cyp6t3-IR*; *UAS-Cyp6t3-IR* was generated from *UAS-Cyp6t3-IR* strains (#30896 and #109703) obtained from the VDRC Stock Center. *UAS-spok-IR*; *UAS-spok-IR* was described in Ono et al., 2012. *Phm-Gal4-22/TM3*, *Sb*, *GFP* was obtained from M.B. O'Connor. Oregon-R was obtained from the Drosophila Genetic Resource Center at

Kyoto Institute of Technology. Flies were cultured on a standard cornmeal/yeast extract/dextrose medium.

2.2. Chemicals

3-Oxo- $\Delta^{4,7}\text{C}$, 3-oxo- $\Delta^{4,6,8(14)}\text{C}$, $\Delta^{4,7}\text{C}$ -3,6-dione, 3-oxo-5 β - $\Delta^7\text{C}$ and Δ^4 -diketol were synthesized from 7dC as described previously (Dolle et al., 1991; Kinnear et al., 1979). Δ^4 -Ketodiol was synthesized from Δ^4 -diketol by reduction using NaBH_4 , and then purified by reverse-phase HPLC. (3 α ,25,26,26,26,27,27,27- ^2H)-Cholesterol (cholesterol- d_8) was synthesized from (25,26,26,26,27,27,27- ^2H)-cholesterol (cholesterol- d_7) by Jones oxidation at 3-position and following reduction using NaBD_4 , and then purified by reverse-phase HPLC. Cholesterol- d_7 was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). E and 7dC were purchased from Sigma-Aldrich (St. Louis, MO, USA). Each compound was purified by reverse-phase HPLC before experiments. Chemical structures of steroids are shown in Fig. 1.

2.3. Sample preparation and analyses of ecdysteroids using an LC/MS/MS system

Drosophila larvae (Oregon-R) were fed with yeast paste containing cholesterol or deuterium-labeled cholesterol. For preparation of yeast paste, 50 mg of dry yeast was thoroughly mixed with 90 μl of water and 10 μl of 10 mM steroid in ethanol. Twenty of developed pupae were thoroughly washed and homogenized in 1 ml ethanol by hand with a plastic pestle. The homogenate was pretreated using Sep-Pak C18 plus cartridge (Waters, MA, USA) as described previously (Ono et al., 2012) and dissolved in 50 μl of ethanol.

Ecdysteroids were analyzed in an LC/MS/MS system, as described previously (Hikiba et al., 2013). Briefly, ecdysteroids were separated by reverse-phase HPLC using a PEGASIL ODS column (3 μ m, 2 x 50 mm, Senshu-pak, Senshu-kagaku, Tokyo, Japan) with gradient elution of acetonitrile/water, and quantified with the QTRAP5500 MS/MS system (AB SCIEX, Foster City, CA, USA) using MRM mode.

2.4. Feeding-rescue experiments

Feeding-rescue experiments were done as described previously (Ono et al., 2012). Briefly, L1 larvae were fed with yeast paste which was prepared from 50 mg of dry yeast mixed with 90 μ l of water and 10 μ l of 10 mM steroid in ethanol or else only solvent. Supplied steroids were recovered from yeast paste after incubation for 24 hrs at 29°C under dark conditions, as described previously with minor modification. Briefly, each sample was extracted with ethanol and the eluate was applied to ODS column (Cosmosil 140C140-OPN, Nacalai Tesque, Inc., Kyoto, Japan) or Sep-Pak Plus C18 cartridge (Waters, MA, USA), which was then eluted with 5 ml of water and subsequently with 5 ml of methanol or ethanol, respectively. The eluate with methanol containing 3-oxo- $\Delta^{4,7}$ C or 3-oxo- $\Delta^{4,6,8(14)}$ C was chromatographed on a reverse-phase HPLC column (YMC-Pack CN-A-523, 10 X 250mm, YMC Co., Ltd., Kyoto, Japan) at a flow rate of 2.5 ml/min with 76% methanol in water or 72% methanol in water, respectively. The eluate with ethanol containing $\Delta^{4,7}$ C-3,6-dione or Δ^4 -ketodiol was chromatographed on a reverse-phase HPLC column (YMC-Pack CN, 6 X 150mm, YMC Co., Ltd., Kyoto, Japan) at a flow rate of 1.5 ml/min with 63% methanol in water or at a flow rate of 1.0 ml/min with 60% methanol in water, respectively (Fig. S1). For

generation of RNAi-mediated knockdown larvae, *phm-Gal4-22/TM3, Sb, GFP* was crossed to *UAS-transgene* containing inverted repeat of a gene coding for an ecdysteroid biosynthetic enzyme. RNAi-mediated knockdown larvae without balancer marker were picked after hatching and reared at 29°C in the experiments. Reduction of transcriptional level of a target gene was confirmed by quantitative RT-PCR (Fig. S2).

2.5. Quantitative RT-PCR

Purification of total RNA, reverse transcription and quantitative RT-PCR were performed as described previously (Ono et al., 2012). Transcription levels were normalized with RpL23 transcription levels in the same samples. The primers used for quantitative RT-PCR are listed in Table S1.

3. Results

3.1. Oxidation of 3 β -alcohol at the 3-position is essential for ecdysteroidogenesis

To clarify the formation of 3-oxo-steroids during ecdysteroidogenesis, we fed food containing cholesterol-*d8* possessing deuterium at the 3-position or cholesterol-*d7* without deuterium at the 3-position to *Drosophila* larvae. Whole bodies of the developed pupae were extracted in order to analyze for 20E using the LC/MS/MS system, as 20E was contained as a major ecdysteroid in the pupal extracts. If the 3 α -²H atom is eliminated during ecdysteroidogenesis, cholesterol-*d8* is converted into (26,26,26,27,27,27-²H)-ecdysone (ecdysone-*d6*) in the PG, and then into 20E-*d6* in peripheral tissues. On the other hand, if the 3 α -²H atom is retained, cholesterol-*d8* is

converted into (3α , 26,26,26,27,27,27- ^2H)-ecdysone (ecdysone-*d*7) in the PG, and then into 20E-*d*7 by subsequent oxidation. To clarify the products derived from the labelled cholesterol, fragments with a loss of two water molecules derived from 20E-*d*6 and 20E-*d*7 were analyzed by multiple reaction monitoring (MRM) (Table 1).

While we detected 20E in cholesterol-fed animals, we detected 20E-*d*6 and 20E-*d*7, but not 20E in both the cholesterol-*d*7- and cholesterol-*d*8-fed animals, presumably due to exclusive incorporation of labeled cholesterol into animals by feeding substrate at high concentration (Table 2). We calculated the peak area ratio of fragment derived from 20E-*d*7 to that from 20E-*d*6 (20E-*d*7/20E-*d*6). We assumed that the detection of 20E-*d*7 as a minor product in the cholesterol-*d*7-fed animals, as the ratio of 20E-*d*7/20E-*d*6 ratio was 0.284, was derived from isotope effects. To confirm it, isotope effect of 20E was examined by comparison of the relative peak area of 20E and 20E-*d*1 derived from standard 20E. We observed their peak areas derived from 20E and 20E-*d*1, 1.66×10^4 and 5.75×10^5 , respectively. Hence, the ratio of 20E-*d*1/20E, 0.289, was similar to that of 20E-*d*7/20E-*d*6, 0.284, indicating that the detection of 20E-*d*7 was derived from isotope effects.

We next analyzed products of the cholesterol-*d*8-fed animals. If the 3α - ^2H atom is retained during ecdysteroidogenesis, the ratio must be more than 1 due to production of 20E-*d*7 in the cholesterol-*d*8-fed animals, but this is not the case. The ratio in the cholesterol-*d*8-fed animals was significantly larger than that in the cholesterol-*d*7-fed animals. The excess deuterium is likely due to reintroduction of deuterium during subsequent reductive process as reported in previous study (Davies et al., 1981). Indeed, the observation of approximately 30% excess percentage of deuterium in the cholesterol-

d8-fed animals relative to the cholesterol-*d7*-fed animals is agreement with the results in the locust, *S. gregaria*, where the retention of up to 30% of tritium by administration of (3 α -³H)-cholesterol in larvae was observed (Davies et al., 1981). Therefore, we concluded that 3 α -²H of cholesterol-*d8* was eliminated during ecdysteroid biosynthesis in *D. melanogaster* as shown in the locust.

3.2 Administration of 3-oxo- $\Delta^{4,7}$ C, but not 3-oxo- $\Delta^{4,6,8(14)}$ C, triggered molting of *nvd*-RNAi larvae

As Nvd catalyzes the first reaction step, from cholesterol to 7dC, in ecdysone biosynthesis (Yoshiyama-Yanagawa et al., 2011; Yoshiyama et al., 2006), we assumed that feeding-rescue experiments for *nvd*-RNAi larvae could be applicable to clarify components in the Black Box. We focused on oxidative products of 7dC and their related compounds (Fig. 2A). 7dC is oxidized into 3-oxo-7dC but this product is immediately isomerized into 3-oxo- $\Delta^{4,7}$ C (Warren et al., 2009). 3-Oxo- $\Delta^{4,7}$ C is stable but further converted into the highly conjugated 3-oxo- $\Delta^{4,6,8(14)}$ C by further oxidation. We first tested if these 3-oxo-steroids can overcome the molting arrest of *nvd*-RNAi larvae. More than 80% of larvae died at L1 stage without steroidal supplement and all of the remaining larvae died at L1/L2 or L2 stage by reduction of *nvd* expression (Table 3, Fig. 2B). We confirmed that the developmental arrest was rescued by administration of E and 7dC, but not by administration of C, (Table 3, Table S2), as shown in the previous paper (Yoshiyama et al., 2006). When 3-oxo- $\Delta^{4,7}$ C was applied to *nvd*-RNAi larvae, approximately 65% of them attained L1 molting, the percentage of which is significantly higher than that of unsupplied larvae. Approximately 10% of 3-oxo- $\Delta^{4,7}$ C-fed larvae

further attained L2 molting and two of them pupariated but died at this stage. In contrast, feeding of 3-oxo- $\Delta^{4,6,8(14)}\text{C}$ did not rescue the developmental arrest, as more than 90% of L1 larvae died without molting. We further tested if developmental arrest could be rescued by administration of a 3-oxo-steroid, 5 β -cholest-7-en-3-one (3-oxo-5 β - $\Delta^7\text{C}$), which has a *cis* junction of rings A and B (Fig. 2A), but this compound also did not overcome the arrest of molting. Taken together, the developmental arrest of *nvd*-RNAi larvae was rescued by administration of 3-oxo- $\Delta^{4,7}\text{C}$, but not of another 3-oxo-steroids tested, suggesting that 3-oxo- $\Delta^{4,7}\text{C}$ was metabolized into ecdysteroid or ecdysteroid-like compound possessing molting activity.

3.3 Administration of 3-oxo- $\Delta^{4,7}\text{C}$ inhibited molting of *sro*- and *Cyp6t3*-RNAi larvae

Because *Sro* likely catalyzes an early step in the Black Box, we anticipated that administration of 3-oxo- $\Delta^{4,7}\text{C}$ could rescue the molting arrest of *sro*-RNAi larvae. We found that approximately 70 % of larvae containing two copies of *UAS-sro-IR* and one copy of *phm-GAL4* died at L1 stage without molting and the remaining larvae mostly died at L1/L2 or L2 stage by reduction of *sro* expression (Table 4, Fig. 2C). We confirmed that administration of C did not rescue the developmental arrest (Table S3). The developmental arrest was rescued by feeding E, as 80 % of them developed to L3 stage and two animals further attained pupariation (Table 4). In contrast, all *sro*-RNAi larvae unexpectedly died at L1 stage by feeding food containing 3-oxo- $\Delta^{4,7}\text{C}$ at 1 mM final concentration. Next, we examined the lethal phase of larvae fed with 3-oxo- $\Delta^{4,7}\text{C}$ at different concentrations. Significant difference in L1 lethality from control unsupplied-larvae was observed by feeding 3-oxo- $\Delta^{4,7}\text{C}$ at 1mM, but not at 0.1mM (Table 5, χ^2 test,

$p < 0.05$). Administration of 3-oxo- $\Delta^{4,6,8(14)}\text{C}$ did not rescue the developmental arrest but rather inhibited larval molting, as the percentage of L1 molting was significantly reduced than that of unsupplied control larvae (Fig. 2C).

Next, we focused on CYP6T3 which is another component in the Black Box. By RNAi-mediated knockdown of *Cyp6t3* in the PG, all larvae molted to L2 stage, but almost all of them did not develop to L3 stage. Instead the animals died at L2 stage or developed into L2 prepupae in which L2 larvae underwent precocious metamorphosis without L2-L3 transition (Table 6), as reported previously (Ou et al., 2011). We confirmed that administration of C did not rescue the developmental arrest to L3 or prepupal stage (Table S4). The developmental arrest was rescued by administration of E, as all larvae developed to L3 stage (Table 6, Fig. 2D). When *Cyp6t3*-RNAi larvae were fed with food containing 3-oxo- $\Delta^{4,7}\text{C}$, more than 20% of them died at L1 stage, indicating that this compound inhibited L1 molting as shown in *sro*-RNAi larvae. We also found that more than 20% of *Cyp6t3*-RNAi larvae died at L1 stage by administration of 3-oxo- $\Delta^{4,6,8(14)}\text{C}$, as shown by administration of 3-oxo- $\Delta^{4,7}\text{C}$.

3.4 Administration of neither 3-oxo- $\Delta^{4,7}\text{C}$ nor 3-oxo- $\Delta^{4,6,8(14)}\text{C}$ triggered molting of *spok*-RNAi larvae

We further tested if administration of 3-oxo- $\Delta^{4,7}\text{C}$ or 3-oxo- $\Delta^{4,6,8(14)}\text{C}$ can rescue the developmental arrest of *spok*-RNAi larvae. All of *spok*-RNAi larvae died at L1 stage, when they fed with food containing either 3-oxo- $\Delta^{4,7}\text{C}$ or 3-oxo- $\Delta^{4,6,8(14)}\text{C}$ as well as food without steroidal supplement (Fig. 2E).

315 3.5 14α -Hydroxyl moiety of Δ^4 -steroids is required to rescue the developmental arrest 316 of ecdysteroid-defective larvae

317 Because the 14α -hydroxyl moiety is characteristic of the structure of
318 ecdysteroids, we tested if this function is essential to rescue developmental arrest of
319 ecdysteroid-defective larvae. We confirmed that administration of the proposed 3-oxo- Δ^4
320 intermediate with the 14α -hydroxyl moiety, i.e. the Δ^4 -diketol (Fig. 3A), triggered
321 molting of ecdysteroid-defective larvae. As expected, administration of the Δ^4 -diketol
322 rescued developmental arrest of *nvd*- and *sro*-RNAi larvae, as approximately 40% and
323 65% of Δ^4 -diketol-fed larvae attained L1 molting, respectively, the percentages of which
324 are significantly higher than those of unsupplied larvae. (Table 3, 4, Fig. 3B, C). The
325 attainment of L1 molting by administration of the Δ^4 -diketol was also confirmed in *spok*-
326 RNAi larvae as reported previously (Table 7, Fig. 3E) (Ono et al., 2012). When *Cyp6t3*-
327 RNAi larvae were fed with food containing the Δ^4 -diketol, no L2 prepupa was observed.
328 Instead approximately 46% of tested animals showed molting behavior to the L3 stage
329 (Table 6, Fig. 3D). These results indicate that developmental arrest of *Cyp6t3*-RNAi
330 animals at the L2 or L2 prepupal stage was rescued by administration of the Δ^4 -diketol.

331 Next, we focused on 3β -hydroxy- Δ^4 -steroids with a 14α -hydroxyl moiety, Δ^4 -
332 ketodiol, and 3-oxo- Δ^4 -steroid without 14α -hydroxyl moiety, $\Delta^{4,7}$ C-3,6-dione (Fig. 3A).
333 Administration of the Δ^4 -ketodiol rescued the developmental arrest of *nvd*-, *sro*-, *Cyp6t3*-
334 and *spok*-RNAi larvae as shown in that of the Δ^4 -diketol (Fig. 3B-E). The significant
335 elevations of percentage of L1 or L2 molting, relative to unsupplied larvae, were
336 observed by feeding the Δ^4 -ketodiol in *nvd*-, *sro*-, *spok*- or *Cyp6t3*-RNAi larvae,
337 respectively. By administration of $\Delta^{4,7}$ C-3,6-dione, all *nvd*-, *sro*- and *spok*-RNAi larvae

died at L1 stage. It should be noted that not all *nvd*- and *sro*-RNAi larvae fed with unsupplied food died at L1 stage, indicating that administration of $\Delta^{4,7}\text{C}$ -3,6-dione inhibited larval molting in both *nvd*- and *sro*-RNAi larvae as shown in *sro*-RNAi larvae fed with 3-oxo- $\Delta^{4,7}\text{C}$. We further examined the lethal phase of *sro*-RNAi larvae fed with $\Delta^{4,7}\text{C}$ -3,6-dione at different concentration (Table 5). Significant difference in L1 lethality from control unsupplied-larvae was observed at 0.1mM, but not at 0.01mM (χ^2 test, $p < 0.01$). We also found that 20% of *Cyp6t3*-RNAi larvae died at L1 stage by administration of $\Delta^{4,7}\text{C}$ -3,6-dione, while all *Cyp6t3*-RNAi larvae developed to L2 stage fed with unsupplied food (Table 6), indicating that the administration of $\Delta^{4,7}\text{C}$ -3,6-dione also inhibited larval molting of *Cyp6t3*-RNAi larvae. While the application of $\Delta^{4,7}\text{C}$ -3,6-dione inhibited larval molting of ecdysteroids-defective larvae, this compound did not show any growth defect, including molting inhibition and reduced body size in wild-type animals (Fig. S3).

While both the Δ^4 -diketol and Δ^4 -ketodiol rescued the developmental arrest of ecdysteroid-defective larvae, their effects on RNAi-mediated knockdown animals were considerably different between the four target genes, *nvd*, *sro*, *cyp6t3* and *spok*. By application of the Δ^4 -diketol or Δ^4 -ketodiol, approximately 35% of *nvd*-RNAi L1 larvae successfully molted to L2 stage. In contrast, a substantial proportion of *sro*- and *spok*-RNAi larvae, from 29% to 63% of animals, died during molting from L1 to L2 stage, while a small proportion of them, from 2% to 15% of animals, successfully molted to L2 stage. For *Cyp6t3*-RNAi animals, approximately half of animals died during molting from L2 to L3 stage by feeding the Δ^4 -diketol or Δ^4 -ketodiol, while only one larva fed with the Δ^4 -diketol successfully molted to L3 stage.

361

362 4. Discussion

363

364 Rapid reactions of unstable intermediates in the small ecdysteroid-producing
365 organ have been considered an obstacle to the elucidation of the reaction mechanism that
366 builds the ecdysteroid skeleton in the Black Box. In fact, even the true intermediates,
367 such as the ketodiol and ketotriol, have not been detected from the PG of *B. mori* by
368 LC/MS/MS analysis in a previous study (Hikiba et al., 2013). It is interesting to note that
369 7dC has been detected from the PG of *B. mori*, but not from that of *D. melanogaster*
370 (Enya et al., 2014), suggesting that 7dC is metabolized into the following intermediate at
371 different reaction rates between these species. We have failed to detect candidate
372 intermediates, such as the Δ^4 -diketol and diketol, as well as oxidative products of 7dC
373 including 3-oxo- $\Delta^{4,7}$ C from the *Drosophila* whole bodies and *Bombyx* PG by LC/MS/MS
374 analysis (data not shown). We, therefore, have taken strategy using feeding-rescue
375 experiments for ecdysteroid-defective larvae in which the expression of an ecdysteroid
376 biosynthetic enzyme is knocked down in order to characterize conceivable intermediates.

377 The previous study (Davies et al., 1981) and this study have shown that
378 oxidation at C-3 unambiguously occurs during ecdysteroidogenesis in different insect
379 species, i.e. the locust and fruitfly. Thus, 3DE is the product of ecdysteroid biosynthesis,
380 but facile 3β -OH reduction must occur before it can be secreted or else earlier in the
381 synthesis. The possible candidate, 3-oxo-7dC, which is a product of the oxidation of 7dC
382 at C-3, is very unstable. However, it is interesting to note that 3-oxo-7dC can be protected
383 as a photosensitive ketal, and then released by irradiation with UV-light. By taking

384 advantage of this reaction, conversions of 3-oxo-7dC into ecdysteroid conjugates in adult
 385 *Drosophila* and into precursors of E in the PG of *M. sexta* have been confirmed (Warren
 386 et al., 2009). Because 3-oxo-7dC is rapidly isomerized into 3-oxo- $\Delta^{4,7}$ C and then
 387 oxygenated into the highly conjugated 3-oxo- $\Delta^{4,6,8(14)}$ C, we anticipated that these
 388 compounds could be candidate intermediates in the Black Box. It is plausible that 3-oxo-
 389 $\Delta^{4,6,8(14)}$ C can be converted into the diketol by concerted addition of oxygen or peroxide
 390 across C-6 and C-14 (Gilbert et al., 2002), however, application of this compound did not
 391 show any molting activity in ecdysteroid-defective larvae i.e. *nvd*-, *sro*- and *Cyt6t3*-RNAi
 392 larvae, but instead partially inhibited L1 molting of *sro*- and *Cyt6t3*-RNAi larvae. On the
 393 other hand, administration of 3-oxo- $\Delta^{4,7}$ C rescued the molting arrest of *nvd*-RNAi larvae,
 394 as 65% of them developed to L2 stage and some of them further developed to L3 or
 395 prepupal stage. These results could provide a convincing hypothesis that this compound
 396 is the bona fide intermediate in the Black Box in which concomitant oxidation of 3-oxo-
 397 $\Delta^{4,7}$ C at C-6 and C-14 catalyzed by Spo/Spok leads to the Δ^4 -diketol. In this hypothesis,
 398 Sro/Nm-g catalyze the oxidation from 7dC to 3-oxo- $\Delta^{4,7}$ C, as its homolog DHS-16
 399 catalyzes oxidation of lathosterol at C-3 in *C. elegans* (Wollam et al., 2012). However,
 400 administration of 3-oxo- $\Delta^{4,7}$ C unexpectedly did not rescue the developmental arrest of
 401 *sro*-RNAi larvae, but rather markedly inhibited their molting. To explain this
 402 phenomenon, two possibilities could exist. One possibility is that 3-oxo- $\Delta^{4,7}$ C cannot be
 403 converted into any metabolite in loss of function of Sro, and so then inhibits molting of
 404 L1 larvae by itself. To examine this possibility, we fed 3-oxo- $\Delta^{4,7}$ C to wild-type larvae,
 405 but did not see any growth defect such as elevation of lethality or reduction of body size
 406 (Fig. S3), indicating 3-oxo- $\Delta^{4,7}$ C itself does not have a detrimental effect on larval

development. Alternatively, a critical reaction to otherwise build an ecdysteroid structure would not proceed in loss of function of *Sro*, so that 3-oxo- $\Delta^{4,7}\text{C}$ is instead converted into an undesirable by-product which has a negative effect on production of ecdysteroids in the PG or antagonistically inhibits 20E signaling to trigger molting (Fig. 4). A previous study has suggested that an unknown metabolite which can be recognized by an anti-ecdysone antibody was accumulated in *sro* mutant embryos of *D. melanogaster* (Chavez et al., 2000; Niwa et al., 2010). Because such metabolite possibly has an ecdysteroid-like structure, it could compete with ecdysteroids as an antagonist.

To clarify whether 3-oxo- $\Delta^{4,7}\text{C}$ is the true intermediate in the Black Box, we have further examined if deuterium-labeled 3-oxo- $\Delta^{4,7}\text{C}$ can be converted into deuterium-labeled ecdysteroids in whole bodies of *Drosophila*, however no labeled- intermediate, E nor 20E has been detected as products derived from the administrated 3-oxo- $\Delta^{4,7}\text{C}$ (data not shown). If 3-oxo- $\Delta^{4,7}\text{C}$ is not an intermediate in the Black Box, it was likely converted into an ecdysteroid-like compound which has a potential activity to trigger molting in *nvd*-RNAi larvae (Fig. 4). Previous studies have shown that the terminal hydroxylations, C-25, C-22 and C-2, do not have strict substrate specificities, i.e. the 5 α -ketodiol and 5 β -cholest-7-ene-3 β ,6 α ,14 α -triol were hydroxylated at C-25, C-22 and C-2 in the PG as shown in 5 β -ketodiol, but neither of them were converted into E by isomerization at C-5 or oxidation at C-6, respectively (Bollenbacher et al., 1977; Schwab and Hetru, 1991). Hence, 3-oxo- $\Delta^{4,7}\text{C}$ might be converted to a ketodiol-like compound by enzymes in the Black Box, and then hydroxylated to an uncharacterized compound possessing a molting activity as shown in 14-deoxyecdysone derived from 3 β -hydroxy-5 β -cholest-7-en-6-one (ketol) (Bollenbacher et al., 1977; Ono et al., 2012). Regardless of

whether 3-oxo- $\Delta^{4,7}\text{C}$ is the intermediate or not, identification of any metabolite derived from 3-oxo- $\Delta^{4,7}\text{C}$ could provide a critical clue to understand reactions in the Black Box.

Although the 14 α -hydroxylation of a precursor of ecdysteroids is essential to the ecdysteroid skeleton, it is not clear which step in the Black Box is involved in this hydroxylation. Considering that the ketol is not hydroxylated at C-14 in both in *vivo* and in *vitro* (Bollenbacher et al., 1977; Haag et al., 1987), the 14 α -hydroxylation must precede 5 β -reduction and/or formation of a 7-en-6-one chromophore. We showed that the developmental arrest of *nvd*-, *sro*- and *spok*-RNAi larvae were rescued by feeding the Δ^4 -diketol and Δ^4 -ketodiol, suggesting that oxidation at C-14 is indispensable for conversion of these Δ^4 -steroids into ecdysteroids via 5 β -reduction regardless of 3-dehydroxy- or 3 β -hydroxy moiety (Fig. 5). In contrast, both steroids lacking 14 α -hydroxyl moiety, 3-oxo- $\Delta^{4,7}\text{C}$ and $\Delta^{4,7}\text{C}$ -3,6-dione, inhibited molting of *sro*-RNAi larvae, and $\Delta^{4,7}\text{C}$ -3,6-dione further inhibited molting of *nvd*-RNAi. It should also be noted that application of these steroids lacking 14 α -hydroxyl moiety, 3-oxo- $\Delta^{4,7}\text{C}$ and $\Delta^{4,7}\text{C}$ -3,6-dione partially inhibited L1 molting of *Cyp6t3*-RNAi larvae. These results suggest that Δ^4 -steroids lacking 14 α -hydroxyl moiety could be converted into a detrimental by-product inhibiting larval molting in ecdysteroids-defective larvae. Although the idea that concomitant oxidation of 3-oxo- $\Delta^{4,6,8(14)}\text{C}$ at C-6 and C-14 leads to the Δ^4 -diketol is plausible (Gilbert et al., 2002), our trials of feeding-rescue experiments did not show consistent results as discussed above. Rather, application of 3-oxo- $\Delta^{4,6,8(14)}\text{C}$ inhibited L1 molting of *sro*- and *Cyp6t3*-RNAi larvae, suggesting that formation of 3-oxo- $\Delta^{4,6,8(14)}\text{C}$ must be circumvented in order to build the ecdysteroid skeleton. One speculation is that, to this end, 14 α -hydroxylation at an early step in the Black Box is required to build the 5 β -7-en-6-one

structure without formation of 3-oxo- $\Delta^{4,6,8(14)}\text{C}$, nevertheless any positive evidence for this is lacking.

If this highly conjugated 3-oxo-steroid is not included in the Black Box, in which step does 14 α -hydroxylation occur? One possibility is that 3-oxo- $\Delta^{4,7}\text{C}$ is concomitantly oxidized at C-6 and C-14, if this steroid is the true intermediate. Another possibility is that oxidation of 3-oxo-7dC at C-14 takes place before the formation of 3-oxo- $\Delta^{4,7}\text{C}$. Otherwise, 14 α -hydroxylation precedes oxidation at C-3, as 7dC first oxygenated at C-14, and then other oxidation reactions including C-3 oxidation follow. In both cases, it is possible that 14 α -hydroxylation plays a role to prevent the formation of undesirable by-products causing molting inhibition.

We observed the different effects of the same steroids on larval development among the different RNAi-treated animals. For example, approximately 40% of *nvd*-RNAi larvae were rescued by administration of the 14 α -hydroxy-steroids, the Δ^4 -diketol and Δ^4 -ketodiol, but a substantial proportion of *sro*-, *spok*- and *Cyp6t3*- RNAi larvae fed with these steroids failed to progress to the next stage, as they died during molting from L1 to L2 or from L2 to L3 stage. It is conceivable that these results could give clues to speculate a sequential position of uncharacterized enzymes, but comparison of the extent of development among the different RNAi-treated animals is not necessarily appropriate, because phenotypes of RNAi-treated animals were different from each other likely due to the different extent of loss of function of targeted genes. While the application of the Δ^4 -diketol and Δ^4 -ketodiol significantly rescued the developmental arrest of *nvd*-RNAi animals, a substantial proportion of *sro*-, *spok*- and *Cyp6t3*-RNAi larvae failed to progress to the next stage, as they died during molting from L1 to L2 or from L2 to L3

stage. One possible explanation for the incomplete developmental progression is that larvae could not produce enough E from the ingested Δ^4 -diketol and Δ^4 -ketodiol in the PG to complete a series of molting process due to complex pharmacokinetics underlying the oral administration. In these cases, low ecdysteroid titer could induce the formation of second larval mouth hook and new cuticle, but not complete molting process. Indeed, the developmental arrest during molting was observed in *nvd*- and *spok*- RNAi larvae even by E feeding regimen, suggesting that ecdysteroid titers were not elevated at an appropriate time in these larvae. Another explanation is that the ingested Δ^4 -diketol and Δ^4 -ketodiol were not converted into E in the PG, but rather into ecdysteroid-like compounds having competence to bind to ecdysone receptor. It should be pointed out that ecdysteroid titer must increase, then decrease to attain the normal molting cascade including the formation of new cuticle, the digestion of old cuticle, and the completion of molting. Escape from the old cuticle is initiated by the release of the peptide hormones triggered by the decline of 20E titer to a basal level (Truman, 2005). Therefore, larvae could not complete molting without the decline of 20E titer, as an ecdysteroid mimic was applied to lepidopteran larvae where ecdysteroid agonist activity was persisted in larval tissues (Dhadialla et al., 1998). There is a possibility that the ingested Δ^4 -diketol and Δ^4 -ketodiol were converted into ecdysteroid agonists not to be degraded in *sro*-, *spok*- and *Cyp6t3*-RNAi larval tissues, thereby, larvae fed with these steroids could not complete molting. Besides, it should be pointed out that a small number of ecdysteroid-defective larvae successfully progress to the next stage by feeding the Δ^4 -diketol and Δ^4 -ketodiol, suggesting that a possibility of a production of a small amounts of E in the PG.

498 Now, insect genome engineering using the CRISPR/Cas9 system is rapidly
499 prevailing (Daimon et al., 2014), therefore, knockout of a gene encoding for an
500 ecdysteroid biosynthetic enzyme in large size insects such as *B. mori* will give us a clue
501 to elucidate the Black Box, i.e. identification of an accumulated metabolite caused by loss
502 of function of a target enzyme will unveil the biosynthetic pathway of the Black Box.

503

504 **Acknowledgments**

505 We are grateful to M. Iga and H. Kataoka for LC/MS/MS analyses. We are
506 grateful to J.T. Warren for critical reading. We thank R. Niwa, M.B. O'Connor, the
507 Genetic Resource Center at Kyoto Institute of Technology and the Vienna Drosophila
508 RNAi Centre for fly stocks. This work was supported in part by JSPS KAKENHI Grant
509 Number 26450466.

510

511 Figure legends

512

513 **Fig. 1.** Chemical structures of steroids.

514

515 **Fig. 2.** Molting activities of oxidative products derived from 7dC in the ecdysteroid-
516 defective larvae. (A) Synthetic pathway of oxidative products of 7dC. 3-Oxo- $\Delta^{4,7}$ C and 3-
517 oxo- $\Delta^{4,6,8(14)}$ C were synthesized from 7dC by successive oxidation. 3-Oxo- 5β - Δ^7 C was
518 synthesized from 3-oxo- $\Delta^{4,7}$ C by reduction reaction. (B) Percentage of *nvd*-RNAi larvae
519 attained L1 molting. (C) Percentage of *sro*-RNAi larvae attained L1 molting. (D)
520 Percentage of *Cyp6t3*-RNAi larvae attained L2 molting. (E) Percentage of *spok*-RNAi
521 larvae attained L1 molting. Asterisk indicates a statistically significant difference of L1 or
522 L2 molting between steroid-supplied and unsupplied animals. χ^2 test: $**p < 0.01$; $*p <$
523 0.05.

524

525 **Fig. 3.** Molting activities of Δ^4 -diketol analogs in the ecdysteroid-defective larvae. (A)
526 Synthetic pathway of Δ^4 -diketol analogs. $\Delta^{4,7}$ C-3,6-dione and Δ^4 -diketol were
527 synthesized from 3-oxo- $\Delta^{4,7}$ C by successive oxidation. The Δ^4 -ketodiol was synthesized
528 from the Δ^4 -diketol by reduction reaction. (B) Percentage of *nvd*-RNAi larvae attained L1
529 molting. (C) Percentage of *sro*-RNAi larvae attained L1 molting. (D) Percentage of
530 *Cyp6t3*-RNAi larvae attained L2 molting. (E) Percentage of *spok*-RNAi larvae attained
531 L1 molting. Asterisk indicates a statistically significant difference of L1 or L2 molting
532 between steroid-supplied and unsupplied animals. χ^2 test: $**p < 0.01$; $*p < 0.05$.

533

534 Fig. 4. Possible metabolic pathways of 3-oxo- $\Delta^{4,7}$ C in *Drosophila*. The oxidative product
535 of 7dC, 3-oxo- $\Delta^{4,7}$ C, could be converted into ecdysteroid or ecdysteroid-like compound
536 via a step catalyzed by Sro. Loss of function of Sro leads production of ecdysteroid-
537 antagonist from 3-oxo- $\Delta^{4,7}$ C due to an unfavorable metabolic conversion.

538

539 Fig. 5. Possible metabolic pathways of Δ^4 -steroids in *Drosophila*. As the developmental
540 arrest of ecdysteroid-defective larvae were rescued by feeding the 14 α -hydroxy-steroids,
541 oxidation at C-14 is indispensable for conversion of Δ^4 -steroids into ecdysteroids
542 regardless of 3-dehydro- or 3 β -hydroxy moiety. In contrast, Δ^4 -steroids lacking 14 α -
543 hydroxyl moiety could be converted into a detrimental by-product inhibiting larval
544 molting in ecdysteroids-defective larvae.

545

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676
677

Table 1

Parameters for MRM analysis of steroids.

20E	Q1/Q3	Retention time in LC (min)
20E	481.3/445.3	1.69
20E- <i>d6</i>	487.3/451.3	1.67
20E- <i>d7</i>	488.3/452.3	1.67

Table 2

Analysis of 20E extracted from *Drosophila* pupae.

Substrate	Peak area ($\times 10^4$, mean $\pm$ SD, $n = 3$)			Ratio (mean $\pm$ SD, $n = 3$)
	20E	20E- <i>d6</i>	20E- <i>d7</i>	20E- <i>d7</i> /20E- <i>d6</i>
Cholesterol	19.9 $\pm$ 5.89	ND	ND	
Cholesterol- <i>d7</i>	ND	14.1 $\pm$ 9.37	3.82 $\pm$ 2.24	0.284 $\pm$ 0.0255
Cholesterol- <i>d8</i>	ND	8.18 $\pm$ 3.76	3.05 $\pm$ 1.31	0.378 $\pm$ 0.0152

ND: not detected

Table 3

Lethal phase of *nvd*-RNAi animals fed with steroid or none.

Steroid	Percentage of animals which died at each stage					
	Lethal stage					
	L1	L1/L2	L2	L2/L3	L3	Purepupa
EtOH	83 (92)	9 (10)	8 (9)	0 (0)	0 (0)	0 (0)
7dC	16 (3)	0 (0)	47 (9)	11 (2)	21 (4)	5 (1)
3-Oxo- $\Delta^{4,7}$ C	35 (19)	4 (2)	53 (29)	4 (2)	4 (2)	2 (1)
3-Oxo- $\Delta^{4,6,8(14)}$ C	94 (45)	4 (2)	2 (1)	0 (0)	0 (0)	0 (0)
3-Oxo-5 β -7-ene	84 (26)	13 (4)	3 (1)	0 (0)	0 (0)	0 (0)
Δ^4 -Diketol	61 (19)	0 (0)	35 (11)	0 (0)	3 (1)	0 (0)
Δ^4 -Ketodiol	33 (19)	30 (17)	37 (21)	0 (0)	0 (0)	0 (0)
$\Delta^{4,7}$ C-3,6-dione	100 (68)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ecdysone	6 (2)	0 (0)	16 (5)	16 (5)	48 (15)	13 (4)

Each number in parentheses refers to the number of animals which died at each stage.

L1/L2 and L2/L3 refer to larvae that died while molting from L1 to L2 and from L2 to L3, respectively.

Table 4

Lethal phase of *sro*-RNAi animals fed with steroid or none.

Steroid	Percentage of animals which died at each stage					
	Lethal stage					
	L1	L1/L2	L2	L2/L3	L3	Purepupa
EtOH	67 (59)	26 (23)	7 (6)	0 (0)	0 (0)	0 (0)
3-Oxo- $\Delta^{4,7}$ C	100 (95)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3-Oxo- $\Delta^{4,6,8(14)}$ C	88 (37)	10 (4)	2 (1)	0 (0)	0 (0)	0 (0)
Δ^4 -Diketol	34 (14)	63 (26)	2 (1)	0 (0)	0 (0)	0 (0)
Δ^4 -Ketodiol	43 (13)	47 (14)	10 (3)	0 (0)	0 (0)	0 (0)
$\Delta^{4,7}$ C-3,6-dione	100 (53)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ecdysone	0 (0)	0 (0)	20 (5)	0 (0)	72 (18)	8 (2)

Each number in parentheses refers to the number of animals which died at each stage.

L1/L2 and L2/L3 refer to larvae that died while molting from L1 to L2 and from L2 to L3, respectively.

Table 5

Lethal phase of *sro*-RNAi animals fed with 3-oxo- $\Delta^{4,7}\text{C}$ at different concentration.

Steroid	Concentration	Percentage of animals which died at each stage					
		Lethal stage					
		L1	L1/L2	L2	L2/L3	L3	Purepupa
3-oxo- $\Delta^{4,7}\text{C}$	1 mM	99 (180)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)
	0.1 mM	83 (156)	12 (22)	5 (9)	0 (0)	0 (0)	0 (0)
	0.01 mM	86 (76)	11 (10)	2 (2)	0 (0)	0 (0)	0 (0)
	0 mM	75 (128)	12 (21)	13 (22)	0 (0)	0 (0)	0 (0)
$\Delta^{4,7}\text{C}$ -3,6-dione	1 mM	97 (36)	3 (1)	0 (0)	0 (0)	0 (0)	0 (0)
	0.1 mM	82 (28)	18 (6)	0 (0)	0 (0)	0 (0)	0 (0)
	0.01 mM	72 (28)	28 (11)	0 (0)	0 (0)	0 (0)	0 (0)
	0 mM	67 (38)	28 (16)	5 (3)	0 (0)	0 (0)	0 (0)

Each number in parentheses refers to the number of animals which died at each stage.

L1/L2 refers to larvae that died while molting from L1 to L2.

Table 6

Lethal phase of *Cyp6t3*-RNAi animals fed with steroid or none.

Steroid	Percentage of animals which died at each stage						
	Lethal stage						
	L1	L1/L2	L2	L2 prepupa	L2/L3	L3	Purepupa
EtOH	0 (0)	0 (0)	60 (24)	38 (15)	0 (0)	3 (1)	0 (0)
3-Oxo- $\Delta^{4,7}$ C	21 (11)	4 (2)	60 (21)	8 (14)	6 (3)	2 (1)	0 (0)
3-Oxo- $\Delta^{4,6,8(14)}$ C	23 (9)	0 (0)	77 (30)	0 (0)	0 (0)	0 (0)	0 (0)
Δ^4 -Diketol	3 (2)	0 (0)	48 (29)	0 (0)	47 (28)	2 (1)	0 (0)
Δ^4 -Ketodiol	2 (1)	0 (0)	44 (24)	0 (0)	55 (30)	0 (0)	0 (0)
$\Delta^{4,7}$ C-3,6-dione	20 (12)	0 (0)	63 (38)	17 (10)	0 (0)	0 (0)	0 (0)
Ecdysone	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (22)	0 (0)

Each number in parentheses refers to the number of animals which died at each stage.

L1/L2 and L2/L3 refer to larvae that died while molting from L1 to L2 and from L2 to L3, respectively.

Table 7

Lethal phase of *spok*-RNAi animals fed with steroid or none.

Steroid	Percentage of animals which died at each stage					
	Lethal stage					
	L1	L1/L2	L2	L2/L3	L3	Purepupa
EtOH	100 (84)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3-Oxo- $\Delta^{4,7}$ C	100 (117)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3-Oxo- $\Delta^{4,6,8(14)}$ C	100 (65)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Δ^4 -Diketol	56 (33)	37 (22)	7 (4)	0 (0)	0 (0)	0 (0)
Δ^4 -Ketodiol	57 (34)	28 (17)	15 (9)	0 (0)	0 (0)	0 (0)
$\Delta^{4,7}$ C-3,6-dione	100 (60)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ecdysone	8 (4)	0 (0)	14 (7)	2 (1)	65 (33)	12 (6)

Each number in parentheses refers to the number of animals which died at each stage.

L1/L2 refers to larvae that died while molting from L1 to L2.

7dC

3-Oxo-7dC

3-Oxo- $\Delta^{4,7}$ C

3-Oxo- $\Delta^{4,6,8(14)}$ C

3-Oxo-5 β - Δ^7 C

$\Delta^{4,7}$ C-3,6-dione

Δ^4 -Diketol

Δ^4 -Ketodiol

Ketol

Diketol

Ketodiol

3DE

E

20E

Fig. 2

A

3-Oxo- $\Delta^{4,7}C$

$\Delta^{4,7}C$ -3,6-dione

Δ^4 -Diketol

Δ^4 -Ketodiol

B

C

D

E

Fig. 3

Fig. 4

Supplemental Table

Table S1. The primers used for quantitative RT-PCR

gene	forward primer	reverse primer
<i>nvd</i>	5'-ACCTCCCCCTTATCCAAATG-3'	5'-AGCAACGCTTCCACCAATAC-3'
<i>sro</i>	5'-ATGAGCGGCAGTCAACTTCT-3'	5'-CAGGAAATCACGGTCATGTG-3'
<i>Cyp6t3</i>	5'- ACGCTACCGCTGGCTAAGTA-3'	5'-ACTGGCACATTCTTCCCAAC-3'
<i>spok</i>	5'-TATCTCTTGGGCACACTCGCTG-3'	5'-GCCGAGCTAAATTTCTCCGCTT-3'
<i>rpL23</i>	5'-GCTCAGGAAGAAGGTCATGC-3'	5'-GGCTATAGAGCTTGCATTGGA

Table S2

Lethal phase of *nvd*-RNAi animals fed with steroid or none.

Steroid	Percentage of animals which died at each stage					
	Lethal stage					
	L1	L1/L2	L2	L2/L3	L3	Purepupa
EtOH	64 (28)	30 (13)	5 (2)	2 (1)	0 (0)	0 (0)
C	65 (35)	30 (16)	6 (3)	0 (0)	0 (0)	0 (0)
Ecdysone	0 (0)	0 (0)	0 (0)	6 (1)	94 (16)	0 (0)

Each number in parentheses refers to the number of animals which died at each stage.

Table S3

Lethal phase of *sro*-RNAi animals fed with steroid or none.

Steroid	Percentage of animals which died at each stage					
	Lethal stage					
	L1	L1/L2	L2	L2/L3	L3	Purepupa
EtOH	33 (14)	62 (26)	5 (2)	0 (0)	0 (0)	0 (0)
C	46 (16)	54 (19)	0 (0)	0 (0)	0 (0)	0 (0)
Ecdysone	0 (0)	0 (0)	5 (1)	5 (1)	89 (17)	0 (0)

Each number in parentheses refers to the number of animals which died at each stage.

Table S4

Lethal phase of *Cyp6t3*-RNAi animals fed with steroid or none.

Steroid	Percentage of animals which died at each stage						
	Lethal stage						
	L1	L1/L2	L2	L2 prepupa	L2/L3	L3	Purepupa
EtOH	19 (10)	10 (5)	21 (11)	35 (18)	15 (8)	0 (0)	0 (0)
C	7 (3)	0 (0)	46 (19)	22 (9)	24 (10)	0 (0)	0 (0)
Ecdysone	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	47 (7)	53 (8)

Each number in parentheses refers to the number of animals which died at each stage.

1 **Supplementary figure legends**

2

3 **Fig. S1.** HPLC analyses of the stability of tested steroids, 3-oxo- $\Delta^{4,7}$ C (A),
4 3-oxo- $\Delta^{4,6,8(14)}$ C (B), $\Delta^{4,7}$ C-3,6-dione (C) and Δ^4 -ketodiol (D). Approximately, 22, 43, 35
5 or 22% of the original substrate was recovered from yeast paste in (A), (B), (C) and (D),
6 respectively. UV absorption at 237, 348, 280 or 264 nm was monitored for (A), (B), (C)
7 and (D), respectively.

8

9 **Fig. S2.** Transcriptional levels of target genes in RNAi-mediated L1 larvae. (A)

10 Transcriptional levels of *nvd* (A), *sro* (B), *Cyp6t3* (C) and *sro* (D) in each targeted RNAi
11 larvae.

12

13 **Fig. S3.** Pupal length of animals fed with steroid or none. Each number in parentheses
14 refers to the number of animals. All animals normally developed to pupal stage without
15 any molting defects. No significant difference was observed in animals fed with steroid
16 or none (Student's *t*-test: $p > 0.5$).

17

18

Fig. S1

A

B

C

D