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STUDY ON THE BIOSYNTHESIS OF ECDYSONE
PART IV[1] :
Synthesis of high specific activity ($^3H_2$-22,23)-2,22-dideoxyecdysone
Tissue distribution of the C-22 hydroxylase in Locusta migratoria

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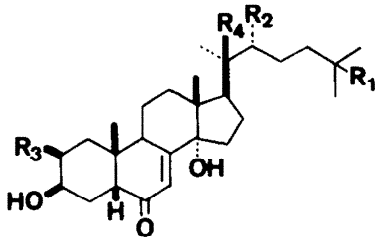
Abstract - We have synthesized a tritiated form of 2,22-dideoxyecdysone (($^3H_2$-22,23)-3β,14α,25-trihydroxy-5β-cholest-7-en-6-one) of high specific activity (2.2 TBq/mmol).We have examined the capacity of various endocrine (prothoracic glands, follicle cells) and peripheral (fat body, Malpighian tubules) tissues of Locusta migratoria to use this molecule as a precursor of ecdysone biosynthesis. Efficient conversion of 2,22-dideoxyecdysone to 2-deoxyecdysone and to ecdysone could principally be monitored in the prothoracic glands and follicle cells.

Ecdysteroids are highly hydroxylated steroid hormones which control moulting in arthropods. Ecdysone 1, the mother compound of this family, is produced during postembryonic development of insects in endocrine glands, generally referred to as prothoracic glands. In adult reproductively competent female insects, it is synthesized in the ovaries.

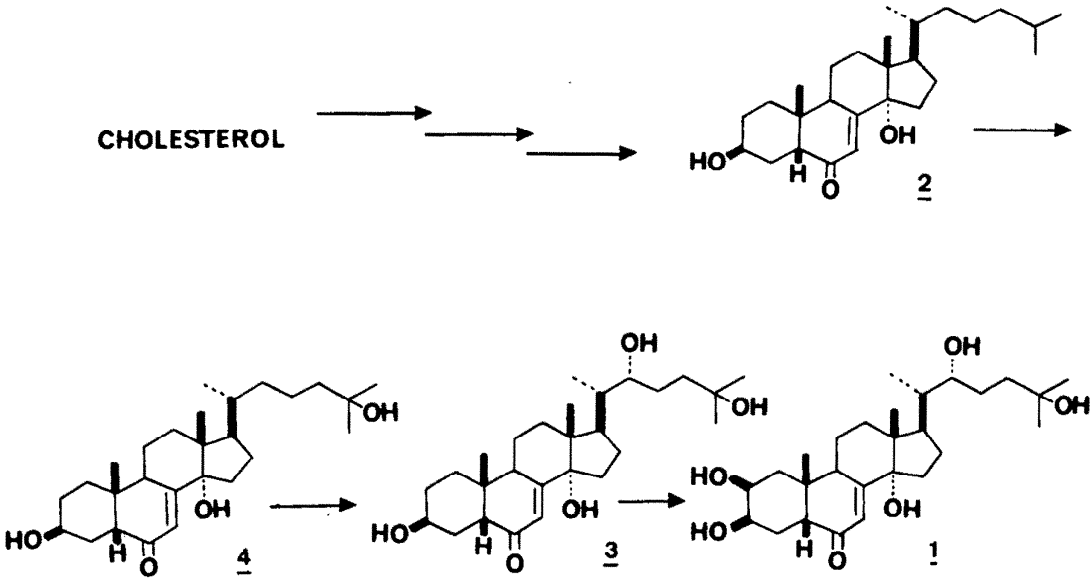
	R_1	R_2	R_3	R_4
Ecdysone 1	OH	OH	OH	H
2,22,25-Trideoxyecdysone 2 (5β-ketodiol)	H	H	H	H
2-Deoxyecdysone 3	OH	OH	H	H
2,22-Dideoxyecdysone 4	OH	H	H	H
20-Hydroxyecdysone 5	OH	OH	OH	OH
22-Deoxyecdysone 6	OH	H	OH	H



Scheme 1

Our understanding of the biosynthetic pathway of ecdysone is still fragmentary. Only the sequence of the last three steps of biosynthesis has been established in several insect species[2,3]. It consists of a series of hydroxylations of 5β-ketodiol 2 in the following order : C-25, C-22 and C-2

(scheme 2). The synthesis of tritium labelled 2-deoxyecdysone 3[4] has allowed the study of the enzyme involved in the last hydroxylation step : the C-2 hydroxylase. The tissue distribution of this enzyme has been established and some of its biochemical characteristics have been defined[5,6]. None of the other enzymes involved in the biosynthesis of ecdysone has as yet been investigated. In this paper we report the synthesis of labelled 2,22-dideoxyecdysone 4 with high specific activity, and we describe first experiments on the tissue distribution of the C-22 hydroxylase in Locusta migratoria undertaken with the new labelled precursor.



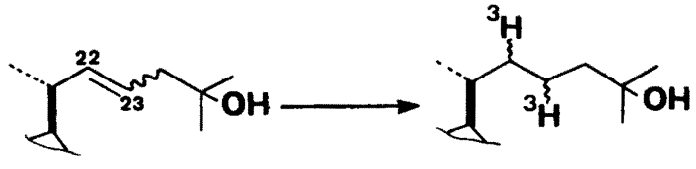
Scheme 2

CHEMICAL RESULTS.

In a previous paper we have described a multi-step synthesis of 2,22-dideoxyecdysone[1]. Several of the biological questions which we are addressing require the use of a labelled substrate, but our attempts to introduce the radioactivity (tritium) during the synthesis were unsuccessful[1]. This has led us to modify the synthetic scheme and we have aimed in particular at introducing the label during the very last step of the synthesis of 2,22-dideoxyecdysone.

The key step in our new approach is the introduction of the label on the side-chain by using a homoallylic alcohol as is shown in figure 1. This strategy allows introduction of two atoms of tritium in positions C-22 and C-23.

Figure 1

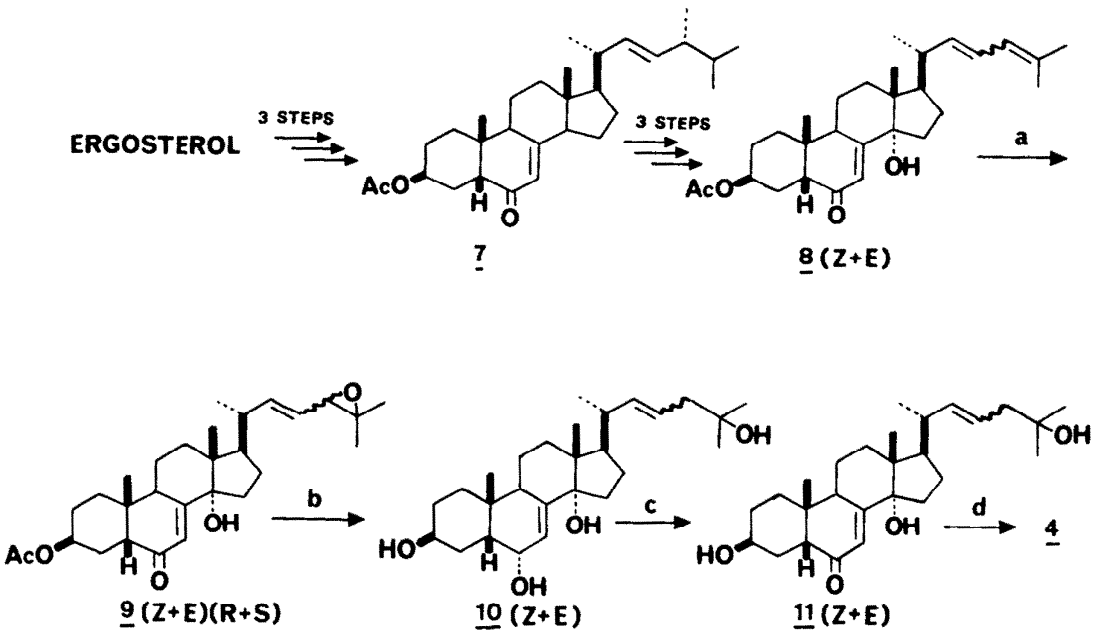


We anticipated that during the stereoselective biological hydroxylation in C-22(R), the tritium introduced in this position would be removed and we therefore decided to use a mixture of the cis and trans isomers of the Δ^{22} unsaturation to increase the statistical probability of equivalent labelling at C-22(R) and C-22(S).

2,22-Dideoxyecdysone 4 belongs to the 5β-H series (A/B cis fused rings) of 2-deoxyecdysteroids. There exists a certain number of difficulties in working

with this type of molecules and we have explained in previous papers[1,7] the methodology which we use to circumvent these difficulties.

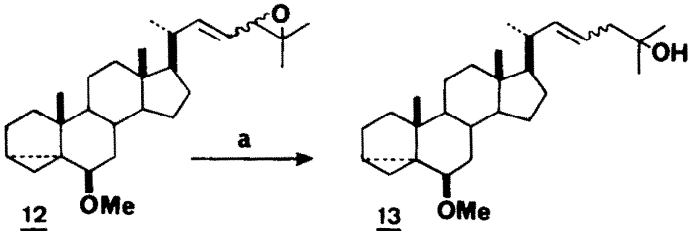
The overall synthesis of ($^3H_2$)-2,22-dideoxyecdysone is depicted in scheme 3. Compound 7[7], with A/B *cis* fused rings was obtained in three steps from ergosterol. Ozonolysis of 7 was then followed by introduction of a 14α-hydroxyl group and a Wittig reaction provided a (Z+E) mixture of compound 8[1]. All the details of these synthetic pathways are given in ref. (1,8).



Scheme 3

a: MCPBA(1 eq.)-NaF(1.5 eq.)-KF(0.5 eq.), CH_2Cl_2, R.T., 40 mn;
b: 1)$LiBHEt_3$, THF, -50°C to +5°C, 4h; 2)$Na_2SO_4.10H_2O$
c: MnO_2-$MgSO_4$, $CHCl_3$-THF-CH_2Cl_2, R.T., 12h;
d: H_2, $^2H_2$ or $^3H_2$, Pd/C 5%, THF, R.T., 3h;

The regiospecific epoxidation[9] of 8 (Z+E) was achieved by use of metachloroperbenzoic acid. Because the resulting allylic epoxide is quite unstable and highly acid sensitive, this oxidation was performed in the presence of a mixture of sodium fluoride and potassium fluoride[10]. Compound 9(Z+E)(R+S) was isolated with a 96% yield after chromatography over 'NEtMe$_2$-deactivated' silicagel. The 1,2-regioselective cleavage of the epoxide has been previously investigated on compound 12 (Z+E)(R+S) with the superhydride $LiBHEt_3$ (Scheme 4).



Scheme 4

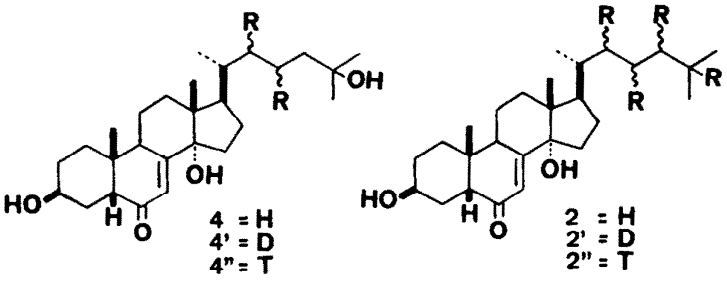
a: 1)$LiBHEt_3$, THF, R.T., 1h; 2)$Na_2SO_4.10H_2O$

This reaction gave quantitatively the homoallylic alcohol 13 (Z+E). When 9 was treated with this reagent, we could perform, in addition to the regiospecific opening of the epoxide, the stereospecific and regiospecific 1,2-reduction of

the C-6 ketone as well as the deprotection of the 3β-alcohol. This reduction allowed the stereospecific introduction of the hydride in the 6β-H configuration, which gave quantitatively the 6 (OH) alcohol[11]. The resulting deactivation of the C-5 position (5β-H stereochemistry) permits an easy handling of this molecule, without any fear of epimerisation during the reaction or even during the work up. In this way, the tetrol 10 (Z+E) was obtained with a 89% yield. A kinetic study showed that the rate-limiting step in this reaction was the opening of the epoxide.

The allylic alcohol 10, which is relatively unstable, was rapidly regenerated to the conjugated ketone by oxidation with manganese dioxide (compound 11 (Z+E),84% yield).

The hydrogenation of the $\Delta^{22(23)}$ double bond in dioxane, at room temperature, with palladium on charcoal (5%), gave the unlabelled 2,22-dideoxyecdysone 4 (R=H) together with 5% of 5β-ketodiol 2(R=H)[8](scheme 5). Hydrogenolysis of the tertiary homoallylic alcohol at C-25 can be avoided by the use of poisoned catalysts (e.g. sodium nitrite). Nevertheless, in order to shorten the critical time of tritiation we have performed the hydrogenation (4 (R=H)) and deuteriation (4' (R=D)) in the same conditions as for the tritiation, that is without poisoning the catalyst.



Scheme 5

Tritiation with $^3H_2$ in these conditions was achieved at the Commissariat à l'Energie Atomique (Saclay, France) and yielded ($^3H_2$)-2,22-dideoxyecdysone 4" (R=T) with a specific activity of 2.2 TBq/mmole (60 Ci/mmole). Besides the reduction of the $\Delta^{22(23)}$ insaturation, the palladium cacatalysed hydrogenation (deuteriation or tritiation) have given also rise to small amount of isotopic exchange in position other than the insaturated one.

The labelled 2,22-dideoxyecdysone has been carefully purified and the traces of labelled 5β-ketodiol 2" (R=T)[8] were removed by TLC. In addition, we routinely purify small amounts of the labelled 2,22-dideoxyecdysone by reverse phase HPLC before each biological experiment (see experimental part).

^1H and ^{13}C-NMR interpretations have been proposed for all the intermediates synthesized (see Table 1 and 2).

BIOLOGICAL RESULTS.

We have incubated the newly synthesized tritiated 2,22-dideoxyecdysone under *in vitro* conditions in the presence of prothoracic glands (the normal site of ecdysone biosynthesis in larvae), fat body body fragments (functional equivalent of vertebrate liver), and Malpighian tubules (functional equivalent of vertebrate kidney). The tissues were excised from last instar larvae of *Locusta* and maintained for 6 h in the presence of the tritiated molecule (185 kBq

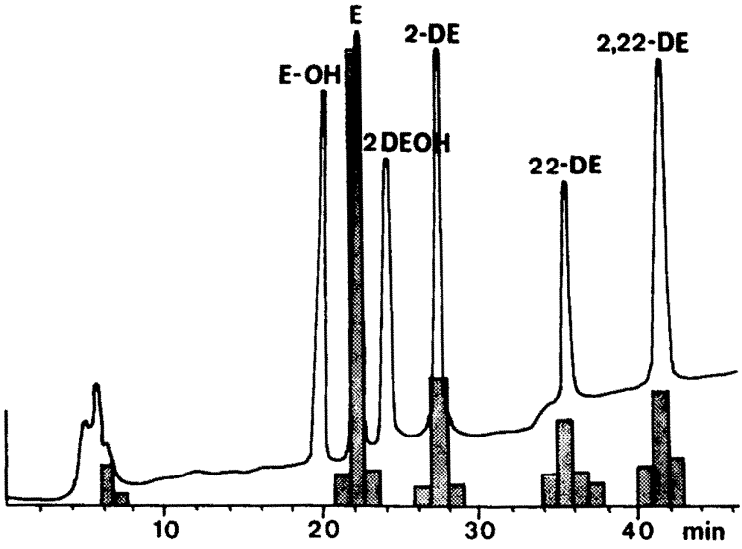


Figure 2

Conversion of 2,22-dideoxyecdysone by larval prothoracic glands of Locusta migratoria. Three pairs of prothoracic glands of fifth instar larvae were incubated for 6 h with 185 kBq (5 μCi) (0.08 μM) of 2,22-dideoxyecdysone after which 20 μl of the incubation medium was coeluted with reference ecdysteroids into a C_{18} reversed-phase HPLC column; elution with a gradient from 0 to 100% methanol in water over 30 min; full line : UV absorbance of reference molecules; columns : radioactivity measurements of aliquots of each fraction.
E-OH, 2-DEOH, E, 22-DE, 2-DE and 2,22-DE : migration of reference 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, ecdysone, 22-deoxyecdysone, 2-deoxyecdysone and 2,22-dideoxyecdysone.

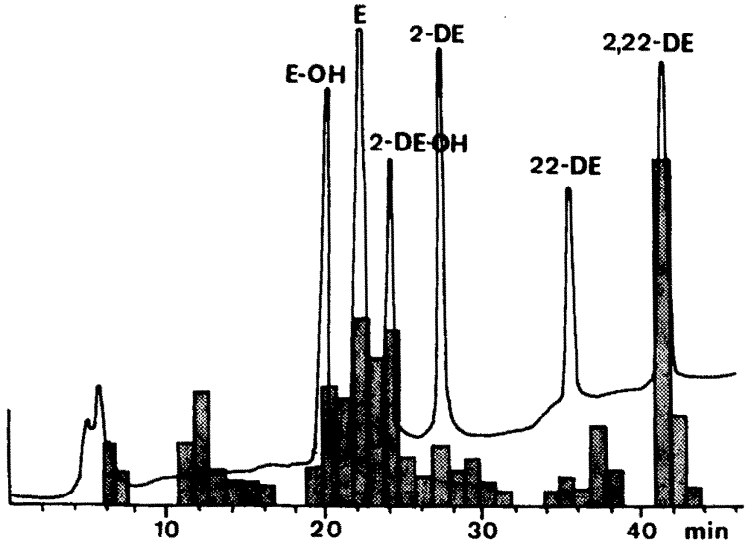


Figure 3

Conversion of 2,22-dideoxyecdysone by larval fat body of Locusta migratoria.

All excisable fat body from three 5-day-old fifth instar larvae was incubated for 6 h with 185 kBq (5 μCi) (0.08 μM) of 2,22-dideoxyecdysone, after which 20 μl of the incubation medium was coeluted with reference ecdysteroids into a C_{18} reversed-phase HPLC column; elution with a gradient from 0 to 100% methanol in water over 30 min; full line : UV absorbance of reference molecules; columns : radioactivity measurements of aliquots of each fraction. It should be noted that in this experiment the radioactive peaks do not necessarily represent pure compounds.
E-OH, 2-DEOH, E, 22-DE, 2-DE and 2,22-DE : migration of reference 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, ecdysone, 22-deoxyecdysone, 2-deoxyecdysone and 2,22-dideoxyecdysone.

in 1 ml of incubation medium), after which the steroids were extracted from either the combined tissues and incubation media or solely from the incubation media.

Figure 2 gives a representative profile of HPLC elution of tritiated ecdysteroids present in the medium after incubation of prothoracic glands with labelled 2,22-dideoxyecdysone. In addition to unconverted tritiated precursor, the chromatogramme shows the presence of three peaks of radioactivity which could be identified as 22-deoxyecdysone, 2-deoxyecdysone and ecdysone (co-elution and co-acetylation with reference compounds as detailed in (2)). Ecdysone is by far the major metabolite of 2,22-dideoxyecdysone in this experiment, which underlines that prothoracic glands have efficient enzyme systems for C-22 and C-2 hydroxylation. In contrast to prothoracic glands, fat body fragments appear (Figure 3) to be able to convert 2,22-dideoxyecdysone to a large variety of metabolites. Among these, we have ascertained the presence of 2-deoxyecdysone, 22-deoxyecdysone, 2-deoxy-20-hydroxyecdysone, ecdysone and 20-hydroxyecdysone by co-elution and co-acetylation with reference compounds (data not shown). Several other radioactive metabolites remain unidentified. The high polarity compounds seen in the chromatogramme could not be hydrolysed by the conventional Helix pomatia enzyme mixture [12].

In adult females of Locusta, prothoracic glands degenerate and an intense ecdysteroid biosynthesis occurs in the follicle cell epithelium in vitellogenic ovaries shortly before ovulation and egg-laying. We have excised follicle cells [13] and incubated them in the presence of tritiated 2,22-dideoxyecdysone for 6 h, after which the incubation medium was processed as

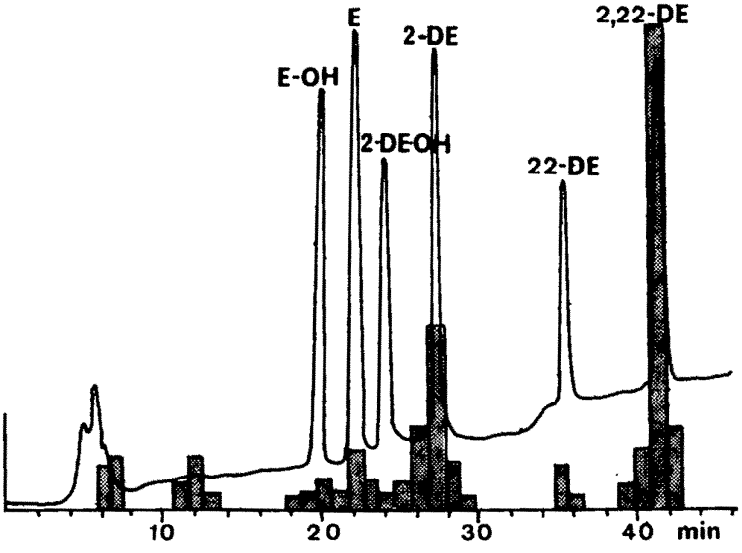


Figure 4

Conversion of 2,22-dideoxyecdysone by adult female follicle cells of Locusta migratoria.

The follicle cells were separated from 150 terminal oocytes from vitellogenic females (terminal oocytes length from 5.3 to 5.7 mm). These follicle cells were incubated for 6 h with 185 kBq (5 µCi, 0.08 µM) of 2,22-dideoxyecdysone, after which 20 µl of the incubation medium was coeluted with reference ecdysteroids into a C_{18} reversed-phase HPLC column; elution with a gradient from 0 to 100% methanol in water over 30 min; full line : UV absorbance of reference molecules; columns : radioactivity measurements of aliquots of each fraction.
E-OH, 2-DEOH, E, 22-DE, 2-DE and 2,22-DE : migration of reference 20-hydroxy-ecdysone, 2-deoxy-20-hydroxyecdysone, ecdysone, 22-deoxyecdysone, 2-deoxy-ecdysone and 2,22-dideoxyecdysone.

in the foregoing experiments. The results are presented in figure 4 and show that one major metabolite was formed in this experiment which we identified as 2-deoxyecdysone. The other products which appear in the chromatogramme were identified as 22-deoxyecdysone, ecdysone and 20-hydroxyecdysone. The polar products seen in figure 4 were hydrolysable by the Helix pomatia enzyme mixture and yielded ecdysone and 2-deoxyecdysone, indicating that they are polar conjugates of these ecdysteroids.

Finally, we have attempted to compare the efficiency of the C-22 hydroxylase in the various in vitro experiments which we have performed with tritiated 2,22-dideoxyecdysone. The sum of the molecules which had been hydroxylated at C-22 in each experiment was plotted against the protein content of the incubated tissues as shown in Table 3. It is obvious that under our conditions the prothoracic glands and the follicle cells, i.e. established biosynthetic tissues for ecdysone, have by far the highest rate of C-22 hydroxylation per mg of protein per tissue.

	Fat body	Malpighian tubules	Prothoracic glands	Follicle cells
pmol/mg protein				
C-22 hydroxylation	2.7	1.2	41.0	18.8

Table 3

Efficiency of hydroxylation at C-22 in various tissues of Locusta. The sum of the labelled precursor molecules which have been hydroxylated at C-22 during the incubation (expressed in pmol) is plotted versus the protein content (expressed in mg) of the incubated tissues.

CONCLUSION.

The chemical synthesis of high specific activity tritiated 2,22-dideoxyecdysone has allowed us to make a first investigation of the distribution of C-22 hydroxylase in an insect. We have shown that both the endocrine tissues and the peripheral tissues of Locusta migratoria have the capacity to hydroxylate 2,22-dideoxyecdysone in position C-22 and in several other positions. The efficiency of the hydroxylation at C-22 is highest in the endocrine tissues. We are now extending our investigations towards the biochemical characterization of the C-22-hydroxylase.

EXPERIMENTAL

CHEMICAL.

Melting points were measured on a Reichert microscope melting point apparatus and are uncorrected. $(\alpha)_D$ were measured on a Perkin-Elmer 141 polarimeter. I.R. spectra were recorded in KBr on a Perkin-Elmer spectrometer and a Pye Unicam SP3-300S infrared spectrophotometer Philips. U.V. Spectra were measured on a Kontron Uvikon 810 u.v.-vis. spectrophotometer. N.M.R. Spectra were recorded on a Bruker SY (200MHz) and a Bruker AM (400MHz) apparatus in $CDCl_3$, CD_2Cl_2 or CD_3OD with TMS as internal standard. M.S. were measured on a Thomson THN 208 by direct introduction for unmodified compounds, and on a LKB 9000 S apparatus coupled to a GC (OV-1 column) for $SiMe_3$-derivatization. High resolution-MS measurements have been done on the Thomson THN 208 by the 'peak-matching method'. TLC were run on pre-coated plates of silica gel 60F254 (Merck) and silica gel (200-63 μm, 40-63 μm or 15 μm , Merck) was used for column chromatography. Microanalyses were performed by the Strasbourg Division of the Service Central de Microanalyses of CNRS. Radioactivity has been determined with

an Intertechnique SL 4000 liquid scintillation counter equipped with external standards and the samples counted in the ACS (Amersham, Great Britain) liquid scintillation cocktail.

We have already described the synthesis of the 5β-isomers 7 to 8Z and 8E, the three first steps (from ergosterol to 7) being reported in (7), and the the last ones (from 7 to 8Z and 8E) being reported in (1).

3-beta-ACETOXY-14-alpha-HYDROXY-5-beta-CHOLESTA-7,22(Z+E)-DIEN-24(R+S)-24,25-EPOXY-6-ONE (9(Z+E)-(R+S))

Diene 8(Z+E) [1] (595 mg, 1.31 mmoles) was dissolved in dry CH_2Cl_2 (10 ml), and a mixture of anhydrous KF (53 mg, 0.9 mmoles, 0.7 eq.), NaF (100 mg, 2.4 mmoles, 1.8 eq.), and 3-chloroperbenzoic acid (Fluka 90%, 270 mg, 1.5 mmoles peracid, 1.1 eq.) was added[10]. The solution was stirred for 40 mn at room temperature and then filtered on celite, and the salts washed with CH_2Cl_2. After silicagel chromatography (0.040-0.063 mm; gradient from Hex-AcOEt 8:2 to Hex-AcOEt 6:4), 96% of the the four isomers 9(Z+E)-(R+S) (590 mg, 1.26 mmoles) was obtained (TLC Hex-AcOEt 1:1, R_f(8(Z+E)) = 0.60-0.65, R_f(9(Z+E)-(R+S)) = 0.30-0.40).

U.V. : λ_{max}(acetonitrile) = 244 nm, ε = 13000. I.R.(cm^{-1}) : 3600(m), 3550-3430(m), 3050((m), 2970(s), 2860(m), 1725(vs), 1660(vs), 1440((m), 1380(m), 1370(m), 1230(s), 1220(s), 1140(m), 1020(m), 870(m). M.S. : m/z 470(29) (M^+, $C_{29}H_{42}O_5$), 452(8), 442(8),437(7), 434(2), 412(2), 410(3), 395(3), 394(2), 382(4), 379(3), 373(3), 372(4), 368(2), 367(3), 354(2), 353(2), 344(3), 339(3), 335(3), 327(11), 322(2), 313(5), 285(5), 276(11), 275(8), 269(4), 268(3), 267(4), 216(14), 215(14), 97(100%). High Res. Mass Spectr. : M^+ $C_{29}H_{42}O_5$ found: 470.3026 ± 0.0017, calc.: 470.3032. Anal. : found C: 73.93 H: 9.16; require (for $C_{29}H_{42}O_5$) C: 74.04 H: 8.94. ^{1}H-NMR in table 1 and ^{13}C-NMR in table 2

3-beta,6-alpha,14-alpha-25-TETRAHYDROXY-5-beta-CHOLESTA-7,22(Z+E)-DIENE (10(Z+E))

Epoxide 9(Z+E)-(R+S) (85 mg, 0.18 mmoles) was dissolved in THF (1 ml, freshly distilled over $LiAlH_4$), cooled to -50°C, and $LiBHEt_3$ 1M (4 ml) was added at this temperature (1 ml/h during 4h). The solution was then allowed to come slowly to +5°C. TLC (Hex-AcOEt-MeOH 1:1:0.2) study of the reaction showed the successive formation of three intermediates during the reduction : the first reaction was the acetate deprotection (R_f= 0.45), then the 6-ketone reduction (R_f=0.3) and finally the epoxide opening (R_f=0.2) at much higher temperature (0°C), that afforded the tetrol 10(Z+E). When after 4h the reaction was completed, the excess of hydride was carefully destroyed by addition of $Na_2SO_4.10H_2O$ in the medium with vigorous stirring at 0-10°C. After filtration on celite, the salts were washed with $CHCl_3$-CH_2Cl_2-MeOH and the compound chromatographed on SiO_2 (0.040-0.063 mm; gradient from Hex-AcOEt 1:1 to Hex-AcOEt-MeOH 1:1:0.1). In this way, 89% of the unstable tetrol 10(Z+E) (69 mg, 0.16 mmoles) was obtained (TLC Hex-AcOEt-MeOH 1:1:0.2 , R_f(9(Z+E)-(R+S)) = 0.85, R_f(10(Z+E)) = 0.2).

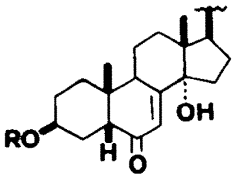
I.R.(cm^{-1}) : 3660(s), 3550-3300(m), 3100(m) 3080(s), 2980(s), 2860(m), 1450(m), 1380(m), 1330(m), 1250(m), 1020(vs), 920(w). M.S. : m/z 432(7) (M^+, $C_{27}H_{44}O_4$), 414(30), 399(15), 396(21), 381(12), 374(25), 363(6), 356(100%), 341(15), 338(15), 323(9), 288(44), 287(37), 269(33), 219(37), 218(26), 217(39). High Res. Mass Spectr. : M^+ $C_{27}H_{44}O_4$ found: 432.3228 ± 0.0017 calc.: 432.3239. ^{1}H-NMR in table 1 and ^{13}C-NMR in table 2

3-beta,14-alpha,25-TRIHYDROXY-5-beta-CHOLESTA-7,22(Z+E)-DIEN-6-ONE (11(Z+E))

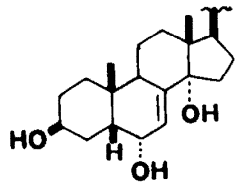
Tetrol 10(Z+E) (302 mg, 0.7 mmoles) was dried overnight under vacuum at 35°C, dissolved (using ultrasons at 60°C) in a mixture of dry $CHCl_3$ (5 ml) and dry THF (5 ml) and then $MgSO_4$ (anhydrous, 1 g) and MnO_2 (Janssen activated, 1 g, 11 mmoles) were added. After one night stirring, the yellow solution was filtered on celite and the oxide washed with $CHCl_3$-CH_2Cl_2-MeOH. SiO_2-chromatography (0.040-0.063 mm; gradient from Hex-AcOEt 1:1 to Hex-AcOEt-MeOH 1:1:0.15) afforded 84% of the ketone 11(Z+E) (252 mg, 0.59 mmoles) (TLC Hex-AcOEt-MeOH 1:1:0.2 , R_f(11(Z+E)) = 0.35, R_f(10(Z+E)) = 0.2).

U.V. : λ_{max}(acetonitrile) = 240 nm , ε = 12000. I.R.(cm^{-1}) : 3520(s), 3500-3200(s), 2980(s), 2940(s), 2880(m), 1630(vs), 1450(m), 1380(m), 1360(m), 1160(m), 1040(m). M.S. : m/z 430(10) (M^+, $C_{27}H_{42}O_4$), 415(4), 412(15), 402(4), 397(24), 394(11), 384(30), 379(6), 372(79), 354(28), 344(33), 339(14), 315(11), 311(10), 303(11), 287(70), 234(100%), 233(98), 59(68). High Res. Mass Spectr. : M^+ $C_{27}H_{42}O_4$ found: 430.3074 ± 0.0012 calc.: 430.3083. Anal. : found C: 73.79 H: 10.24; require (for $C_{27}H_{42}O_4.1/_2MeOH$) C: 73.99 H: 9.87. ^{1}H-NMR in table 1 and 13C-NMR in table 2

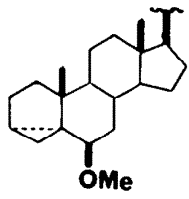
	2	4	9Z		9E	10		11		13	
			R	S		Z	E	Z	E	Z	E
3-H	4.01	4.01	5.03		5.03	4.09		4.02			
4-H										0.44	
4-H										0.66	
5-H	2.45	2.45	2.34		2.34	2.05		2.45			
6-H						4.56				2.78	
7-H	5.83	5.83	5.83		5.83	M		5.83			
9-H	3.23	3.22	3.13		3.13	2.83		3.24			
18-H	0.74	0.74	0.76	0.77	0.73	0.75	0.77	0.78	0.76	0.76	0.74
19-H	1.00	1.00	1.00		1.01	0.97		1.00		1.04	
20-H			2.70		2.24	2.58	2.15	2.60	2.18	2.47	2.15
21-H	0.95	1.00	1.07		1.08	1.01	1.05	1.02	1.07	0.98	1.04
22-H			5.15	5.14	5.80	5.33	5.45	5.36	5.48	5.28	5.38
23-H			5.64	5.59	5.32	5.36	5.45	5.39	5.48	5.32	5.35
24-H			3.45		3.14	2.34*	2.15	2.35	2.18	2.32*	2.15
26-H	0.91	1.21	1.28	1.29	1.27	1.23	1.19	1.22	1.19	1.20	1.25
27-H	0.94	1.21	1.36		1.33	1.23	1.19	1.22	1.19	1.21	1.25
1'-H										3.33	
Solv.	3.34	3.34	5.35		5.35	3.34		3.34		7.27	



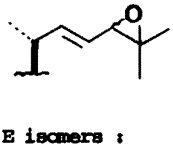
H-3(e): δ = 4.05m(R=H), $w_{1/2} \simeq 15$Hz; δ = 5.03m(R=Ac), $w_{1/2} \simeq 10$Hz
H-5(a): δ = 2.45dd(R=H); δ = 2.34dd(R=Ac); J_{5a-4a} = 12Hz;
J_{5a-4e} = 4Hz
H-7 : δ = 5.85d; J_{7-9a} = 2.5Hz
H-9(a): δ = 3.22dt; J_{9a-11} = 10Hz; J_{9a-7} = 3Hz;
this multiplet is generally not resolved : $w_{1/2} \simeq 20$–25Hz

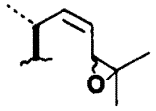


H-3(e) : δ = 4.1m, $w_{1/2} \simeq 7$Hz
H-9(a) : δ = 2.8m, $w_{1/2} \simeq 20$Hz
H-6β(a): δ = 4.6m, $w_{1/2} \simeq 11$Hz
H-7 : δ = 5.42m, $w_{1/2} \simeq 6$Hz



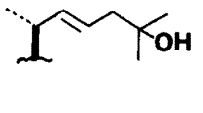
H-4: $\delta_{4\alpha}$ = 0.43dd; $\delta_{4\beta}$ = 0.65dd, $J_{4\alpha-3\alpha}$ = 8Hz,
$J_{4\beta-3\alpha}$ = 5Hz, $J_{4\alpha-4\beta}$ = 5Hz
H-6: δ = 2.77dd, $J_{6-7\alpha} = J_{6-7\beta}$ = 2.7Hz

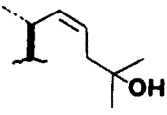




E isomers :
H-21: δ = 1.08, J = 6.5Hz; H-20: δ = 2.24m;
H-22: δ =5.80dd; H-23: δ =5.32dd; H-24: δ =3.14d
J_{23-24} = 8Hz, J_{23-22} = 15Hz, J_{22-20} = 9Hz

Z isomers:
H-21: δ = 1.07d, J =6.5Hz; H-22: δ =5.15dd
H-23: δ = 5.64dd; H-24: δ = 3.45d
J_{20-22} = 8Hz, J_{22-23} = 11Hz, J_{22-24} = 2Hz,
J_{23-24} = 8Hz, J_{23-20} = 1Hz





E isomers:
H-21: δ = 1.07, J=6.5Hz; H-22: δ = 5.48dd;
H-23: δ =5.48td; H-24: δ =2.18m; J_{22-23} =15Hz,
J_{23-24} =5.5Hz, J_{22-20} =7.5Hz, $J_{24A-24B}$ =15Hz
(from irradiation at 2.22ppm)

Z isomers:
H-21: δ = 1.02, J = 6.5Hz
H-22: δ = 5.36dd; H-23: δ = 5.39m;
H-24A: δ = 2.35dd; H-24B: δ = 2.24dd
J_{22-23} =10.6Hz, J_{22-20} =6.2, $J_{24A-24B}$ =15Hz

TABLE 1: ^{1}H-NMR chemical shifts
(δ ppm from TMS)

a = axial, e = equatorial referring to the A-cycle for H-3 and H-5, and to the B-cycle for H-6; d = doublet; m = multiplet; t = triplet
* : for the H-24A proton, and δ = 2.23ppm for H-24B; M : masked by H_2O

3-beta,14-alpha,25-TRIHYDROXY-5-beta-CHOLEST-7-ENE-6-ONE (4(R=H))

Compound 11(Z+E) (61 mg, 0.14 mmoles) was dissolved in freshly distilled dioxane (10 ml) by ultrasonication at 90°C, till the product was completely dissolved. Hydrogenation can then be conducted over Pd/C 5% (palladium) at room temperature during 3h (to make sure that there is no starting material left). TLC and HPLC analysis showed the presence of 5% of the 25-OH hydrogenolysis product, identified by comparison with an authentic sample of 3-beta,14-alpha-DIHYDROXY-5-beta-CHOLEST-7-EN-6-ONE 2(R=H)[8] (= 5β-ketodiol). After filtration through a millipore membrane (0.5 µm, solvent resistant), the hydrogenation product was chromatographed on SiO_2 (0.063-0.200 mm; gradient from Hex-AcOEt 1:1 to Hex-AcOEt-MeOH 1:1:0.06), which yielded 87% of the 2,22-dideoxy-ecdysone 4(R=H) (53 mg, 0.12 mmoles) (TLC Hex-AcOEt-MeOH 1:1:0.2 , R_f(4) = R_f(11(Z+E)) = 0.35, R_f(2) = 0.45; mild revelation with a solution of 1% H_2SO_4 in EtOH and 1 g of vanilin led to a grey-brown spot for 11(Z+E) and a red spot for 4).

m.p.(AcOEt-Hex-MeOH) : 262-263°C. U.V. : λ_{max}(acetonitrile) = 239 nm , ε = 12000. $(\alpha)_D^{22}$= +66° (c = 7.6 , MeOH). I.R.(cm^{-1}) : 3550-3300(s), 2980(s), 2970(s), 2880(m), 1650(s), 1460(m), 1440(m), 1380(m), 1140(m), 1050(w).
M.S. : m/z 432(9) (M^+, $C_{27}H_{44}O_4$), 414(30), 404(17), 399(26), 396(22), 386(84), 381(14), 353(9), 341(4), 338(3), 325(4), 323(4), 315(6), 285(13), 234(50), 233(100%), 215(18), 207(16), 194(11).
G.C.-M.S. ($SiMe_3$-derivatization) :

a) Trisilylated compound: m/z 648(22) (M^+, $C_{36}H_{68}O_4Si_3$), 633(19), 620(29), 558(22), 543(12), 468(11), 453(16), 377(17), 131(100%)
Isotopic peaks in the molecular area : M^+ : 648(22); $(M+1)^+$: 649(11) ((M)/(M)+(M+1) = 67%; (M+1)/(M)+(M+1) = 33%)

b) Disilylated compound : m/z 576(4) (M^+, $C_{33}H_{60}O_4Si_2$), 561(23), 543(3), 533(10), 518(6), 486(18), 471(9), 468(10), 396(6), 381(7), 305(9), 131(100)

High Res. Mass Spectr. : M^+ $C_{27}H_{44}O_4$ found: 432.3240 ± 0.0012; calc.: 432.3239
Anal. : found C: 75.19 H: 10.28; require (for $C_{27}H_{44}O_4$) C: 75.00 H: 10.19
^{1}H-NMR in table 1 and ^{13}C-NMR in table 2

($^2H_2$-22,23)-3-beta,14-alpha,25-TRIHYDROXY-5-beta-CHOLEST-7-ENE-6-ONE (4'(R=D))

Same procedure as for 4(R=H) was run on 11(Z+E) (10.5 mg, 24.4 µmoles) in the presence of Pd/C 5% and D_2 (deuterium gas) during one night. 83% of the deuteriated compound 4'(R=D) (8.5 mg, 20 µmoles) was obtained (TLC : same as for 4(R=H)).

M.S. : m/z 434(5) (M^+, $C_{27}H_{42}D_2O_4$), 416(13), 406(12), 401(19), 388(58), 355(6), 343(4), 327(3), 317(4), 285(11), 234(55), 233(100%), 215(12), 207(16), 194(12)

- Isotopic peaks in the molecular area : $M^+(C_{27}H_{42}D_2O_4)$: 434(5); $(M+1)^+(C_{27}H_{41}D_3O_4)$: 435(5).
- Corrected intensities by comparison with the natural isotopic ratio of 4(R=H): (M)=66% (= % of dideuteriated compound), (M+1)=34% (= % of trideuteriated compound) (this isotopic ratio, relative to the molecular peak, is compatible with that of each of the eight first fragmentation peaks)

G.C.-M.S. ($SiMe_3$-derivatization) : m/z 650(20) (M^+, $C_{36}H_{66}D_2O_4Si_3$), 635(22), 622(32), 560(27), 545(19), 470(28), 455(29), 377(36), 131(100%).

- Isotopic peaks in the molecular area : $(M+2)^+(C_{36}H_{64}D_4O_4Si_3)$: 652(14); $(M+1)^+(C_{36}H_{65}D_3O_4Si_3)$: 651(21); $M^+(C_{36}H_{66}D_2O_4Si_3)$: 650(22); $(M-1)^+(C_{36}H_{67}D_1O_4Si_3)$: 649(11).
- Corrected intensities by comparison with the natural isotopic ratio of "4(R=H)-($SiMe_3$)$_3$" : if ((M-1)+(M)+(M+1)+(M+2))=100%, then (M-1)=25% (= % of monodeuteriated compound); (M) = 40% (= % of dideuteriated compound); (M+1)=25% (= % of trideuteriated compound); (M+2)=10% (= % of tetradeuteriated compound); and the ratio of (dideuteriated compound)/(trideuteriated compound) is 6/4 ((M)/(M)+(M+1)=62%, (M+1)/(M)+(M+1)=38%) (these relative deuteriation ratios, calculated for the molecular peak area, are also compatible with those of each of the six first fragmentation peaks areas of the spectrum).

High Res. Mass Spectr. : M^+ $C_{27}H_{42}D_2O_4$ found: 434.3365 ± 0.0012; calc.: 434.3365
^{1}H-NMR : same as for 4(R=H). ^{2}H-NMR : (δ ppm from TMS) H-23: 1.2(m, $w_{1/2}$≃20Hz); H-22: 1.4(m, $w_{1/2}$ ≃ 20Hz); solvent: (CH_3OH) 3.34

($^3H_2$-22,23)-3-beta,14-alpha,25-TRIHYDROXY-5-beta-CHOLEST-7-ENE-6-ONE (4"(R=T))

Same procedure as for 4(R=H) was run on 11(Z+E) (4.1 mg, 0.95 mmoles) in the presence of Pd/C 5% and $^3H_2$ (tritium gas) for 4h. Identification of the products has been made by TLC and HPLC, by comparison with the unlabelled references 4(R=H) and 2(R=H)[8] and with the labelled reference 2"(R=T)[8].
In this way, we have confirmed that the 25-(OH) hydrogenolysis side product

2"(R=T) was present in 7% yield after the tritiation. After filtration through a millipore membrane (0.5 µm, solvent resistant) and the consecutive purification procedure, the labelled 2,22-dideoxyecdysone 4"(R=T) was obtained with a specific activity of 2.2 TBq/mmole (60 Ci/mmole) (TLC Hex-AcOEt-MeOH 1:1:0.2 , R_f(4"(R=T))=0.35, R_f(2"(R=T))=0.45).

^{3}H-NMR : (δ ppm from TMS; we give also an estimation of the % of labelling for each position) H-22 : δ = 1.2 (30%); H-23 : δ = 1.7 (40%); (we also have labelling at unidentified positions : δ = 1.0 (18%), δ = 2.0 (12%)).
HPLC-Techniques: Purification of small amounts of the labelled 2,22-dideoxyecdysone 4"(R=T) has been performed by use of HPLC before each biological experiment. Conditions: isocratic (methanol/water 70/30) reverse phase (uBondapak C_{18}), retention time 17 mn.

25-HYDROXY-6-beta-METHOXY-3,5-alpha-CYCLO-CHOLEST-22(Z+E)-ENE (13(Z+E))

An isomeric mixture of 12(Z+E)-(R+S) (prepared in the same way as for 9) (125 mg, 0.30 mmoles) was dissolved in freshly distilled THF (5 ml), and a large excess of $LiBHEt_3$ 1N (3 ml, 3 mmoles) was added at room temperature. When after 1h the reduction was complete, the excess of hydride was carefully hydrolysed with $Na_2SO_4.10H_2O$ (1 g) at 0°C, and the mixture was then filtered through a small amount of SiO_2. 95% of TLC-pure compound 13(Z+E) (119 mg, 0.29 mmoles) was obtained (TLC Hex-AcOEt 8:2, R_f(12(Z+E)-(R+S)) = 0.80-0.85, R_f(13(Z+E)) = 0.35).

M.S. : m/z 414(2) (M^+, $C_{28}H_{46}O_2$), 399(6), 396(3), 382(3), 367(8), 359(11), 356(100%), 341(29), 324(100%), 301(46), 298(25), 256(39), 255(36). High Res. Mass Spectr. : M^+ $C_{28}H_{46}O_2$ found: 414.3477 ± 0.0017; calc.: 414.3498. ^{1}H-NMR in table 1 and ^{13}C-NMR in table 2

	9Z R*	9Z S*	9E R*	9E S*	10 Z	10 E	11 Z	11 E	4	2	13 Z	13 E
1	25.4°		25.4°		28.8°		28.4°		28.8°	30.0*	33.2	
2	26.6°		26.6°		30.0°		30.7°		28.8°	28.8*	24.8	
3	67.9		67.9		67.9		66.3		66.3	66.4	21.3	
4	29.3+		29.3+		29.0°		34.1°		34.1°	38.4	13.0	
5	51.8*		51.8*		33.4		52.5+		52.7*	53.2	43.2	
6	202.7		202.7		68.4		207.3		207.5	207.3	82.3	
7	121.1		121.1		124.0		122.6		122.6	122.6	35.1	
8	165.1		165.1		144.5		169.2		169.3	169.3	30.4	
9	36.4		36.4		44.2		36.0		35.7		47.9	
10					37.6		38.5		38.4		34.9	
11	21.3		21.3		22.5		22.9		22.8+	23.0	22.6	
12	29.4+		29.4+		32.4+		29.0°		29.9°	32.7+	40.1	
13	47.0				48.9				45.7	solv	42.6	
14	84.9		84.9		87.7		86.7		86.7	86.8	56.4	
15	31.7+		31.7+		33.9+		33.1°		33.2°	34.2+	24.1	
16	31.0+		31.0+		33.8+		32.7°		32.6°	33.3+	28.0	
17	50.17	50.45	49.96	50.07	52.7	53.1	53.2+	52.9+	52.7*	52.8	55.7	
18	16.02	16.11	15.9		18.1	18.3	17.5	17.6	17.2	17.2	12.5	
19	23.8		23.8		26.9		25.3		25.3	25.3	19.2	
20	34.86	34.92	39.9		42.8	36.5	36.0	42.2	37.8	37.8	34.2	
21	20.58	20.85	20.3	20.5	22.4	22.7	22.4	22.1	20.3	20.3	20.6	
22	122.5	122.6	123.3		125.9	124.5	124.6	126.0	38.8	38.4	121.1	122.3
23	143.1		142.7		142.6	140.8	140.3	142.0	22.5+	25.9	139.9	141.8
24	59.88	60.12	64.19	64.24	49.5	43.8	43.6	48.9	46.2	41.6	41.6	46.7
25					72.7		72.5		72.4	30.1	70.4	70.8
26	24.58		24.5		30.0	30.4	30.1	30.0	30.0	24.0	29.0	
27	19.1		18.8		30.1	30.2	29.95	29.9	30.2	23.8	28.9	
28												
1'	170.5		170.5								56.4	
2'	21.3		21.3									
solv.	53.80		53.80		49.90		49.89		49.90	50.34	76.92	

TABLE 2 : ^{13}C-NMR chemical shifts (δ ppm from TMS)
(*, +, ° : interchangeable assignments)

BIOLOGICAL.

Studies of the metabolism of ($^3H_2$)-2,22-dideoxyecdysone.

In vitro incubations : All dissections were performed under sterile conditions. The various excised tissues were rinsed repeatedly in Landureau's medium (7) and incubated in 1 ml of the same medium in the presence of 185 kBq

(0.08µM) of ($^3H_2$)-2,22-dideoxyecdysone for 6 h at 33°C.

Extraction and purification : The incubated tissues were homogenized together with the incubation medium in 95% aqueous ethanol and heated at 60°C for 10 min. After centrifugation, the pellet was reextracted repeatedly with 95% ethanol; the combined supernatants were dried under nitrogen and redissolved in methanol. The compounds were then purified by HPLC by co-elution with reference molecules on a C_{18} reversed-phase column (µBondapack, 10µm, Waters) and eluted with a non linear gradient of 0 to 100% of methanol in water (curve 4; gradient controller 680 Waters). Fractions of 500 µl were collected and elution of the reference substances was monitored by UV absorption at 254 nm.

The identity of labelled ecdysteroids was ascertained by co-acetylation with unlabelled reference molecules in a mixture of 100 µl anhydrous pyridine and 50 µl acetic anhydride for 1 h at room temperature. The reaction was stopped by addition of 300 µl methanol and the hydrolysis of acetic anhydride was allowed to proceed for over night at 4°C. After dessication of the mixture under nitrogen, the products were chromatographied by reversed-phase HPLC in appropriate solvent systems[2].

Protein concentration was determined according to Lowry *et al.*[14].

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(11) No detectable amount of the 6α-H (= 6β(OH)) isomer was observed, neither by 400 ^{1}H-NMR, nor by TLC. By comparison, the use of $NaBH_4$-$CeCl_3$ in THF resulted in a mixture of the two C-6 isomers in the ratio $6\beta\text{-H}/6\alpha\text{-H} = 6\alpha(OH)/6\beta(OH) > 9/1$ [1].

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