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SYNTHESIS OF HIGH SPECIFIC ACTIVITY [$^3H_2$-1,2]-7-DEHYDROCHOLESTEROL. CONVERSION TO ECDYSONE IN FOLLICLE CELLS OF *LOCUSTA* (INSECTS)

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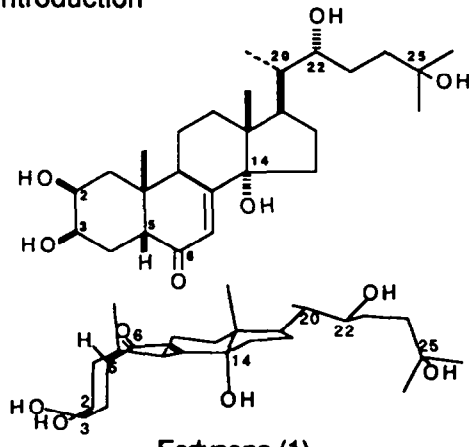
Summary-In previous studies we have characterized the last steps of the biosynthetic pathway of the insect moulting hormone ecdysone. We are now extending our studies to the early steps and have postulated that 7-dehydrocholesterol is one of the earliest precursor molecules. To probe this hypothesis we have synthesized this molecule under tritiated form with high specific activity (2 TBq/mmol). We have devised an original pathway which includes, as a last step, the tritiation at positions 1α and 2α. In a first series of investigations, we have demonstrated that injected 7-dehydrocholesterol was efficiently converted *in vivo* by the ovaries of adult females of *Locusta migratoria* into conjugated ecdysone and 2-deoxyecdysone. *In vitro* incubations of 7-dehydrocholesterol with a 1,000g supernatant of a follicle cell homogenate have occasionnaly yielded conversion rates into ecdysone which were as high as 10%.

Introduction



Ecdysone (1)

The development and the reproduction of insects are controlled by several hormones among which ecdysteroids play a key role. These are polyhydroxylated steroids which are synthesized in endocrine glands (namely prothoracic glands or homologous structures) during larval and pupal development. Their primary target tissue is the epidermis in which they control the molting cycles. In adult females of several insect species the follicle cells surrounding the oocytes synthesize ecdysteroids[1] which are believed to control meiotic reinitiation[2] and some events of early embryonic development[3].

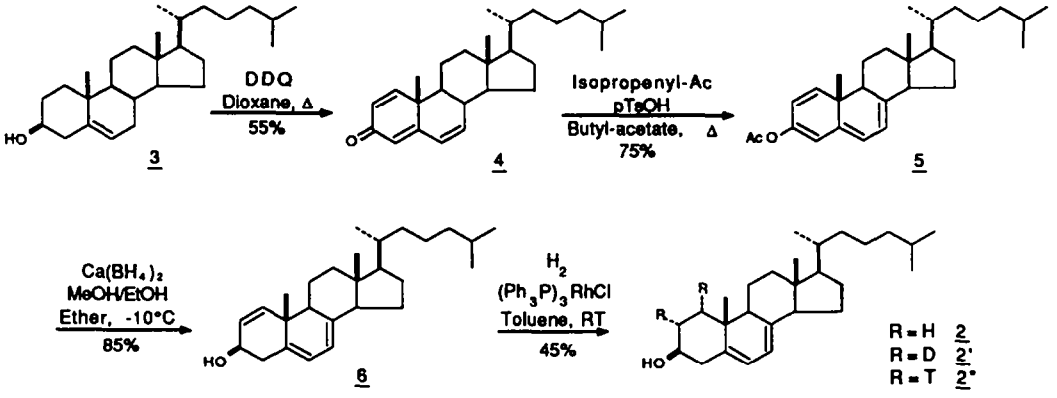
Insects, like the other Arthropods, are unable to synthesize *de novo* sterols and rely upon a dietary source of these compounds for normal growth and development[4]. It is now well established that the initial transformations leading from a poorly oxygenated sterol to ecdysteroids first occur on

the polycycle and namely on the B ring[5]. They are followed by hydroxylations on the side chain[5,6].

We are at present investigating the early steps of biosynthesis of ecdysone, the mother ecdysteroid which was also the first to be isolated by Karlson and associates in 1954[7]. It has been repeatedly proposed that 7-dehydrocholesterol is the earliest intermediate in the biosynthesis from cholesterol to ecdysone[8-10]. The unequivocal demonstration that 7-dehydrocholesterol plays this role required experiments based on the use of a labelled compound in large quantities and at high specific activity.

The synthesis of labelled 7-dehydrocholesterol had already been performed by several authors but under conditions which proved inappropriate for our studies. The label had firstly been introduced by the non-specific Wilzbach technique for the study of vitamin D. Low yields and very low specific activity (396MBq/mmole[11]) were obtained. Labelling had also been performed in similar studies by an early tritiation of a double bond, followed by 3 or 4 reactions on the radioactive compounds. Such microscale reactions with radiolabelled compounds necessitated dilution with unlabelled material which markedly decreased both the specific activity and the final yield (6.6GBq/mmole[12], 18.8GBq/mmole[13]). Studies on a putative precursor role of 7-dehydrocholesterol in ecdysone biosynthesis were routinely based on the use of tritiated cholesterol[8,9] as starting material for labelled 7-dehydrocholesterol. This inevitably yielded low amounts of the expected material and necessitated tedious preparative conditions.

The strategy which we propose in the present paper for the synthesis of labelled 7-dehydrocholesterol has not been described before. Our aims were to fulfill three requirements (1) synthesize the labelled molecule with a high specific activity; (2) introduce the label at the very last step of the chemical synthesis; (3) recover sufficient amounts of labelled compound to allow a reasonable number of experiments. We have introduced the radioactivity through tritiation of double bond at the last step of the synthetic sequence and obtained a large quantity of tritiated material with a high specific activity. The key-step of our synthesis was the selective hydrogenation of a newly formed double bond without interfering with the diene of the B ring.



Scheme 1

Synthesis of [$^3H_2$-1,2]-7-dehydrocholesterol

Compound **4** was obtained by reacting unlabelled cholesterol (**3**) with DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone)[14]. Dioxane was chosen as solvent because it dissolved completely the benzoquinone (180g/l) but only very poorly the reduced hydroquinone (1.8g/l)[15]. An overall yield of 55% was obtained after crystallization of the pure ketotriene **4**.

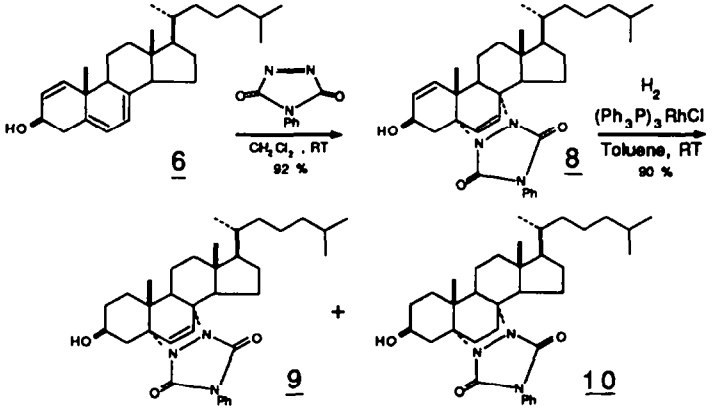
Acidic treatment of **4** favoured the delocalisation of electrons in ring B leading to the formation of an enol which was trapped as acetate **5** by isopropenyl acetate[16]. A yield of 75% was obtained when working in very strict anhydrous conditions

The reduction of the enol ester **5** was performed by reacting with calcium borohydride[17]. The temperature was maintained below -10°C to avoid the solvolysis of the hydride[18]. This reaction was stereo- and regioselective and gave compound **6** with a yield of 85%. The configuration of the allylic alcohol at C-3 was shown to be exclusively β.

The selective reduction of the Δ^1 double bond in **6**, without any reaction with the conjugated $\Delta^{5,7}$ diene, had not been reported previously. As the positions C-1 and C-2 are more accessible in the nucleus than the positions around the conjugated diene, we selected the Wilkinson catalyst [$(Ph_3P)_3RhCl$] which is known to be highly sensitive to steric hinderance[19]. The best reaction conditions were in toluene, at room temperature, under a H_2 atmosphere. The preferential reduction of the Δ^1 double bond was indeed observed but it was followed to a considerably lesser degree by reduction of the Δ^5 double bond. As a result, 7-dehydrocholesterol (**2**) was obtained together with lathosterol (**7**) in a ratio of 4/1 when half of the starting material had been consumed.

The separation of 7-dehydrocholesterol, lathosterol and the starting material was easily performed on a preparative silica gel column and also on preparative TLC.

Our results suggested that an increased selectivity could be expected if the $\Delta^{5,7}$ diene was protected, which would lead to an increased hinderance in this part of the molecule obtained. We have therefore used the classical protection with 4-phenyl-1,2,4-triazoline-3,5-dione and obtained compound **8** with a high yield (92%)[20].



Scheme 2

The hydrogenation carried out under the same conditions as described above gave only small amounts of the desired compound **9** together with compound **10**. In all reaction conditions investigated, the selectivity of the hydrogenation of the Δ^1 double bond was low as compared to that of the Δ^5 double bond. Therefore, we have chosen to perform the tritiation directly on compound **6**.

Before performing the actual tritiation, we have analysed the positions and the number of the hydrogens susceptible to be introduced : for this purpose we have undertaken a deuteriation in the conditions which we anticipated to use for tritiation.

^{1}H NMR analysis of deuteriated 7-dehydrocholesterol showed that two deuteriums had been introduced at positions C-1 and C-2. The coupling constants of the proton at position C-3α (ddd, $J_{H3ax-H2ax}$ and $J_{H3ax-H4ax'}$ = 10.9Hz, $J_{H3ax-H4eq}$ = 4.55Hz, $J_{D2eq-H3ax}$ = very weak) were consistent with the incorporation of one deuterium at position C-2α. As the Wilkinson catalyst is highly sensitive to the steric hinderance, the second deuterium had probably been introduced at position C-1α. ^{2}H NMR analysis corroborated the introduction of two deuteriums (coupling constants of ^{2}H at C1 and at C2, δ = 1.29 ppm, m, $w_{1/2}$ = 15Hz, ^{2}H-1 and δ = 1.87 ppm, $w_{1/2}$ = 15Hz, ^{2}H-2). The analysis by mass spectrometry of the reaction products gave the following proportions : without deuterium, 17%; one deuterium, 7%; two deuteriums, 66%; three deuteriums, 3%; four deuteriums, 6%.

Tritiation was performed at the Commissariat à l'Energie Atomique (Saclay, France). The ratio of 7-dehydrocholesterol to lathosterol under the conditions which are particular for tritiation was 1/1. The specific activity of 7-dehydrocholesterol was 2.2 TBq/mmole.

Conversion studies of 7-dehydrocholesterol to ecdysone

Conversion of tritiated 7-dehydrocholesterol by ovaries of female adults of *Locusta*.

Adult females were isolated at the beginning of vitellogenesis. They received a daily injection of 37kBq of tritiated 7-dehydrocholesterol over a 10-day period which allowed the progression of three successive cycles of oocyte maturation. Consequently, several egg-pods were produced by these females and were collected immediately after egg-laying, homogenized in ethanol and the extracts were subjected to the analysis of their steroid content. Eventually, after the third egg-laying, the adult insects were homogenized in ethanol and their steroids were extracted. The figures of the radioactivity present in the egg-pods and in the adult females showed that approximatively 50% of the label had been transferred from the females into their eggs. The extracts of the steroids present in the egg pods were partitioned between hexane and ethanol and the ethanol phase was subjected to thin layer chromatography. Highly polar compounds were eluted from the thin layer plates and submitted to enzymatic hydrolysis. The hydrolysate was further subjected to reversed phase HPLC. Radioactive compounds were observed to co-elute in HPLC in our conditions with reference ecdysone and 2-deoxyecdysone. The presumed radioactive ecdysone and 2-deoxyecdysone were therefore subjected to co-acetylation with reference molecules : the profiles obtained for the various acetates were similar for the radioactive compounds and the references, which established the identity of radioactive ecdysone and 2-deoxyecdysone as conversion products of tritiated 7-dehydrocholesterol in adult females. Conversion rates were variable in these

experiments; the highest rate was 3% for ecdysone and 1% of 2-deoxyecdysone (of the total radioactivity recovered from the eggs). The analysis of the hexane phase showed the presence of large amounts of cholesterol and of unconverted 7-dehydrocholesterol.

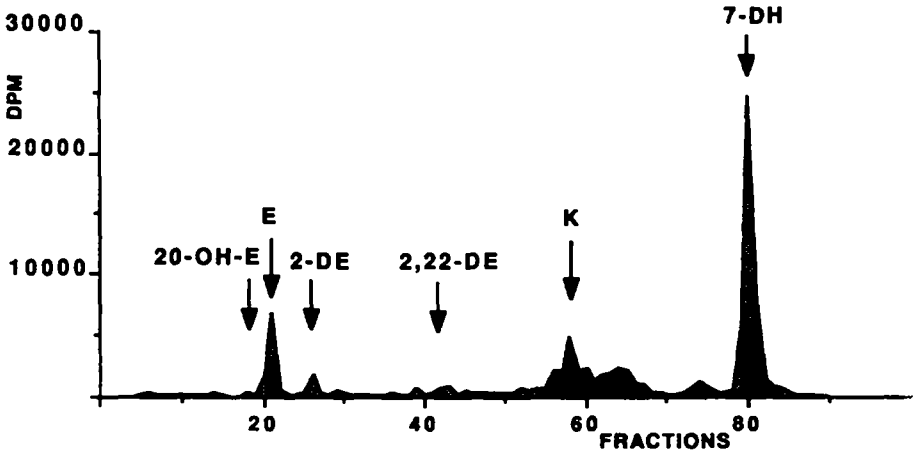


Figure 1 : Conversion of tritiated 7-dehydrocholesterol by 1000g supernatant of follicle cell homogenates of Locusta migratoria. Radiochromatogram of the HPLC separation of the ethanolic extract on a RP C-18 column by a gradient of 0 to 100% methanol in water in 30 min. 20-OH-E, E, 2-DE, 2,22-DE, K, 7-DH : migration of reference 20-hydroxyecdysone, ecdysone, 2-deoxyecdysone, 2,22-dideoxyecdysone, ketodiol, 7-dehydrocholesterol.

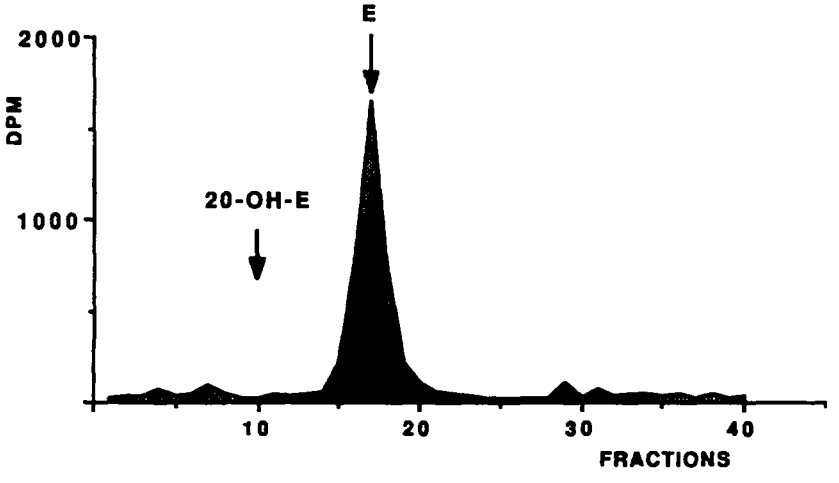


Figure 2 : Radiochromatogram of the HPLC separation of the compound co-migrating with reference ecdysone in figure 1, injected together with reference molecules on a RP C-18 column; isocratic elution with a methanol-water (45/55, v/v) solvent system. 20-OH-E, E : migration of reference 20-hydroxyecdysone, ecdysone

Incubation of follicle cells with tritiated 7-dehydrocholesterol

Follicle cells were separated from 150 terminal oocytes of vitellogenic females (terminal oocytes length 5.3 to 5.7mm cf. [21]). and incubated for 6h with 185kBq (0.08µM) of 7-dehydrocholesterol. An aliquot of the incubation medium was coinjected with reference ecdysteroids into a reversed phase HPLC. Numerous labelled compounds were apparent in these conditions; none of them corresponded to cholesterol or to any known ecdysteroid. This experiment was repeated several times with the same negative result.

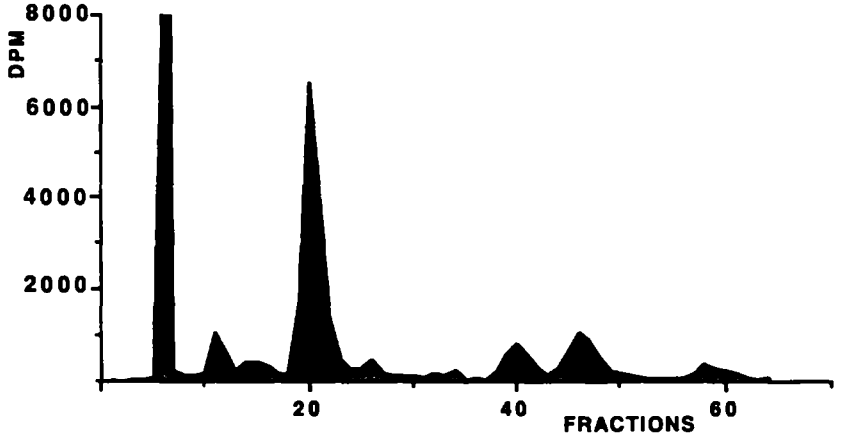


Figure 3 : Co-acetylation of reference ecdysone with the fraction co-eluting with ecdysone. Separation of acetates on a RP C-18 column; isocratic elution with a methanol-water (6/4 v/v) solvent system. ■ UV absorbance; ▒ radioactivity measurements of aliquots of each fraction.

Conversion studies with tritiated 7-dehydrocholesterol in a 1000g supernatant of follicle cell homogenates prepared from follicle cells.

Follicle cells were prepared as above in roughly equivalent amounts per experiment, and served for the preparation of 1000g surpernatants (see Experimental). Supernatants were incubated for 1.5h at 37°C under shaking with 55kBq (0.02µM) of 7-dehydrocholesterol in the presence of NADPH. The methanolic extract of the incubation medium was injected into a reversed phase HPLC and eluted by a methanol/water gradient (fig 1). Some radioactivity clearly co-eluted with reference ecdysone. This presumed radioactive ecdysone was rechromatographed in isocratic conditions (fig 2): the labelled material coeluted with reference ecdysone. The fractions containing the tritiated compound were subjected to co-acetylation with unlabelled ecdysone and, as shown in fig 3, profiles of the various acetates were similar for the radioactive compound and the reference. This result established the identity of radioactive ecdysone as a conversion product of tritiated 7-dehydrocholesterol in a 1000g supernatant of follicle cells. The identities of radioactive 2-deoxyecdysone and 20-hydroxyecdysone were established by the same protocol. Several experiments were run with irregular conversion rates. In some cases, up to 10% of the radioactivity corresponded to ecdysone while in other experiments conversion to ecdysteroids was barely

detectable. We have never observed conversion of 7-dehydrocholesterol into cholesterol by 1000g supernatants of follicle cell homogenates.

Conclusion

We have synthesized radiolabelled 7-dehydrocholesterol according to a novel strategy and obtained significant amounts of tritiated compound with a high specific activity. This compound is very efficiently converted to ecdysone and other ecdysteroids in follicle cell homogenates (1000g supernatant) of vitellogenic females of *Locusta*. These cells are an established site of ecdysone biosynthesis and the efficient conversion of 7-dehydrocholesterol potentially opens new possibilities for the elucidation of the early steps of ecdysone biosynthesis.

Experimental

Melting points were measured on a Reichert hot stage microscope and are uncorrected. $[\alpha]_D$ were measured on a Perkin-Elmer 141 polarimeter in $CHCl_3$. IR spectra were recorded in KBr on a Perkin-Elmer infrared spectrophotometer. UV spectra were measured on a Kontron-Uvikon 810 UV-vis spectrophotometer. NMR spectra were recorded on Brucker SY (200 MHz) apparatus with $CHCl_3$ (δ = 7.26 ppm) as internal standard for ^1H NMR, $CDCl_3$ (δ = 77.02 ppm) as internal standard for ^{13}C NMR and $CHCl_3$ (δ = 7.26 ppm) as internal standard for ^2H NMR. The chemical shifts are reported in ppm downfield from TMS (°, ◊, •, ^ or # = interchangeable assignement). MS were measured on a LKB 9000 S apparatus by direct introduction, or coupled to a GC (OV-1 column); an ionization potential of 70 eV was used. Microanalyses were performed by the Strasbourg Division of the Service Central de Microanalyse of CNRS. TLC were run on pre-coated plates of silica gel 60 F 254 (Merck), dipped in a solution of vanillin (1 g) in EtOH/H_2SO_4 (95/5, 1 l) and heated on a hot plate to reveal the compounds. Medium pressure chromatography (P = 0.3 - 0.7 bars) was conducted on silica gel (40 - 63 mm, Merck) columns. Radioactivity has been determined with an Kontron Betamatic V counter (equipped with external standards). All solvents were freshly distilled before use. Air- or moisture- sensitive reactions were conducted in flame-dried glassware.

1,4,6-Cholestatrien-3-one (4)

A dioxane solution (180 ml) containing 3 (5.22 g, 13.5 mmol) and DDQ (2,3-dichloro-5,6 -dicyano-benzoquinone, 10.75 g, 47.3 mmol, 3.5 eq.) was refluxed for 8 h. After filtration of the hydroquinone, the solution was washed with aq. NaOH 1% solution and brine, dried with sodium sulfate and filtered. The solution was concentrated to dryness under reduced pressure. Crystallization from acetone gave 1,4,6-cholestatrien-3-one (4) 2.87 g (55%).

Mp 88-89°C (litt.[16] mp 88-89°C) ; $[\alpha]_D^{19}$ = - 10 (c = 27.5) ; **UV** λ_{max} (CH_3CN) = 299 nm, ε = 13900 (litt.[16] λ_{max}(EtOH) = 300 nm, ε = 13400) ; **IR** ν (cm^{-1}) : 2940, 1600, 1580, 1470, 1390, 1290, 890, 775, 700 ; **NMR** ^1H δ : 0.79 (s, 3H, H-18); 0.87 (d, J = 6.13 Hz, 6H, H-26,27); 0.92 (d, J = 5.47 Hz, 3H, H-21); 1.20 (s, 3H, H-19); 6.04 (d, J = 14.49 Hz, 2H, H-6,7); 6.22 (m, $w_{1/2}$ = 10.0 Hz, 1H, H-4); 6.28 (m, $w_{1/2}$ = 7.5 Hz, 1H, H-2); 7.10 (d, J = 10.15 Hz, 1H, H-1) ; **NMR** ^{13}C δ : 11.9 (CH_3-18); 18.5 (CH_3-21); 20.6 (CH_3-19); 21.6 (CH_2-11); 22.4° (CH_3-26); 22.9° (CH_3-27); 23.7 (CH_2-23); 23.8° (CH_2-15); 27.9# (CH-25); 28.0# (CH_2-16); 35.7 (CH-20); 36.0° (CH_2-12); 36.0° (CH_2-22); 38.1 (CH-8); 39.4 (CH_2-24); 41.1 (C-10); 42.9 (C-13); 48.3 (CH-14); 53.6 (CH-9); 55.9 (CH-17); 123.6 (C-4); 126.8 (CH-5); 127.4 (CH-2); 128.0 (CH-

6); 138.6 (CH-7); 152.8 (CH-1); 186.2 (C-3) ; **MS** m/z : 380 (62.2) ($M^{+\cdot}C_{27}H_{40}O$), 364 (12.4), 247 (67.9), 172 (50.2), 159 (53.7), 158 (50.1), 147 (40.6), 134 (47.6), 133 (53.6), 121 (45.6), 107 (46.1) ; **Microanalysis** : calc. for $C_{27}H_{40}O$ (380.6120) C: 85.20 ; H: 10.59 , found C: 85.26 ; H: 10.73.

3-Acetoxy-cholesta-1,3,5,7-tetraen (5)

A mixture of (4) (1.00 g, 2.64 mmol), p-toluen-sulphonic acid (556 mg, 2.92 mmol, 1.1 eq.) in butyl-acetate (12 ml) and isopropenyl-acetate (11 ml, 38 eq.) was refluxed for 8 h. The butyl-acetate solution was washed with water and brine, dried with sodium sulfate and filtered. The solution was concentrated to dryness under reduced pressure. Crystallization from acetone gave 3-acetoxy-cholesta-1,3,5,7-tetraen (5) as yellow needles 651 mg (75%).

Mp 122-125 °C (**litt.**[16] 122-124°C) ; $[\alpha]_D^{19}$ = - 519 (c = 16,5) ; **UV** λ_{max} (CH_3CN) = 366 nm, ε = 9000 and 257 nm, ε = 11500 ; **IR** ν (cm^{-1}) : 885, 930, 1140, 1200, 1370, 1650, 1760, 2960 ; **NMR** ^1H δ : 0.65 (s, 3H, H-18); 0.79 (s, 3H, H-19); 0.88 (d, J = 6.55 Hz, 6H, H-26,27); 0.95 (d, J = 6.21 Hz, 1H, H-21); 5.89 (m, $w_{1/2}$ = 10.0 Hz, 1-H, H-7°); 5.86 to 6.04 (broad, 4H, H-1,2,4,6°) ; **NMR** ^{13}C δ : 12.1 (CH_3-18); 18.9 (CH_3-2°); 21.1 (CH_3-19); 21.8 (CH_2-11); 22.9° (CH_3-26); 22.9° (CH_3-27); 22.9 (CH_2-15); 23.9 (CH_2-23); 28.1 (CH_2-16); 28.1 (CH-25); 36.2 (CH-20); 36.2 (CH_2-22); 38.6 (C-10); 39.2 (CH_2-12); 39.6 (CH_2-24); 41.9 (CH-14); 41.9 (CH_3-21); 44.0 (C-13); 55.1 (CH-9); 56.1 (CH-17); 115.4° (CH-7); 117.8° (CH-6); 120.2° (CH-4); 122.9° (CH-2); 134.9° (CH-1); 137.6° (C-8); 138.7° (C-5); 144.7 (C-3); 169.1 (C-1') ; **MS** m/z : 422 (19.5) ($M^{+\cdot}C_{29}H_{42}O_2$), 380 (43.3), 362 (80.4), 172 (27.0), 171 (31.2), 157 (40.5), 155 (62.1), 133 (34.1), 121 (39.3), **Microanalysis** : calc. for $C_{29}H_{42}O_2$ (422.3588) C: 82.47 ; H: 10.02 , found C: 82.36 ; H: 10.15.

1,5,7-Cholestatrien-3β-ol (6)

A solution of the crude acetate (5) (1.75 g, 4.15 mmol) in ether (100 ml) was added dropwise at - 10°C to a stirred solution of calcium borohydride in ethanol-methanol [prepared at -78 °C from sodium borohydride (2.8 g) in ethanol (50 ml) and calcium chloride (5.53 g) in methanol (50 ml)]. The mixture was stirred at - 10°C for 3 h. and allowed to overnight. After addition of 50% acetic acid to dissolve the resulting precipitate, the product was extracted with ether. The extract was washed with aq. sodium hydrogen-carbonate, water and dried with sodium sulfate. The ether was evaporated to dryness under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/ether 9/1 gave 1,5,7-cholestatrien-3β-ol (6) 1.65 g (94 %)

Mp 128-129 °C ; $[\alpha]_D^{19}$ = -121 (c = 12.5) ; **UV** λ_{max} (CH_3CN) : 275 nm (8900) and 285 nm (9200) ; **IR** ν (cm^{-1}): 3420, 2950, 1470, 1390, 1320, 1040, 840, 570 ; **NMR** ^1H δ : 0.62 (s, 3H, H-18); 0.87 (d, J = 6.48 Hz, 6H, H-26,27); 0.93 (d, J = 6.33 Hz, 3H, H-21); 1.00 (s, 3H, H-19); 4.30 (m, $w_{1/2}$ = 20.0 Hz, 1H, H-3); 5.46 (m, $w_{1/2}$ = 10.0 Hz, 1H, H-7°); 5.64 (m, 1H, H-6°); 5.70 (b, 2H, H-1,2) ; **NMR** ^{13}C δ : 11.9 (CH_3-18); 18.2 (CH_3-19); 18.9 (CH_3-21); 21.0 (CH_2-11); 23.9 (CH_2-23); 22.6° (CH_3-27); 22.9° (CH_3-26); 23.1 (CH_2-15); 28.0 (CH_2-16); 28.0 (CH-25); 36.2 (CH_2-22); 36.2 (CH-20); 38.6 (C-10); 39.1 (CH_2-12); 39.6# (CH_2-4) ; 39.6# (CH_2-24); 42.9 (C-13); 43.3 (CH-14); 54.8 (CH-9); 56.0 (CH-17); 68.9 (CH-3); 116.8° (CH-7); 119.5° (CH-6); 130.2° (CH-2); 136.3° (CH-1); 137.9 (C-8); 143.0 (C-5) ; **MS** m/z : 382 (22.4) ($M^{+\cdot}C_{27}H_{42}O$), 364 (15.9), 349 (18.9), 339 (8.5), 141 (100.0) ; **Microanalysis** : calc. for $C_{27}H_{42}O$ (382.3578) C: 84.82 ; H: 11.07 , found C: 84.73 ; H: 11.12.

5,7-Cholestadien-3β-ol (2). Catalytic hydrogenation of 1,5,7-cholestatrien-3β-ol (6) with chloro(triphenyl-phosphine) rhodium.

The compound (6) (100 mg, 0.26 mmol) was dissolved in toluene (8 ml). Hydrogenation was conducted over $(Ph_3P)_3RhCl$ (100 mg) at room temperature during 2 h. After removing the solvent under reduced pressure, the

residue was chromatographed on silica gel. Elution with hexane/ethyl acetate 95/5 gave a mixture of 1,5,7-cholestatrien-3β-ol (6) 45 mg, 5,7-cholestadien-3β-ol (2) 45 mg (45 %) and 7-cholesten-3β-ol (7) 5 mg (5%). The separation of the two products of the reaction has been made on TLC, on silica gel impregnated with silver nitrate (elution with hexane/ethyl acetate 6/4).

(2) **NMR ^1H** δ : 0.62 (s, 3H, H-18); 0.87 (d, J = 6.49 Hz, 6H, H-26,27); 0.93 (d, J = 6.14 Hz, 3H, H-21); 0.94 (s, 3H, H-19); 3.64 (m, $w_{1/2}$ = 25.0 Hz, 1H, H-3); 5.36 (m, $w_{1/2}$ = 10.0 Hz, 1H, H-7*); 5.57 (m, $w_{1/2}$ = 10.0 Hz, 1H, H-6*) ; **NMR ^{13}C** δ : 11.8 (CH_3-18); 16.3 (CH_3-19); 18.9 (CH_3-21); 21.2 (CH_2-11); 22.7* (CH_3-27); 22.8* (CH_3-26); 23.0 (CH_2-15); 23.9 (CH_2-23); 28.0 (CH-25); 28.1 (CH_2-16); 32.0* (CH_2-2); 36.2 (CH_2-22); 36.2 (CH-20); 37.0 (C-10); 38.4 (CH_2-4); 39.2 (CH_2-12); 39.5 (CH_2-24); 40.8* (CH_2-1); 43.0 (C-13); 46.3 (CH-14); 54.5 (CH-9); 55.9 (CH-17); 70.5 (CH-3); 116.3° (CH-7); 119.6° (CH-6); 139.8 (C-8); 141.4 (C-5).

(7) **NMR ^1H** δ : 0.53 (s, 3H, H-18); 0.79 (s, 3H, H-19); 0.86 (d, J = 6.56 Hz, 6H, H-26,27); 0.94 (d, J = 5.97 Hz, 3H, H-21); 3.64 (m, $w_{1/2}$ = 25.0 Hz, 1H, H-3); 5.16 (m, $w_{1/2}$ = 6.7 Hz, 1H, H-7).

[1,2-$^2H_2$]-5,7-Cholestadien-3β-ol (2') Catalytic deuteration of 1,5,7-cholestatrien-3β-ol (6) with chloro(triphenyl-phosphine) rhodium.

The previously described procedure was carried out, under deuterium atmosphere (with 100 mg of cholesta-1,5,7-trien-3β-ol (6)). Elution with hexane/ethyl acetate 95/5 gave a mixture of 1,5,7-cholestatrien-3β-ol 40 mg, [1,2-$^2H_2$]-5,7-cholestadien-3β-ol (2') 48 mg (48 %) and (1,2,5,6-$^2H_4$)-7-cholesten-3β-ol (7') 7 mg (7 %). The separation of the two products of the reaction has been made by TLC, on silica gel impregnated with silver nitrate (elution with toluene/ethyl acetate 6/4).

(2') **NMR ^1H 400 MHz** δ : 0.62 (s, 3H, H-18); 0.87 (d, J = 6.49 Hz, 6H, H-26,27); 0.93 (d, J = 6.14 Hz, 3H, H-21); 0.94 (s, 3H, H-19); 3.58 (ddd, J = 10.86 Hz et J = 4.55 Hz, 1H, H-3); 5.39 (m, $w_{1/2}$ = 10.0 Hz, 1H, H-7*); 5.57 (m, $w_{1/2}$ = 10.0 Hz, 1H, H-6*) ; **NMR ^2H 61.4 MHz** δ : 1.29 (m, $w_{1/2}$ = 15.0 Hz, 1^2H, ^2H-1); 1.87 (m, $w_{1/2}$ = 15.0 Hz, 1^2H, ^2H-2) ; **MS** m/z : 386 (92.1) (M^+, $C_{27}H_{42}O^2H_2$) 387 (31.8), 354 (20.8), 353 (100.0), 160 (25.6), 147 (27.0), 145 (29.4), 119 (22.9).

(7') **NMR ^1H** δ : 0.53 (s, 3H, H-18); 0.86 (d, J = 6.56 Hz, 6H, H-26,27); 0.79 (s, 3H, H-19); 0.94 (d, J = 5.97 Hz, 3H, H-21); 3.64 (m, $w_{1/2}$ = 25.0 Hz, 1H, H-3); 5.16 (m, $w_{1/2}$ = 6.7 Hz, 1H, H-7) ; **NMR ^2H 61.4 MHz** δ : 1.06 (m, $w_{1/2}$ = 15.0 Hz, 1^2H, ^2H-1*); 1.38 (m, $w_{1/2}$ = 15.0 Hz, 1^2H, ^2H-5*); 1.79 (m, $w_{1/2}$ = 15.0 Hz, 2^2H, ^2H-2,6) ; **MS** m/z : 390 (100.0) (M^+, $C_{27}H_{42}O^2H_4$) 391 (32.0), 375 (24.9), 276 (11.8), 259 (43.6).

[1,2-$^3H_2$]-5,7-Cholestadien-3β-ol (2") Catalytic tritiation of 1,5,7-cholestatrien-3β-ol (6) with chloro(triphenyl-phosphine) rhodium.

The previously described procedure was carried out, under tritium atmosphere, at the C.E.A. (Saclay). Tritiated 7-dehydrocholesterol ([1,2-$^3H_2$]-5,7-cholestadien-3β-ol) (2") was obtained over 50% yield after purification on silica gel impregnated with silver nitrate.

Specific activity : 60 Ci/mmol (2.2 TBq /mmol).

5α,8α-(4-phenyl-1,2-urazolo)-1,6-Cholestadien-3β-ol (8)

1,5,7-Cholestatrien-3β-ol (6) (170 mg, 0,45 mmol) was dissolved in CH_2Cl_2 (4 ml) and 4-phenyl-1,2,4-triazoline-3,5-dione was added in small portions (90 mg, 0.49 mmol, 1.1 eq.) until discharge of the red color terminated. After stirring for 1 h. at room temperature, the solvent was evaporated. The residue was chromatographed on silica gel. Elution with

hexane/ethyl acetate 8/2 gave 5α,8α-(4-phenyl-1,2-urazolo)-1,6-cholestadien-3β-ol (8) 226 mg (92 %).
$[\alpha]_D^{19}$ = -39 (c = 3.4) ; UV λ_{max} (CH_3CN) = 262 nm, ε = 3200 (litt.[17] λ_{max} (EtOH) = 255 nm, ε = 4260) ; IR ν (cm^{-1}) : 3440, 2950, 1755, 1700, 1500, 1460, 1040, 750 ; **NMR** ^1H δ : 0.81 (s, 3H, H-18); 0.86 (d, J = 6.63 Hz, 6H, H-26,27); 0.93 (d, J = 6.01 Hz, 3H, H-21); 1.10 (s, 3H, H-19); 2.2 to 2.4 (b, 3H, H-4β, 9, 14); 3.39 (dd, J = 7.89 and J = 14.72, 1H, H-4α) ; 5.07 (m, $w_{1/2}$ = 20.0 Hz; 1H, H-3); 5.76 (br s, $w_{1/2}$ = 5.0 Hz, 2H, H-1,2); 6.29 (d, J = 8.30 Hz, 1H, H-7°); 6.47 (d, J = 8.30 Hz, 1H, H-6°); 7.34 (b, 3H, H-1,2,3) ; **NMR** ^{13}C δ : 12.9 (CH_3-18); 19.0 (CH_3-19); 22.3° (CH_3-26); 22.6° (CH_3-27); 22.8 (CH_2-11); 22.8 (CH_3-21); 23.2 (CH_2-15); 23.7 (CH_2-23); 27.5 (CH_2-16); 28.0 (CH-25); 33.6 (CH_2-4); 35.2 (CH-20); 35.9 (CH_2-22); 38.3 (CH_2-12); 39.5 (CH_2-24); 43.2 (C-10); 44.0 (C-13); 49.2 (CH-14); 51.5 (CH-9); 55.5 (CH-17); 64.5° (C-5); 64.8 (CH-3); 65.6° (C-8); 126.2° (CH-6); 127.8° (CH-7); 128.2# (CH-9'); 128.8# (CH-8'); 130.0 (CH-2); 131.9 (C-6'); 135.1 (CH-7'); 135.4 (CH-1); 146.1^(C-3'); 148.7^ (C-5') ; **MS** m/z : 559 (0.3) (m^+, $C_{35}H_{47}O_3N_3$), 557 (1.3), 408 (1.2), 382 (22.8), 364 (10.2), 349 (11.4), 149 (25.4), 141 (33.5) ; **Microanalysis** : calc. for $C_{35}H_{47}O_3N_3$ (559.7743) C: 75.37 ; H: 8.49 , found C: 75.24 ; H: 8.53.

5α,8α-(4-phenyl-1,2-urazolo)-6-Cholestaen-3β-ol (9)

The coumpound (9) was prepared as described for (8) using 4-phenyl-1,2,4-triazoline-3,5-dione (35 mg, 0.20 mmol) and commercial 5,7-cholestadien-3β-ol (70 mg, 0.18 mmol). The residue was chromatographed on silica gel. Elution with hexane/ethyl acetate 8/2 gave 6-cholesten-5α,8α-(4-phenyl-1,2-urazolo)-3β-ol (9) 93 mg (91 %).
NMR ^1H δ : 0.80 (s, 3H, H-18); 0.86 (d, J = 6.68 Hz, 6H, H-26,27); 0.93 (d, J = 6.16 Hz, 3H, H-21); 0.97 (s, 3H, H-19); 2.2 to 2.4 (b, 3H, H-4β, 9, 14); 3.16 (dd, J = 4.28 and J = 14.41, 1H, H-4α); 4.45 (m, $w_{1/2}$ = 20.0 Hz; 1H, H-3); 6.23 (d, J = 8.34 Hz, 1H, H-7°); 6.41 (d, J = 8.34 Hz, 1H, H-6°); 7.34 (b, 3H, H-1,2,3) ; **NMR** ^{13}C δ : 13.0 (CH_3-18) ; 19.0 (CH_3-19); 22.5° (CH_3-26); 22.5° (CH_3-27); 22.5 (CH_2-11); 22.8 (CH_3-21); 23.3 (CH_2-15); 23.7 (CH_2-23); 27.5 (CH_2-16); 28.0 (CH-25); 29.1° (CH_2-1); 34.3 (C-4); 34.8° (CH_2-2); 35.3 (CH-20); 35.9 (CH_2-22); 38.3 (CH_2-12); 39.5 (CH_2-24); 41.3 (C-10); 44.0 (C-13); 49.3 (CH-14); 53.0 (CH-9); 55.2 (CH-17); 65.1° (C-5); 67.3° (C-8); 67.4 (CH-3); 126.2^ (CH-6); 127.8^ (CH-7); 128.8^ (CH-9'); 129.1^ (CH-8'); 131.9 (C-6'); 135.7^ (CH-7'); 146.1# (C-3'); 148.9# (C-5').

Catalytic reduction of the cycloadduct (8) with chloro(triphenylphosphine) rhodium

The coumpound (8) (200 mg, 0.36 mmol) was dissolved in toluene (5 ml). Hydrogenation was conducted over $(Ph_3P)_3RhCl$ (200 mg) at room temperature during 2 h. The solvent was then removed under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/ethyl acetate 8/2 gave 5α,8α-(4-phenyl-1,2-urazolo)-cholest-3β-ol (12) 190 mg (94 %).
NMR ^1H δ : 0.83 (s, 3H, H-18); 0.85 (d, J = 6.63 Hz, 6H, H-26,27); 0.91 (d, J = 6.48 Hz, 3H, H-21); 1.10 (s, 3H, H-19); 2.2 to 2.4 (b, 3H, H-4β, 9, 14); 2.74 (dd, J = 4.10 and J = 14.21, 1H, H-4α); 4.58 (m, $w_{1/2}$ = 20.0 Hz; 1H, H-3); 7.34 (b, 3H, H-1,2,3) ; **NMR** ^{13}C δ : 14.0 (CH_3-18); 17.7 (CH_3-19); 22.5 (CH_2-11); 22.8 (CH_3-21); 23.1 (CH_2-15); 23.1° (CH_3-26); 23.1° (CH_3-27); 23.6 (CH_2-23); 27.1 (C-4); 28.0 (CH_2-16) ; 28.5 (CH-25); 31.5 (CH_2-1); 35.8 (CH-20); 35.9° (CH_2-22); 37.4° (CH_2-2); 38.2° (CH_2-6); 38.2 (CH_2-12) ; 38.4° (CH_2-7); 39.4° (CH_2-24); 41.8 (C-10); 43.6 (C-13); 50.5 (CH-14); 53.6 (CH-9); 55.7 (CH-17); 63.8° (C-5); 64.8° (C-8); 67.6 (CH-3); 126.4^ (CH-9'); 128.5^ (CH-8'); 131.9 (C-6'); 133.0 (CH-7'); 144.9# (C-3'); 148.8# (C-5').

Injection techniques

The labelled compound in solution in ethanol was diluted with aqueous Ringer solution to a final concentration of 1% in ethanol. 10 µl of this solution containing 185 kBq of 7-dehydrocholesterol were injected into the females twice a

day.

***In vitro* techniques**

All dissections were performed under sterile conditions in a laminar flow hood (Mecaplex). The excised tissues were repeatedly rinsed in Landureau's medium[10] and transferred into incubation vials. The incubations were performed in Landureau's medium containing ethanol at a final concentration of 1% necessary to dissolve the labelled precursor. Incuabations were carried out at a temperature of 33°C with gentle shaking and lasted for 16h.

Subcellular incubation

The follicle cells were suspended in freshly prepared 100mM phosphate buffer, pH 7.4 containing 0.1% bovine serum albumine, 1mM mercaptoethanol, 15% sucrose and homogenized in a Teflon-glass Potter-Evehjem homogenized in the same buffer. The homogenate was centrifuged at 1000g for 10min. All opérations were carried out at 0-4°C. To 400µl of supernatant were added 3 µl of 60% ethanol containing the labelled precursor, 50 µl of a NADH solution (mM), 50 µl of isocitrate solution (mM)/ The solubilization of the tritiated molecule was shecked before and after incubation. Incubation was carried out at a temperature of 37°C with gentle shaking for 1.5h.

Extraction and purification

The tissues were homogenized in 95% ethanol. After centrifugation, the pellet was re-extracted repeatedly with 95% ethanol. The combined supernatants were partitioned with hexane. The ethanol phase was deposited on silica gel plates (Merck, HF 254, 0.25mm) which were developed twice in a chloroform-ethanol (4:1, v/v) solvent system. The plates were scanned for radioactivity with a Berthold scanner, and the different labelled compounds were scraped off and extracted from the silica gel with 95% ethanol. These compounds, dried under nitrogen flow were redissolved either in a buffer for enzymatic hydrolysis or in methanol for HPLC analysis.

For identification, the labelled compounds were purified by co-injection with reference substances into a C-18 reversed phase HPLC column (Merck, Lichrosorb, 10µm; length 25 cm; i.d. 4mm). Two types of solvent system were used. (1) a 30 min gradient from 0 to 100% methanol in water (v/v) isocratic system for the compounds which co-migrate with 2-deoxyecdysone, ecdysone or 20-hydroxyecdysone. The flow rate was 1 ml/min. Fractions of 500 µl were collected. Elution of reference substances was monitored by U.V. absorption at 254 nm (Waters Associates M440 spectrometer).

Enzymatic hydrolysis

Highly polar radioactive compounds presumed to be ecdysteroid conjugates were subjected to enzymatic hydrolysis with β-glucuronidase (Sigma) in a 50 mM acetate buffer (pH 5.3) at 37°C for 18h under shaking.

Acetylation

The identity of the labelled ecdysteroids was ascertained by co-acetylation with unlabelled reference molecules in a mixture of 100µl of anhydrous pyridine and 50 µl of acetic anhydride for 1h at room temperature. The reaction was stopped by addition of 300µl of methanol and the hydrolysis of acetic anhydride was allowed to proceed for one night at 4°C. After dessication of the mixture under nitrogen flow the products were chromatographed by HPLC in a isocratic solvent system (methanol-water 6/4, v/v for ecdysone acetates, 7/3, v/v for 2-deoxyecdysone and 1/1 v/v for 20-hydroxyecdysone).

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