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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

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ABSTRACT

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

oxidation at C-14 is indispensable for conversion of these Δ^4-steroids into ecdysteroids via 5β-reduction.

***Keywords*:** Ecdysteroidogenesis; Ecdysone; Black Box; 7-Dehydrocholesterol; Cholesta-4,7-dien-3-one (3-oxo-$\Delta^{4,7}$C); *Drosophila melanogaster*

***Abbreviations*:** 7dC, 7-dehydrocholesterol; 3-oxo-7dC, cholesta-5,7-dien-3-one; 3-oxo-$\Delta^{4,7}$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-5β-Δ^7C, 5β-cholest-7-en-3-one; $\Delta^{4,7}$C-3,6-dione, cholesta-4,7-diene-3,6-dione; Δ^4-diketol, 14α-hydroxy-cholesta-4,7-diene-3,6-dione; Δ^4-ketodiol, 3β,14α-dihydroxy-cholesta-4,7-dien-6-one; ketol, 3β-hydroxy-5β-cholest-7-en-6-one; diketol, 14α-hydroxy-5β-cholest-7-ene-3,6-dione, 3-oxo-2,22,25-trideoxyecdysone; ketodiol, 3β,14α-dihydroxy-5β-cholest-7-en-6-one, 2,22,25-trideoxyecdysone; 3DE, 3-dehydroecdysone; E, ecdysone; 20E, 20-hydroxyecdysone; nvd, neverland; sro, shroud; spo, spook; spok, spookier; PG, prothoracic gland

1. Introduction

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

The first step in the ecdysteroid biosynthetic pathway is the conversion of cholesterol into 7-dehydrocholesterol (7dC), catalyzed by a Rieske oxygenase, Neverland (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 14α-hydroxy-5β-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 3α-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

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

The 4β-H of cholesterol is eliminated during the biosynthesis of E in the blow-fly *Calliphora erythrocephala* and *S. gregaria* (Davies et al., 1981; Lockley et al., 1975), suggesting the presence of a 3-oxo-Δ^4 intermediate in the Black Box. An ecdysteroid-like steroid, 14α-hydroxy-cholesta-4,7-diene-3,6-dione (Δ^4-diketol), is the possible intermediate, because this compound is converted into ecdysteroids in crustacean Y-organs and showed molting activity in ecdysteroid-defective *Drosophila* larvae (Blais et al., 1996; Ono et al., 2012). If the Δ^4-diketol is an intermediate in ecdysteroid biosynthesis, the 5β-reduction which leads to the diketol is the final step of the Black Box. However, there is no direct evidence in this reduction reaction to generate the A/B *cis* ring junction. The diketol is converted into 3-dehydroecdysone (3DE) or E with the additional reduction step at C-3 by successive hydroxylations (Bocking et al., 1993; Dolle et al., 1991). The sequential hydroxylation reactions are catalyzed by three cytochrome P450 monooxygenases, i.e. Phantom, Disembodied and Shadow (Chavez et al., 2000; Niwa et al., 2004; Warren et al., 2002; Warren et al., 2004). While E is secreted from the PG in many insects, 3DE or both of 3DE and E are released from the PG in several other insect species (Kiriishi et al., 1990). Following secretion of 3DE or/and E from the PG into haemolymph, subsequent reduction of 3DE to E occurs which then is rapidly converted into 20E by the final hydroxylation step catalyzed by the cytochrome P450 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 14α-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-$\Delta^{4,7}$C, 3-oxo-$\Delta^{4,6,8(14)}$C**,** $\Delta^{4,7}$C-3,6-dione, 3-oxo-5β-Δ^{7}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. (3α,25,26,26,26,27,27,27-^{2}H)-Cholesterol (cholesterol-*d8*) was synthesized from (25,26,26,26,27,27,27-^{2}H)-cholesterol (cholesterol-*d7*) by Jones oxidation at 3-position and following reduction using $NaBD_4$, and then purified by reverse-phase HPLC. Cholesterol-*d7* 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.

177 Ecdysteroids were analyzed in an LC/MS/MS system, as described previously
178 (Hikiba et al., 2013). Briefly, ecdysteroids were separated by reverse-phase HPLC using
179 a PEGASIL ODS column (3 μm, 2 x 50 mm, Senshu-pak, Senshu-kagaku, Tokyo, Japan)
180 with gradient elution of acetonitrile/water, and quantified with the QTRAP5500 MS/MS
181 system (AB SCIEX, Foster City, CA, USA) using MRM mode.
182
183 ***2.4. Feeding-rescue experiments***
184 Feeding-rescue experiments were done as described previously (Ono et al.,
185 2012). Briefly, L1 larvae were fed with yeast paste which was prepared from 50 mg of
186 dry yeast mixed with 90 μl of water and 10 μl of 10 mM steroid in ethanol or else only
187 solvent. Supplied steroids were recovered from yeast paste after incubation for 24 hrs at
188 29°C under dark conditions, as described previously with minor modification. Briefly,
189 each sample was extracted with ethanol and the eluate was applied to ODS column
190 (Cosmosil 140C140-OPN, Nacalai Tesque, Inc., Kyoto, Japan) or Sep-Pak Plus C18
191 cartridge (Waters, MA, USA), which was then eluted with 5 ml of water and
192 subsequently with 5 ml of methanol or ethanol, respectively. The eluate with methanol
193 containing 3-oxo-$\Delta^{4,7}$C or 3-oxo-$\Delta^{4,6,8(14)}$C was chromatographed on a reverse-phase
194 HPLC column (YMC-Pack CN-A-523, 10 X 250mm, YMC Co., Ltd., Kyoto, Japan) at a
195 flow rate of 2.5 ml/min with 76% methanol in water or 72% methanol in water,
196 respectively. The eluate with ethanol containing $\Delta^{4,7}$C-3,6-dione or Δ^{4}-ketodiol was
197 chromatographed on a reverse-phase HPLC column (YMC-Pack CN, 6 X 150mm, YMC
198 Co., Ltd., Kyoto, Japan) at a flow rate of 1.5 ml/min with 63% methanol in water or at a
199 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 3β-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 3α-^{2}H atom is eliminated during ecdysteroidogenesis, cholesterol-*d8* is converted into (26,26,26,27,27,27-^{2}H)-ecdysone (ecdysone-*d6*) in the PG, and then into 20E-*d6* in peripheral tissues. On the other hand, if the 3α-^{2}H atom is retained, cholesterol-*d8* is

converted into (3α, 26,26,26,27,27,27-^{2}H)-ecdysone (ecdysone-*d7*) in the PG, and then into 20E-*d7* by subsequent oxidation. To clarify the products derived from the labelled cholesterol, fragments with a loss of two water molecules derived from 20E-*d6* and 20E-*d7* were analyzed by multiple reaction monitoring (MRM) (Table 1).

While we detected 20E in cholesterol-fed animals, we detected 20E-*d6* and 20E-*d7*, but not 20E in both the cholesterol-*d7*- and cholesterol-*d8*-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-*d7* to that from 20E-*d6* (20E-*d7*/20E-*d6*). We assumed that the detection of 20E-*d7* as a minor product in the cholesterol-*d7*-fed animals, as the ratio of 20E-*d7*/20E-*d6* 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-*d1* derived from standard 20E. We observed their peak areas derived from 20E and 20E-*d1*, 1.66 X 10^{4} and 5.75 X 10^{5}, respectively. Hence, the ratio of 20E-*d1*/20E, 0.289, was similar to that of 20E-*d7*/20E-*d6*, 0.284, indicating that the detection of 20E-*d7* was derived from isotope effects.

We next analyzed products of the cholesterol-*d8*-fed animals. If the 3α-^{2}H atom is retained during ecdysteroidogenesis, the ratio must be more than 1 due to production of 20E-*d7* in the cholesterol-*d8*-fed animals, but this is not the case. The ratio in the cholesterol-*d8*-fed animals was significantly larger than that in the cholesterol-*d7*-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 (3α-^{3}H)-cholesterol in larvae was observed (Davies et al., 1981). Therefore, we concluded that 3α-^{2}H of cholesterol-*d8* was eliminated during ecdysteroid biosynthesis in *D. melanogaster* as shown in the locust.

3.2 Administration of 3-oxo-Δ^{4,7}C, but not 3-oxo-Δ$^{4,6,8(14)}$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-Δ^{4,7}C (Warren et al., 2009). 3-Oxo-Δ^{4,7}C is stable but further converted into the highly conjugated 3-oxo-Δ$^{4,6,8(14)}$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-Δ^{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-Δ^{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-$\Delta^{4,6,8(14)}$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, 5β-cholest-7-en-3-one (3-oxo-5β-Δ^{7}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-$\Delta^{4,7}$C, but not of another 3-oxo-steroids tested, suggesting that 3-oxo-$\Delta^{4,7}$C was metabolized into ecdysteroid or ecdysteroid-like compound possessing molting activity.

3.3 Administration of 3-oxo-$\Delta^{4,7}$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-$\Delta^{4,7}$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-$\Delta^{4,7}$C at 1 mM final concentration. Next, we examined the lethal phase of larvae fed with 3-oxo-$\Delta^{4,7}$C at different concentrations. Significant difference in L1 lethality from control unsupplied-larvae was observed by feeding 3-oxo-$\Delta^{4,7}$C at 1mM, but not at 0.1mM (Table 5, χ^2 test,

292 $p < 0.05$). Administration of 3-oxo-$\Delta^{4,6,8(14)}$C did not rescue the developmental arrest but
293 rather inhibited larval molting, as the percentage of L1 molting was significantly reduced
294 than that of unsupplied control larvae (Fig. 2C).
295 Next, we focused on CYP6T3 which is another component in the Black Box. By
296 RNAi-mediated knockdown of *Cyp6t3* in the PG, all larvae molted to L2 stage, but
297 almost all of them did not develop to L3 stage. Instead the animals died at L2 stage or
298 developed into L2 prepupae in which L2 larvae underwent precocious metamorphosis
299 without L2-L3 transition (Table 6), as reported previously (Ou et al., 2011). We
300 confirmed that administration of C did not rescue the developmental arrest to L3 or
301 prepupal stage (Table S4). The developmental arrest was rescued by administration of E,
302 as all larvae developed to L3 stage (Table 6, Fig. 2D). When *Cyp6t3*-RNAi larvae were
303 fed with food containing 3-oxo-$\Delta^{4,7}$C, more than 20% of them died at L1 stage, indicating
304 that this compound inhibited L1 molting as shown in *sro*-RNAi larvae. We also found
305 that more than 20% of *Cyp6t3*-RNAi larvae died at L1 stage by administration of 3-oxo-
306 $\Delta^{4,6,8(14)}$C, as shown by administration of 3-oxo-$\Delta^{4,7}$C.
307
308 ***3.4 Administration of neither 3-oxo-$\Delta^{4,7}$C nor 3-oxo-$\Delta^{4,6,8(14)}$C triggered molting of***
309 ***spok-RNAi larvae***
310 We further tested if administration of 3-oxo-$\Delta^{4,7}$C or 3-oxo-$\Delta^{4,6,8(14)}$C can rescue
311 the developmental arrest of *spok*-RNAi larvae. All of *spok*-RNAi larvae died at L1 stage,
312 when they fed with food containing either 3-oxo-$\Delta^{4,7}$C or 3-oxo-$\Delta^{4,6,8(14)}$C as well as food
313 without steroidal supplement (Fig. 2E).
314

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

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

Next, we focused on 3β-hydroxy-Δ^4-steroids with a 14α-hydroxyl moiety, Δ^4-ketodiol, and 3-oxo-Δ^4-steroid without 14α-hydroxyl moiety, Δ^{4,7}C-3,6-dione (Fig. 3A). Administration of the Δ^4-ketodiol rescued the developmental arrest of *nvd*-, *sro*-, *Cyp6t3*- and *spok*-RNAi larvae as shown in that of the Δ^4-diketol (Fig. 3B-E). The significant elevations of percentage of L1 or L2 molting, relative to unsupplied larvae, were observed by feeding the Δ^4-ketodiol in *nvd*-, *sro*-, *spok*- or *Cyp6t3*-RNAi larvae, respectively. By administration of Δ^{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 $\Delta^{4,7}$C-3,6-dione inhibited larval molting in both *nvd*- and *sro*-RNAi larvae as shown in *sro*-RNAi larvae fed with 3-oxo-$\Delta^{4,7}$C. We further examined the lethal phase of *sro*-RNAi larvae fed with $\Delta^{4,7}$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 $\Delta^{4,7}$C-3,6-dione, while all *Cyp6t3*-RNAi larvae developed to L2 stage fed with unsupplied food (Table 6), indicating that the administration of $\Delta^{4,7}$C-3,6-dione also inhibited larval molting of *Cyp6t3*-RNAi larvae. While the application of $\Delta^{4,7}$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.

4. Discussion

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

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

advantage of this reaction, conversions of 3-oxo-7dC into ecdysteroid conjugates in adult *Drosophila* and into precursors of E in the PG of *M. sexta* have been confirmed (Warren et al., 2009). Because 3-oxo-7dC is rapidly isomerized into 3-oxo-$\Delta^{4,7}$C and then oxygenated into the highly conjugated 3-oxo-$\Delta^{4,6,8(14)}$C, we anticipated that these compounds could be candidate intermediates in the Black Box. It is plausible that 3-oxo-$\Delta^{4,6,8(14)}$C can be converted into the diketol by concerted addition of oxygen or peroxide across C-6 and C-14 (Gilbert et al., 2002), however, application of this compound did not show any molting activity in ecdysteroid-defective larvae i.e. *nvd*-, *sro*- and *Cyt6t3*-RNAi larvae, but instead partially inhibited L1 molting of *sro*- and *Cyt6t3*-RNAi larvae. On the other hand, administration of 3-oxo-$\Delta^{4,7}$C rescued the molting arrest of *nvd*-RNAi larvae, as 65% of them developed to L2 stage and some of them further developed to L3 or prepupal stage. These results could provide a convincing hypothesis that this compound is the bona fide intermediate in the Black Box in which concomitant oxidation of 3-oxo-$\Delta^{4,7}$C at C-6 and C-14 catalyzed by Spo/Spok leads to the Δ^{4}-diketol. In this hypothesis, Sro/Nm-g catalyze the oxidation from 7dC to 3-oxo-$\Delta^{4,7}$C, as its homolog DHS-16 catalyzes oxidation of lathosterol at C-3 in *C. elegans* (Wollam et al., 2012). However, administration of 3-oxo-$\Delta^{4,7}$C unexpectedly did not rescue the developmental arrest of *sro*-RNAi larvae, but rather markedly inhibited their molting. To explain this phenomenon, two possibilities could exist. One possibility is that 3-oxo-$\Delta^{4,7}$C cannot be converted into any metabolite in loss of function of Sro, and so then inhibits molting of L1 larvae by itself. To examine this possibility, we fed 3-oxo-$\Delta^{4,7}$C to wild-type larvae, but did not see any growth defect such as elevation of lethality or reduction of body size (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-$\Delta^{4,7}$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-$\Delta^{4,7}$C is the true intermediate in the Black Box, we have further examined if deuterium-labeled 3-oxo-$\Delta^{4,7}$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-$\Delta^{4,7}$C (data not shown). If 3-oxo-$\Delta^{4,7}$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 5α-ketodiol and 5β-cholest-7-ene-3β,6α,14α-triol were hydroxylated at C-25, C-22 and C-2 in the PG as shown in 5β-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-$\Delta^{4,7}$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 3β-hydroxy-5β-cholest-7-en-6-one (ketol) (Bollenbacher et al., 1977; Ono et al., 2012). Regardless of

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

Although the 14α-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 14α-hydroxylation must precede 5β-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 5β-reduction regardless of 3-dehyroxy- or 3β-hydroxy moiety (Fig. 5). In contrast, both steroids lacking 14α-hydroxyl moiety, 3-oxo-$\Delta^{4,7}$C and $\Delta^{4,7}$C-3,6-dione, inhibited molting of *sro*-RNAi larvae, and $\Delta^{4,7}$C-3,6-dione further inhibited molting of *nvd*-RNAi. It should also be noted that application of these steroids lacking 14α-hydroxyl moiety, 3-oxo-$\Delta^{4,7}$C and $\Delta^{4,7}$C-3,6-dione partially inhibited L1 molting of *Cyp6t3*-RNAi larvae. These results suggest that Δ^4-steroids lacking 14α-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-$\Delta^{4,6,8(14)}$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-$\Delta^{4,6,8(14)}$C inhibited L1 molting of *sro*- and *Cyp6t3*-RNAi larvae, suggesting that formation of 3-oxo-$\Delta^{4,6,8(14)}$C must be circumvented in order to build the ecdysteroid skeleton. One speculation is that, to this end, 14α-hydroxylation at an early step in the Black Box is required to build the 5β-7-en-6-one

structure without formation of 3-oxo-$\Delta^{4,6,8(14)}$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 14α-hydroxylation occur? One possibility is that 3-oxo-$\Delta^{4,7}$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-$\Delta^{4,7}$C. Otherwise, 14α-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 14α-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 14α-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.

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

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Figure legends

Fig. 1. Chemical structures of steroids.

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

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

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

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

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Table 1

Parameters for MRM analysis of steroids.

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

Table 2

Analysis of 20E extracted from *Drosophila* pupae.

Substrate	Peak area (X 10^4, mean ± SD, *n* = 3)			Ratio (mean ± SD, *n* = 3)
	20E	20E-*d6*	20E-*d7*	20E-*d7*/20E-*d6*
Cholesterol	19.9 ± 5.89	ND	ND	
Cholesterol-*d7*	ND	14.1 ± 9.37	3.82 ± 2.24	0.284 ± 0.0255
Cholesterol-*d8*	ND	8.18 ± 3.76	3.05 ± 1.31	0.378 ± 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-5β-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-$\Delta^{4,7}$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-$\Delta^{4,7}$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)
$\Delta^{4,7}$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

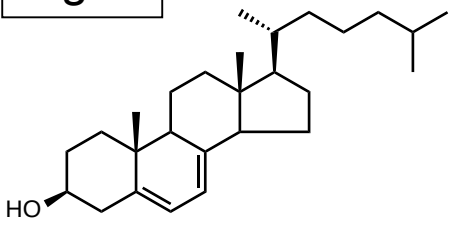
Lethal phase of *spok*-RNAi animals fed with steroid or none.

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)

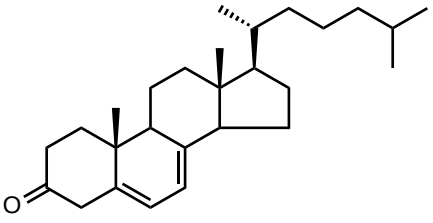
Each number in parentheses refers to the number of animals which died at each stage.

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

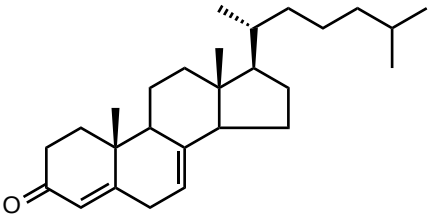
Fig. 1



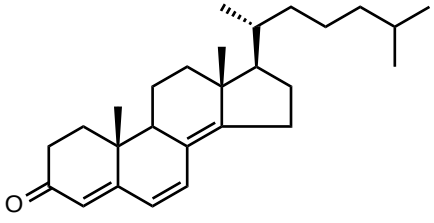
7dC



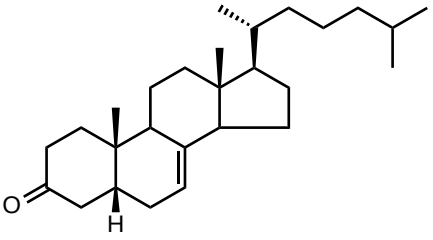
3-Oxo-7dC



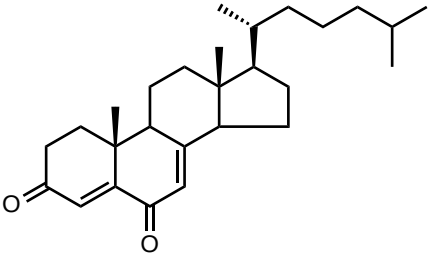
3-Oxo-$\Delta^{4,7}$C



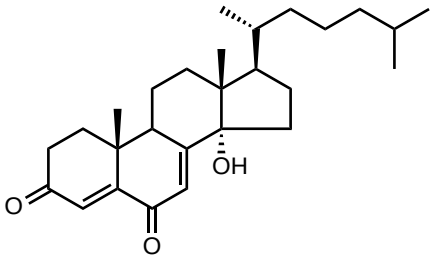
3-Oxo-$\Delta^{4,6,8(14)}$C



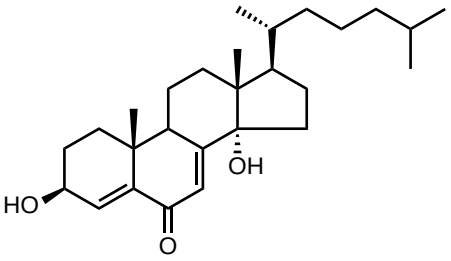
3-Oxo-5β-Δ^{7}C



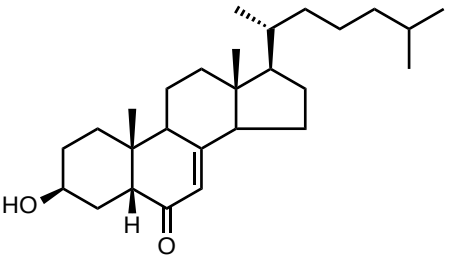
$\Delta^{4,7}$C-3,6-dione



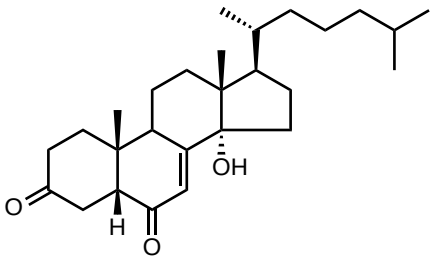
Δ^{4}-Diketol



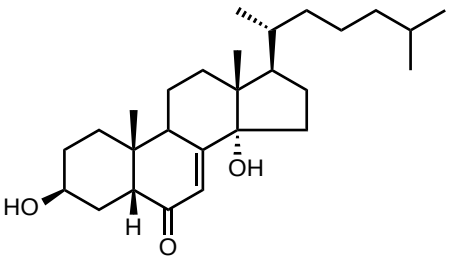
Δ^{4}-Ketodiol



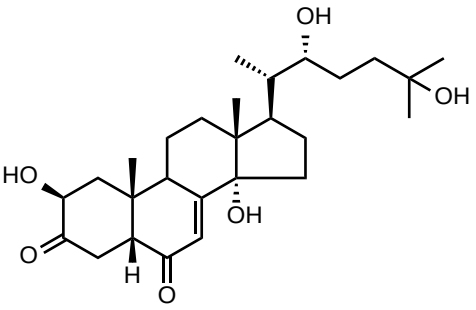
Ketol



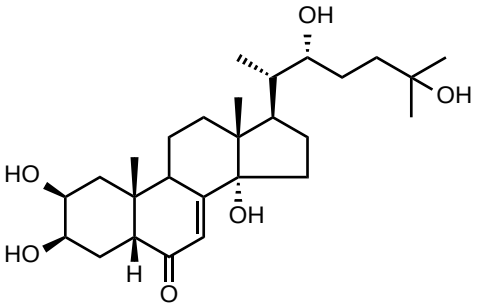
Diketol



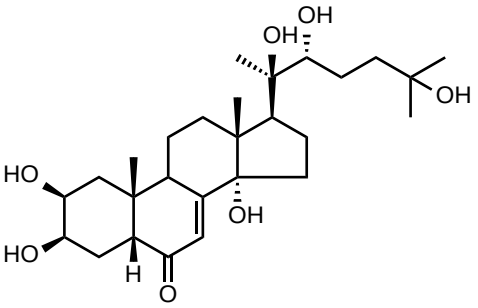
Ketodiol



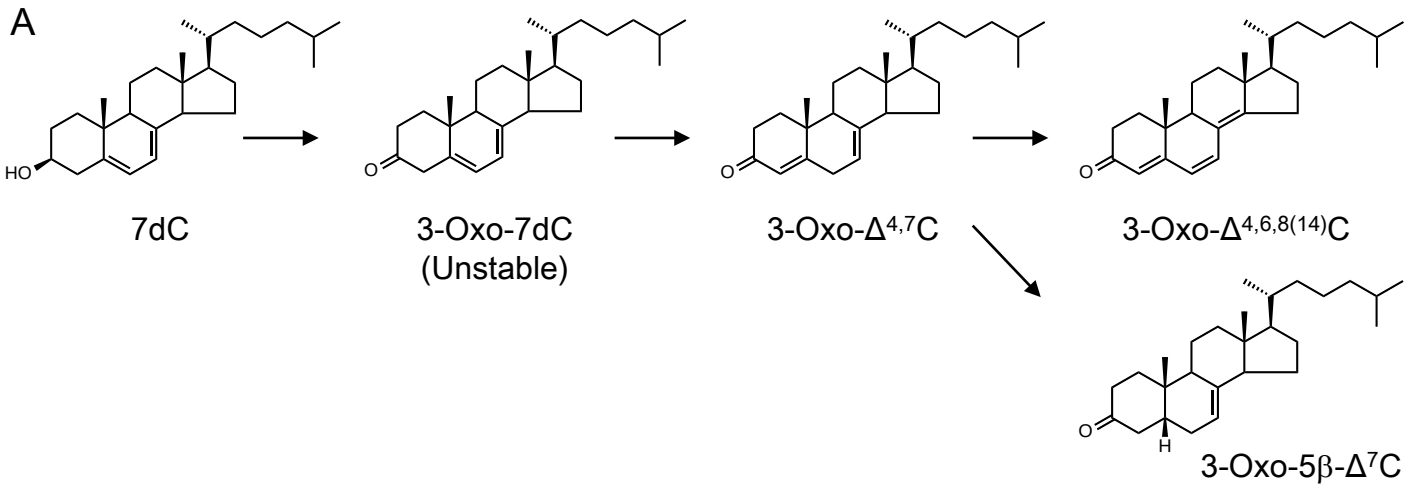
3DE

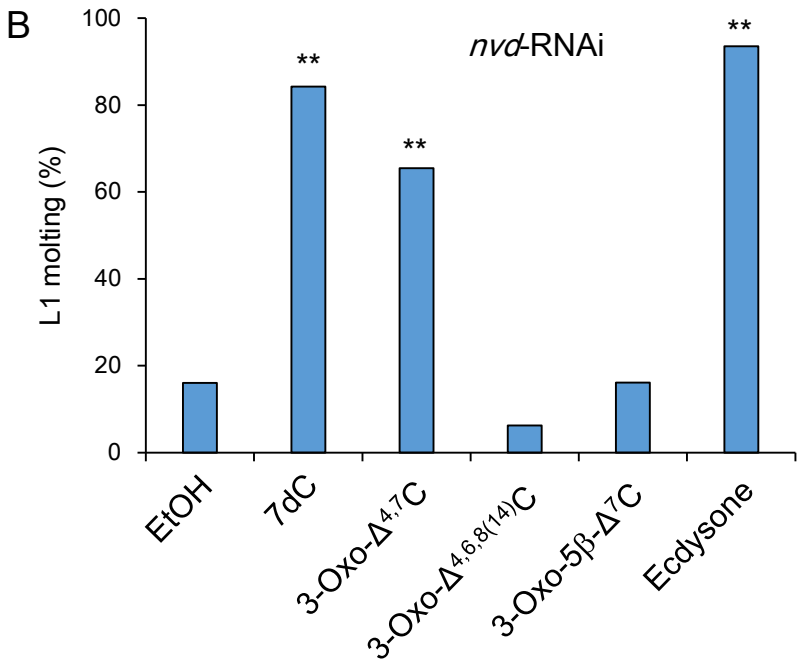


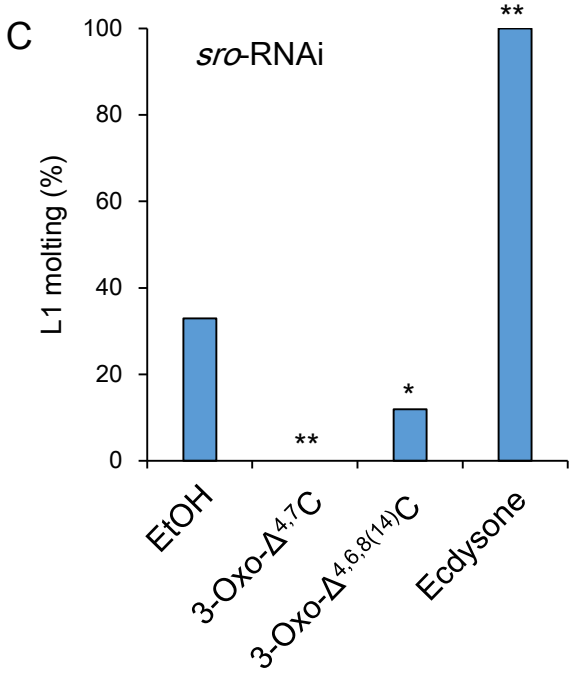
E

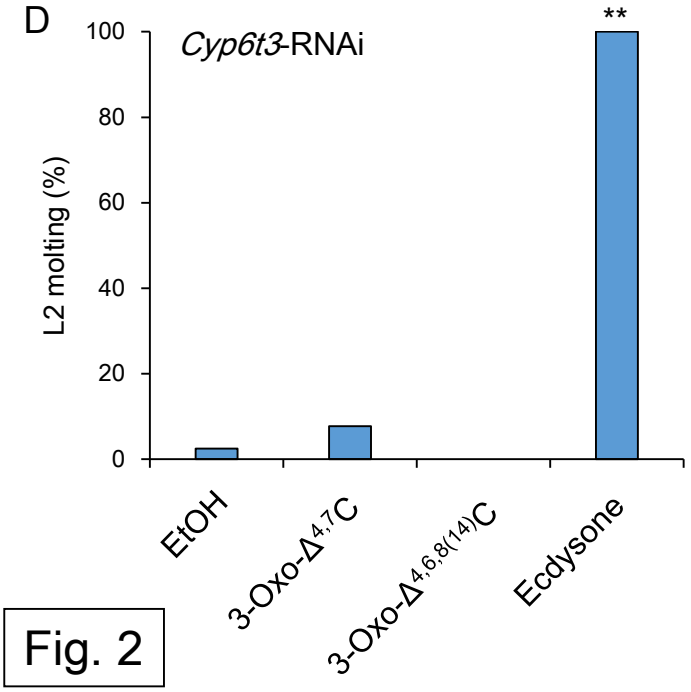


20E


A
HO
O
7dC
3-Oxo-7dC
(Unstable)
3-Oxo-Δ4,7C
3-Oxo-Δ4,6,8(14)C
H
3-Oxo-5β-Δ7C


B
nvd-RNAi
L1 molting (%)
100
80
60
40
20
0
**
**
**
EtOH
7dC
3-Oxo-Δ4,7C
3-Oxo-Δ4,6,8(14)C
3-Oxo-5β-Δ7C
Ecdysone


C
sro-RNAi
L1 molting (%)
100
80
60
40
20
0
**
**
*
EtOH
3-Oxo-Δ4,7C
3-Oxo-Δ4,6,8(14)C
Ecdysone


D
Cyp6t3-RNAi
L2 molting (%)
100
80
60
40
20
0
**
EtOH
3-Oxo-Δ4,7C
3-Oxo-Δ4,6,8(14)C
Ecdysone

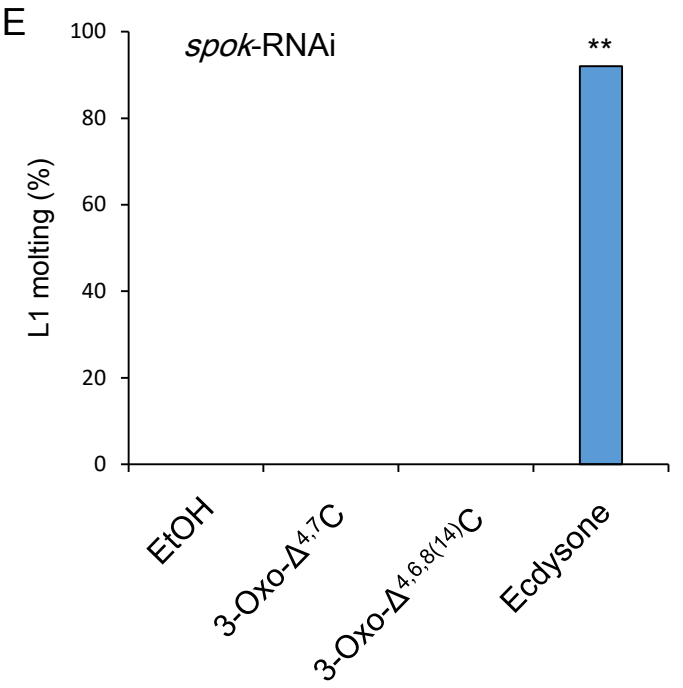
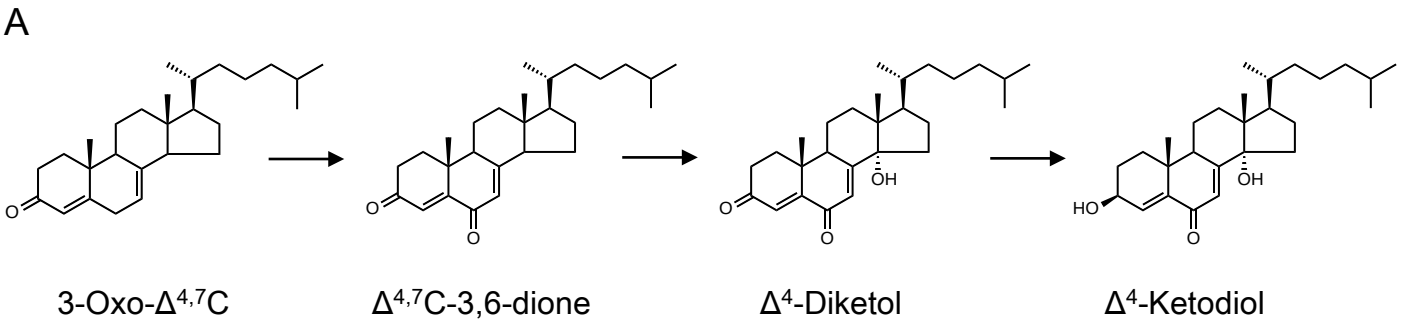
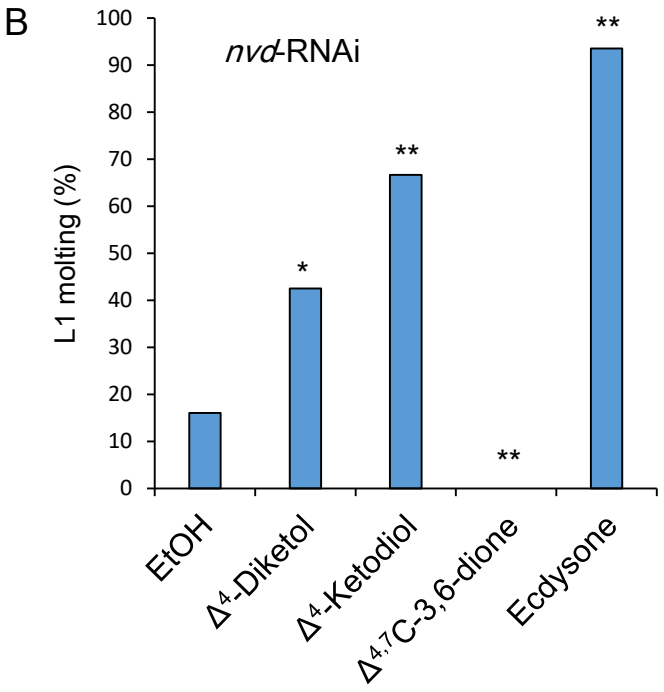
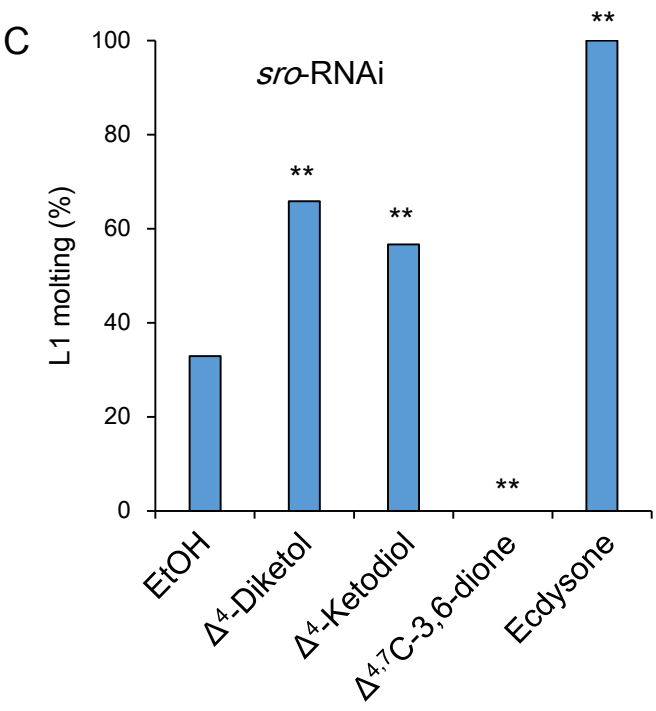
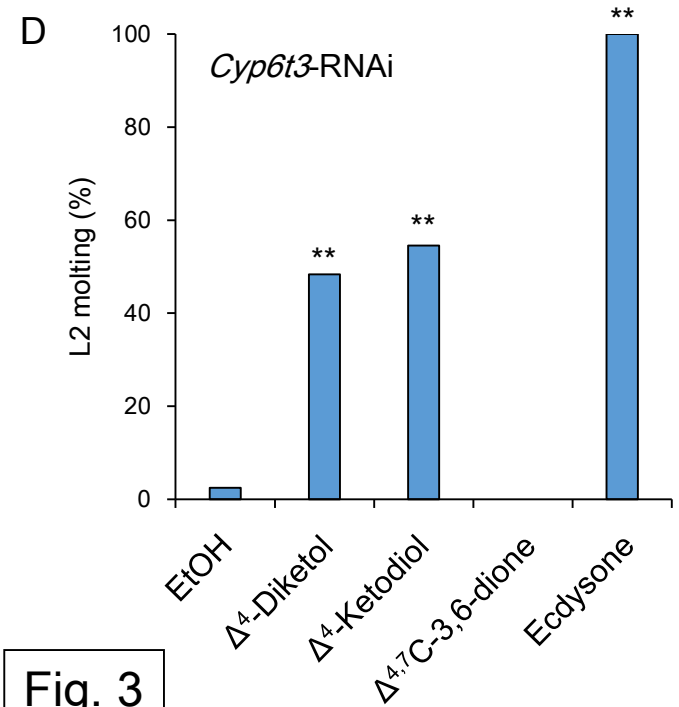

E
spok-RNAi
L1 molting (%)
100
80
60
40
20
0
**
EtOH
3-Oxo-Δ4,7C
3-Oxo-Δ4,6,8(14)C
Ecdysone

Fig. 2


A
3-Oxo-Δ4,7C
Δ4,7C-3,6-dione
Δ4-Diketol
Δ4-Ketodiol


B
nvd-RNAi
L1 molting (%)
EtOH
Δ4-Diketol
Δ4-Ketodiol
Δ4,7C-3,6-dione
Ecdysone


C
sro-RNAi
L1 molting (%)
EtOH
Δ4-Diketol
Δ4-Ketodiol
Δ4,7C-3,6-dione
Ecdysone


D
Cyp6t3-RNAi
L2 molting (%)
EtOH
Δ4-Diketol
Δ4-Ketodiol
Δ4,7C-3,6-dione
Ecdysone

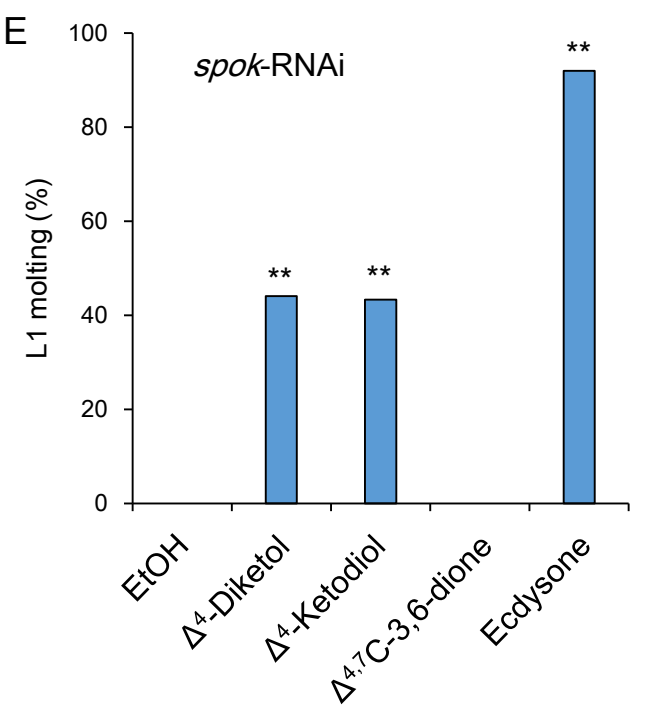
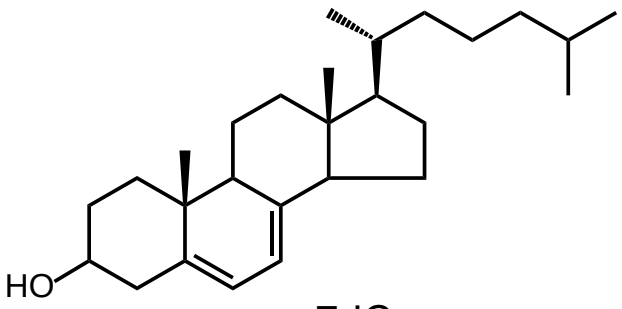
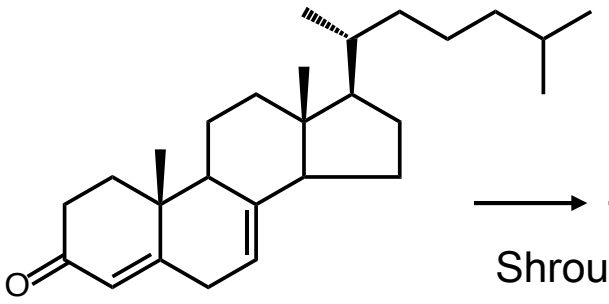

E
spok-RNAi
L1 molting (%)
EtOH
Δ4-Diketol
Δ4-Ketodiol
Δ4,7C-3,6-dione
Ecdysone

Fig. 3



7dC

Oxidation

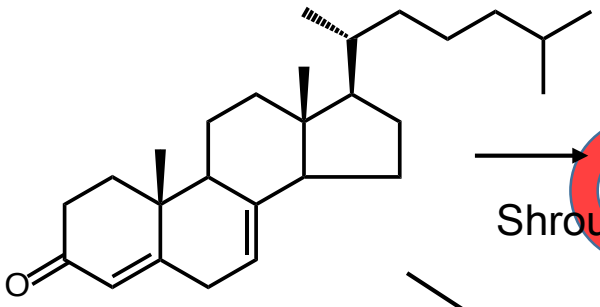


3-Oxo-$\Delta^{4,7}$C

Shroud / CYP6T3

Ecdysteroid
or Ecdysteroid-agonist

Molting



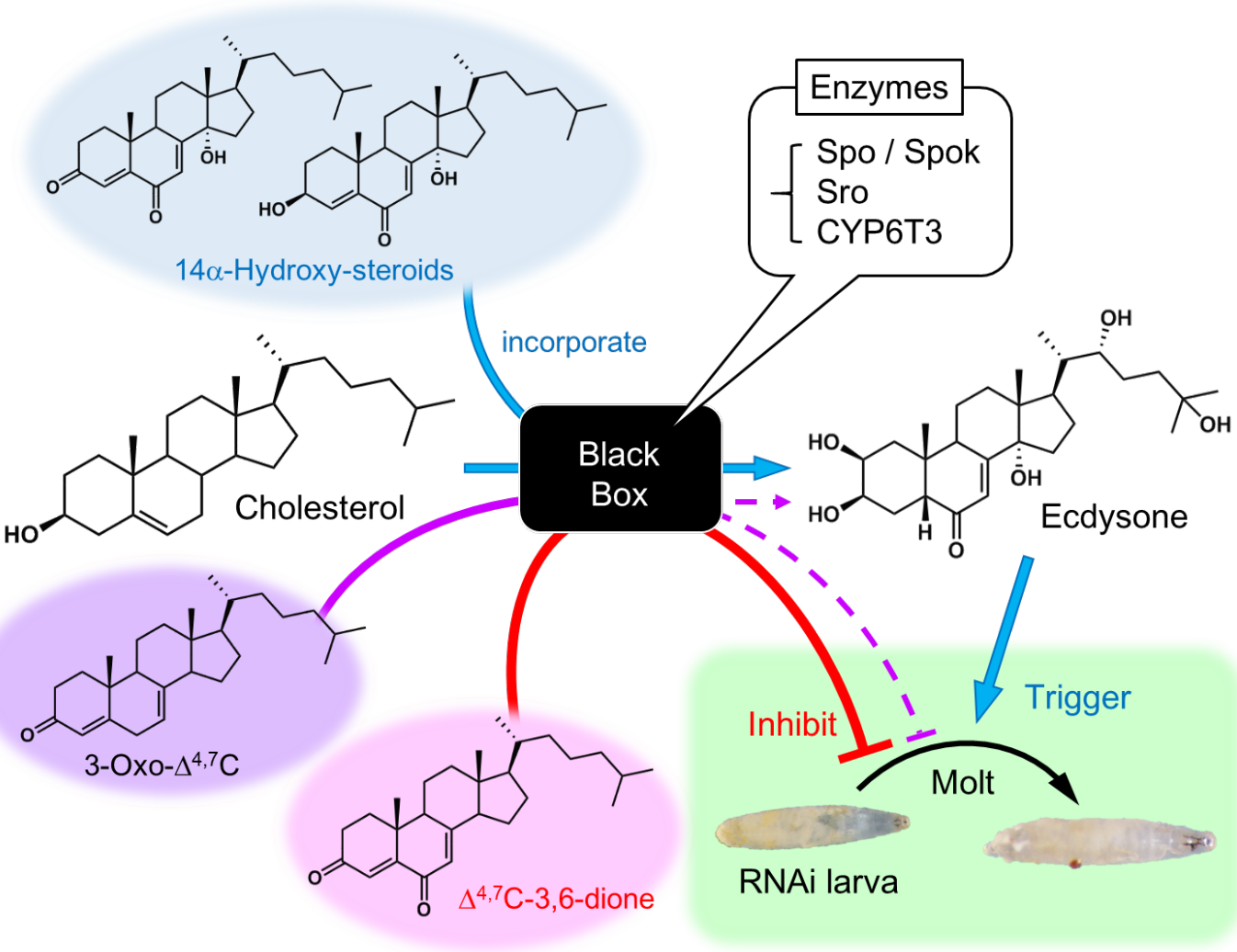
Shroud / CYP6T3

Ecdysteroid-antagonist

Molting

Fig. 4

Fig. 5


14α-Hydroxy-steroids
Enzymes
Spo / Spok
Sro
CYP6T3
incorporate
Cholesterol
Black Box
Ecdysone
3-Oxo-Δ4,7C
Δ4,7C-3,6-dione
Inhibit
Trigger
Molt
RNAi larva

Supplemental Table

Table S1. The primers used for quantitative RT-PCR

gene	forward primer	reverse primer
nvd	5’-ACCTCCCCCTTATCCAAATG-3’	5’-AGCAACGCTTCCACCAATAC-3’
sro	5’-ATGAGCGGCAGTCAACTTCT-3’	5’-CAGGAAATCACGGTCATGTG-3’
Cyp6t3	5’- ACGCTACCGCTGGCTAAGTA-3’	5’-ACTGGCACATTCTTCCCAAC-3’
spok	5’-TATCTCTTGGGCACACTCGCTG-3’	5’-GCCGAGCTAAATTTCTCCGCTT-3’
rpL23	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.

Supplementary figure legends

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

Fig. S2. Transcriptional levels of target genes in RNAi-mediated L1 larvae. (A) Transcriptional levels of *nvd* (A), *sro* (B), *Cyp6t3* (C) and *sro* (D) in each targeted RNAi larvae.

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

Fig. S1

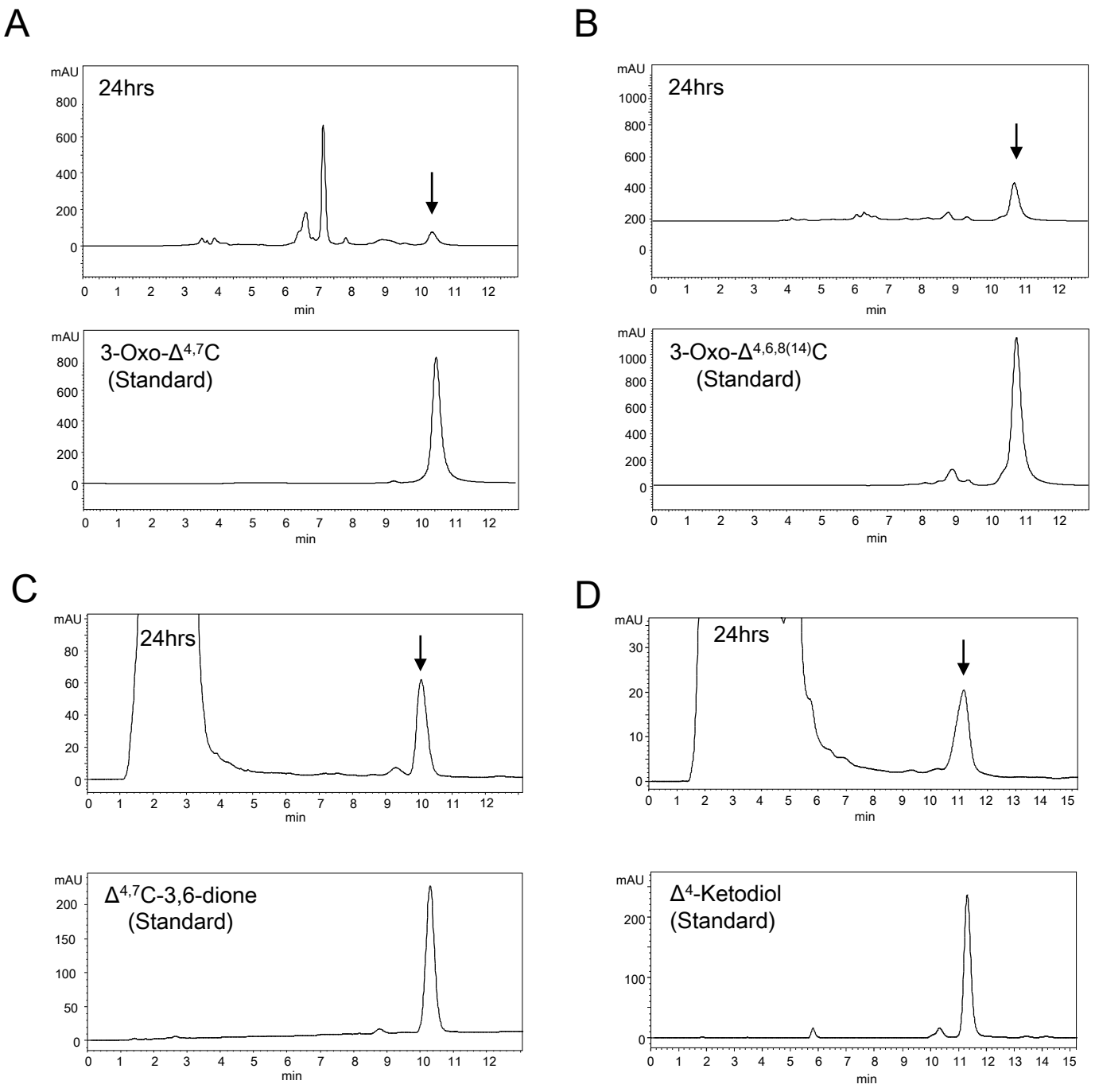

A
mAU
24hrs
min
3-Oxo-Δ4,7C
(Standard)
B
24hrs
3-Oxo-Δ4,6,8(14)C
(Standard)
C
24hrs
Δ4,7C-3,6-dione
(Standard)
D
24hrs
Δ4-Ketodiol
(Standard)

Fig. S2

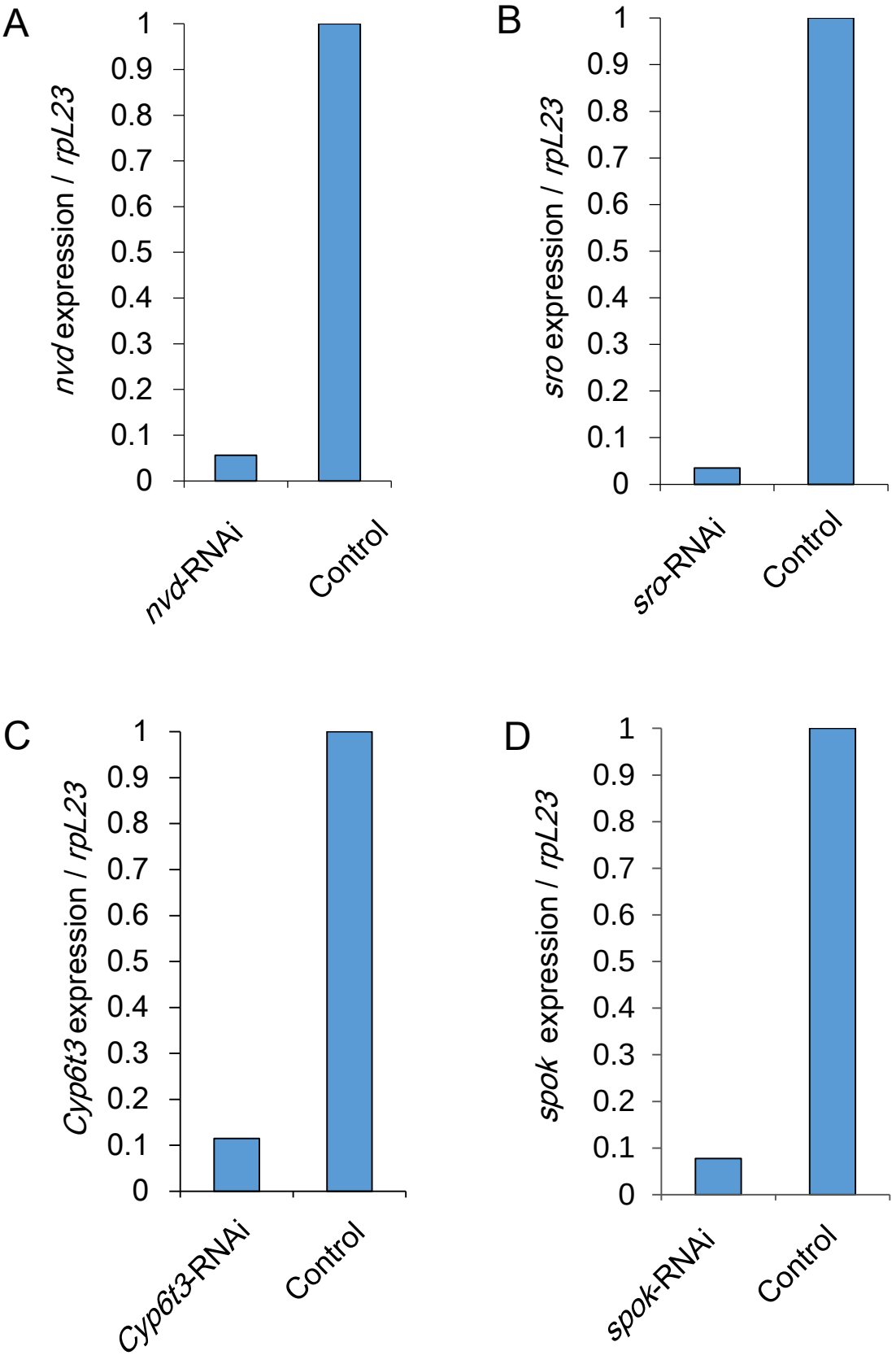
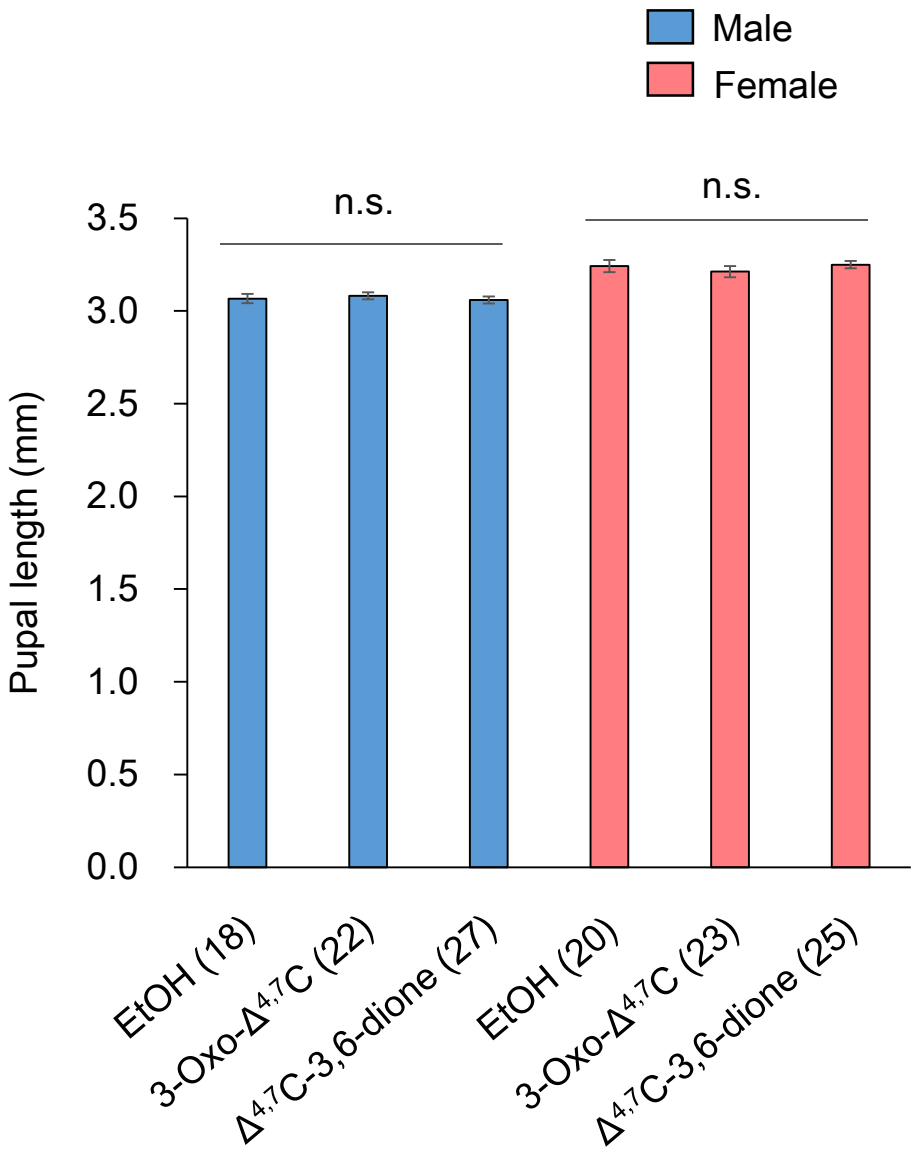

A
nvd expression / rpL23
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
nvd-RNAi
Control
B
sro expression / rpL23
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
sro-RNAi
Control
C
Cyp6t3 expression / rpL23
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cyp6t3-RNAi
Control
D
spok expression / rpL23
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
spok-RNAi
Control

Fig. S3


Male
Female
n.s.
n.s.
Pupal length (mm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EtOH (18)
3-Oxo-Δ4,7C (22)
Δ4,7C-3,6-dione (27)
EtOH (20)
3-Oxo-Δ4,7C (23)
Δ4,7C-3,6-dione (25)