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Studies on the black box: Incorporation of 3-oxo-7-dehydrocholesterol into ecdysteroids by *Drosophila melanogaster* and *Manduca sexta*

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ABSTRACT

It has long been hypothesized that the oxidation of 7-dehydrocholesterol (7dC), made from dietary cholesterol (C), to 3-oxo-7dC (3-oxo-$\Delta^{5,7}$C) immediately precedes the unknown "Black Box" oxidations that lead to the formation of the first up-stream intermediate exhibiting the highly characteristic ecdysteroid structure of the steroid molting hormone of insects, crustaceans and some other arthropods. Perhaps rate-limiting and under the control of the prothoracicotropic hormone (PTTH), the biosynthesis of 3-oxo-7dC and its subsequent oxidative modifications have been difficult to study because of their apparent instability, i.e. no intermediates between 7dC and the diketol (3-oxo-25,22,2-trideoxyecdysone) have ever been observed or identified in insect prothoracic gland incubations with radiolabelled precursors. However, we show that 3-oxo-7dC can be converted into lipophilic, photosensitive, ketone-blocked (PSKB) ketal derivatives which will release 3-oxo-7dC when and where desired following brief irradiation with innocuous long-wave (365 nm) UV-light both in vivo and in vitro. In this manner, 3-oxo-7dC is quickly and efficiently incorporated into ecdysteroids by adult male and female *Drosophila* raised on a diet containing the PSKB ketals and in prothoracic glands of *Manduca sexta* incubated with the ketals emulsified into media. The instability of 3-oxo-7dC and its spontaneous transformation into extensively electron-delocalized intermediates will be discussed in relation to a possible mechanism of the Black Box oxidations eventually leading to the production of the active molting hormone 20-hydroxyecdysone (20E).

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1. Introduction

Growth (molting), development (metamorphosis) and reproduction in insects, crustaceans and arachnids are controlled by the steroid insect molting hormone 20-hydroxyecdysone (20E) (see Lafont et al., 2005). Decorated with six hydroxyl functions, the insect molting hormone is quite polar compared to mammalian steroids, but otherwise seems to function in much the same manner. Yet, in the more than 50 years since its characterization, the complete biosynthetic pathway to 20E, and in particular the all-important rate-limiting step controlled by the prothoracicotropic hormone (PTTH), have not been elucidated (Fig. 1). In the mammal, pituitary ACTH release controls the critical rate-limiting adrenal reaction involving the intra-mitochondrial, facilitative transport of cholesterol (C) to the P450 side-chain cleavage enzyme for conversion to pregnenolone (Privale et al., 1983). Yet, in the prothoracic gland cells of the *Drosophila* ring gland or the prothoracic glands of *Manduca*, the rate-limiting step does not appear to be the initial non-mitochondrial dehydrogenation reaction of C to 7-dehydrocholesterol (7dC). Both ring glands and prothoracic glands contain relatively large amounts of C and 7dC during the critical zenith of ecdysteroid biosynthesis in the last larval instars of both organisms. Consistent with this observation, the rate of C to 7dC conversion remains high throughout most of the last instar in *Manduca* and the adult fruit fly (Sakurai et al., 1986; Grieneisen et al., 1991, 1993; Warren et al., 1996, 1999, 2001). The rate-limiting step does not appear to be mediated by any of the four terminal (C_{25}-, C_{22}-, C_2- and C_{20}-) P450 hydroxylases, the first three of which catalyze the metabolism of the earliest identified ecdysteroid

Abbreviations: C, cholesterol; 7dC, 7-dehydrocholesterol; 3-oxo-7-dehydrocholesterol, 3-oxo-7dC, cholesta-5,7-diene-3-one, 3-oxo-$\Delta^{5,7}$C; cholesta-4,7-diene-3-one, 3-oxo-$\Delta^{4,7}$C; cholesta-4,6,8(14)-triene-3-one, 3-oxo-$\Delta^{4,6,8(14)}$C; PSKB, photosensitive, ketone-blocked; diketol, 5β[H]-cholesta-7-ene-6-one-3β,14α-diol, 3-oxo-2,22,25-trideoxyecdysone; E, ecdysone; 3DE, 3-dehydroecdysone; 20E, 20-hydroxyecdysone; ketotriol, 2,22-dideoxyecdysone; 2dE, 2-deoxyecdysone; 22dE, 22-deoxyecdysone; PTSA, p-toluenesulfonic acid; RP-HPLC, reverse-phase, high-pressure liquid chromatography; RIA, radioimmunoassay.

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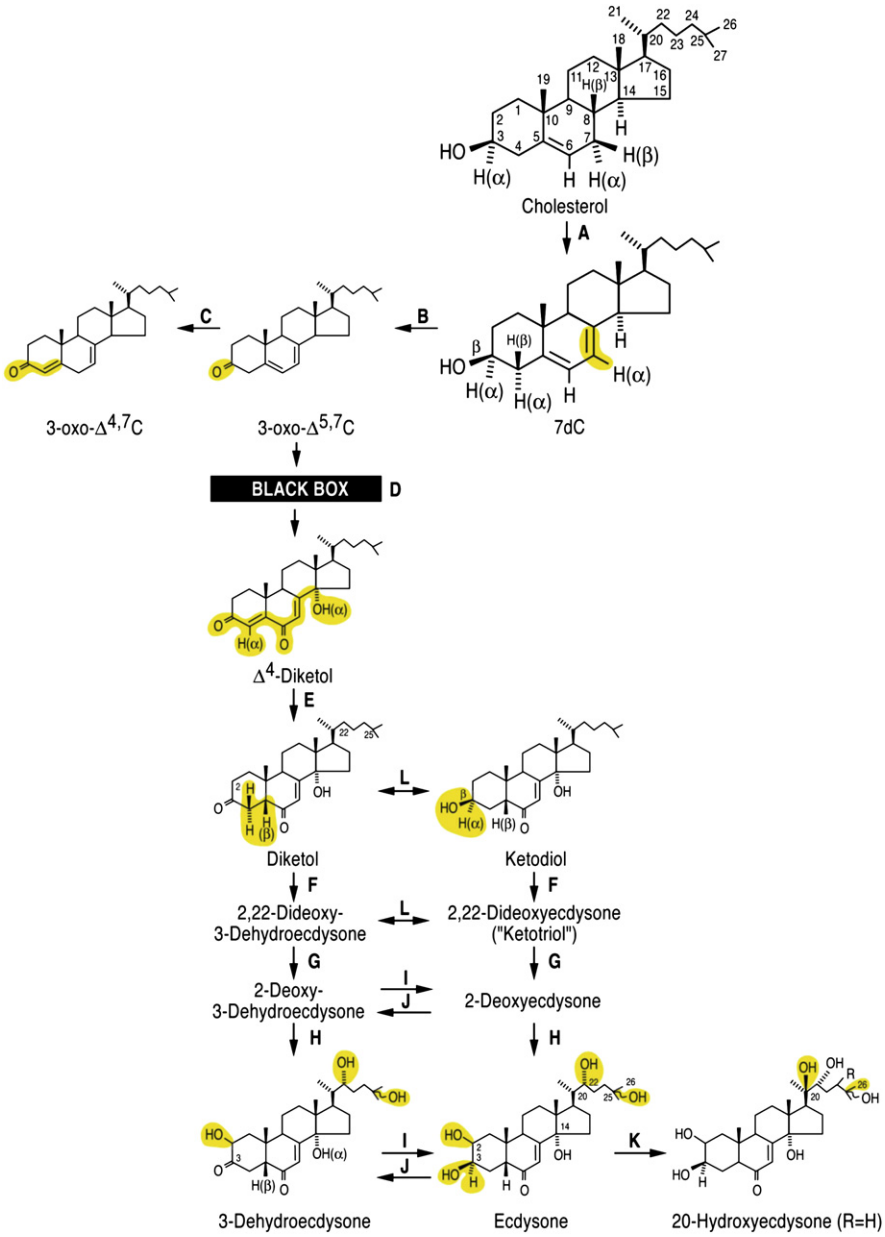

Fig. 1. Scheme of ecdysteroid biosynthesis. (A) 7,8-Dehydrogenase; (B) proposed 3-dehydrogenase of 7dC; (C) chemical isomerization; (D) proposed Spook, Spookier, Spookiest P450 activity; (E) proposed Δ^4-diketol 4-reductase; (F) phantom, 25-hydroxylase; (G) disembodied, 22-hydroxylase; (H) shadow, 2-hydroxylase; (I) 3β-forming 3-dehydroecdysone reductase; (J) ecdysone oxidase; (K) shade, 20-monooxygenase; (L) proposed 3β-hydroxyecdysteroid dehydrogenase/reductase. Starting from the diketol, some insects and crustaceans have been shown to variously reduce each of the subsequent terminal hydroxylated intermediates from the 3-dehydro derivative to the 3β-alcohol. The reaction may be reversible, although in the case of E and 2dE, a separate ecdysone oxidase is known to catalyze the oxidation to the 3-dehydro derivative (Dauphin-Villemant et al., 1997). Not shown is the 3α-forming 3-dehydroecdysone reductase that forms the inactive 3-epiecdysone. Adapted from Gilbert et al. (2002).

precursor, the diketol (5β[H]-cholesta-7-ene-3,6-dione-14α-ol), to the ultimate product of the *Manduca* prothoracic gland, 3-dehydroecdysone (3DE) (Warren et al., 1988; Grieneisen et al., 1993; see Gilbert and Warren, 2005). Following glandular 3DE secretion and subsequent reduction to ecdysone (E) (Sakurai et al., 1989; Kiriishi et al., 1990), rapid uptake of E into target tissues is usually followed rapidly by the final step, significant conversion of E to active 20E by the 20-monooxygenase (e.g. Warren and Gilbert, 1986).

The elusive rate-limiting step may be the yet uncharacterized Δ^4-reductase that mediates the conversion of the hypothetical penultimate up-stream ecdysteroid-like intermediate, the Δ^4-diketol (cholesta-4,7-diene-3,6-dione-14α-ol), to the diketol in a crustacean, but this reaction has not been characterized in insects (Blais et al., 1996). Other candidates for the rate-limiting step are the little understood initial oxidations of 7dC within the prothoracic gland that appear to almost simultaneously oxidize the A-ring 3β-hydroxyl function to the 3-oxo (ketone) and incorporate both the B-ring 6-keto and C/D-ring 14α-ol oxygen atoms into the steroid molecule, i.e. the "Black Box" (Rees, 1985; Warren and Hetru, 1990; Gilbert et al., 2002; Lafont et al., 2005). As such, these complex steroid modification reactions do not appear to occur elsewhere in the animal kingdom. A key hypothesis has long been thought to involve the initial dehydrogenation of 7dC to 3-oxo-7dC (cholesta-5,7-diene-3-one; 3-oxo-$\Delta^{5,7}$C) followed by further extensive rearrangements and oxidations, possibly catalyzed by the very atypical

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