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Characterization of ecdysteroids in *Drosophila melanogaster* by enzyme immunoassay and nano-liquid chromatography–tandem mass spectrometry

Catherine Blais[a,*], Thierry Blasco[b], Annick Maria[a], Chantal Dauphin-Villemant[a], René Lafont[c]

[a] UPMC Univ Paris 06, UMR CNRS 7622, Equipe Biogenèse des signaux hormonaux, Case 29, 7 Quai Saint Bernard, F-75005 Paris, France
[b] UPMC Univ Paris 06, Plate-forme de Spectrométrie de Masse et Protéomique, IFR 83, Case 41, 4 Place Jussieu, F-75005Paris, France
[c] UPMC Univ Paris 06, Laboratoire BIOSIPE ER3, Case 29, 7 Quai Saint Bernard, F-75005 Paris, France

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ABSTRACT

Ecdysteroids are polyhydroxylated steroids that function as molting hormones in insects. 20-Hydroxyecdysone (a 27C-ecdysteroid) is classically considered as the major steroid hormone of *Drosophila melanogaster*, but this insect also contains 28C-ecdysteroids. This arises from both the use of several dietary sterols as precursors for the synthesis of its steroid hormones, and its inability to dealkylate the 28C-phytosterols to produce cholesterol. The nature of Drosophila ecdysteroids has been re-investigated using both high-performance liquid chromatography coupled to enzyme immunoassay and a particularly sensitive nano-liquid chromatography–mass spectrometry methodology, while taking advantage of recently available ecdysteroid standards isolated from plants. *In vitro* incubations of the larval steroidogenic organ, the ring-gland, reveals the synthesis of ecdysone, 20-deoxy-makisterone A and a third less polar compound identified as the 24-epimer of the latter, while wandering larvae contain the three corresponding 20-hydroxylated ecdysteroids. This pattern results from the simultaneous use of higher plant sterols (from maize) and fungal sterols (from yeast). The physiological relevance of all these ecdysteroids, which display different affinities to the ecdysteroid receptors, is still a matter of debate.

1. Introduction

Ecdysteroid hormones control major developmental events and reproduction of insects. The chemical diversity of these polyhydroxylated steroids has been underlined by extensive studies using advanced analytical methods [1]. Even if 20-hydroxyecdysone (20E) is classically considered as the major steroid hormone in the fruit fly *Drosophila melanogaster*, the reality is more complex. As other insects, Drosophila depends exclusively on dietary sterols to produce its steroids. During larval growth of higher Diptera, ecdysteroid biosynthesis takes place in specialized cells of a complex endocrine structure, the ring-gland. Secreted ecdysteroids lack the hydroxy group at C-20, as the enzyme catalyzing 20-hydroxylation, ecdysone 20-monooxygenase, is not expressed in the ring-gland itself, but in various peripheral tissues including fat body [2,3]. Owing to both their inability to dealkylate 24-alkyl sterols to produce cholesterol and their capability to use different sterols as substrates for their steroid hormones, Drosophila larvae consequently contain both 27C- and 28C-ecdysteroids, previously identified as 20E and makisterone A (MaA) [4,5]. The relative proportions of these ecdysteroids depend on the sterol composition of the larval diet [4,5]. Earlier studies demonstrated that Drosophila ring-glands produce both ecdysone and 20-deoxymakisterone A (20dMaA) (Fig. 1), but a third less polar unidentified ecdysteroid was also detected [4,6]. Drosophila larvae feed partly on yeasts, which are known to contain particular 28C-sterols (the major one being ergosterol), differing from those of higher plants by the stereochemistry of their 24-alkyl substituent. Whenever used without dealkylation by insects to produce ecdysteroids, such fungal sterols would not give rise to MaA, but instead to its 24-epimer, as was found to be the case for a leaf-cutting ant [7].

The presence of 24-*epi*-makisterone A (24epiMaA) in Drosophila was therefore an attractive hypothesis, thus prompting us to reinvestigate the nature of Drosophila ecdysteroids. High-performance liquid chromatography coupled to enzyme immunoassay (HPLC-EIA) was used as a first step of purification and pre-identification of ecdysteroids. This very sensitive and specific technique is however limited to ecdysteroids which are recognized by our currently used antisera. Recently, liquid chromatography coupled to electrospray tandem mass spectrometry (LC–MS/MS) has been used to identify ecdysteroids in biological samples from plants [8,9] or animals [10,11]. This specific technique is sensitive enough to quantify

Abbreviations: 20E, 20-hydroxyecdysone; MaA, makisterone A; 20dMaA, 20-deoxymakisterone A; 24epiMaA, 24-*epi*-makisterone A; CE, collision energy; DP, desolvating potential; EcR, ecdysteroid receptor; EIA, enzyme immunoassay; TIC, total ion chromatogram.

* Corresponding author. Tel.: +33 1 44 27 65 84; fax: +33 1 44 27 65 09.
E-mail address: Catherine.Blais@snv.jussieu.fr (C. Blais).

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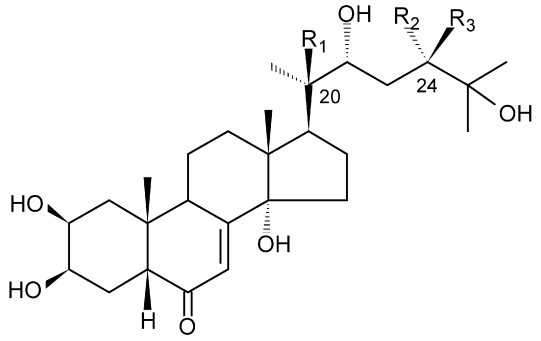

Fig. 1. Structure of ecdysteroids referred to in this paper. $R_1 = R_2 = R_3 = H$: ecdysone; $R_1 = R_2 = H$, $R_3 = Me$: 20-deoxymakisterone A; $R_1 = R_3 = H$, $R_2 = Me$: 24-*epi*-20-deoxymakisterone A; $R_1 = OH$, $R_2 = R_3 = H$: 20-hydroxyecdysone; $R_1 = OH$, $R_2 = H$ $R_3 = Me$: makisterone A; $R_1 = OH$, $R_2 = Me$ $R_3 = H$: 24-*epi*-makisterone A.

steroids at trace levels in biological matrices (i.e. ecdysteroids in the range of $\mu g\,L^{-1}$ [11], or vertebrate corticosteroids in the range of $ng\,L^{-1}$ using micro-LC/MS/MS [12]). Our aim was to precise the chemical nature of ecdysteroids present in Drosophila at the end of larval development, when the hormones peak at about 200 pg per larva, just before pupariation [13], and to estimate their relative quantities. We used an ultra sensitive nanoLC–tandem mass spectrometry (nanoLC–MS/MS), and took advantage of recently available ecdysteroid standards isolated from plants. Our study has focused both on the characterization of ecdysteroids produced by larval ring-glands *in vitro* and of those present in third (last) instar larvae.

2. Experimental

2.1. Animals

Drosophila melanogaster larvae were raised at 24 °C on a standard medium containing (for 1 L) 83.2 g dry baker's yeast, 83.2 g maize flour, 11.2 g agar and 25 mL Moldex®. Cholesterol (Sigma) was added to some media to a final concentration of 0.03% (w/w). Third-instar larvae were collected at the end of the wandering stage, 90–96 h post-hatching, around the time of the large ecdysteroid peak [13]. They were washed, blotted dry, then stored in methanol (10 larvae per 250 μL) at −20 °C until use.

Brain/ring-gland complexes were dissected from late-wandering third-instar larvae, rinsed in ice-cold Schneider's medium (Gibco-BRL) and incubated *in vitro* in new Schneider's medium (10–12 complexes per 100 μL) for 4–5 h, at 25 °C. Another batch of brain/ring-gland complexes was incubated in the presence of fat body from wandering third-instar larvae. After dissection, fat body from 10 larvae was rinsed two times in cold Schneider's medium, transferred to a culture vial with new medium, then 10 brain/ring-gland complexes were added, and incubated for 5 h. Media were then pooled in Eppendorf tubes and stored at −20 °C until further analysis.

2.2. Ecdysteroid extraction

Larvae in each vial were homogenized in methanol by hand using a close-fitting plastic pestle. The mixture was sonicated, centrifuged, and the residue was re-extracted with methanol (250 μL). After evaporation of the methanol phase, a partition between chloroform and water (500 μL each) was performed twice. All aqueous phases were pooled (about 100 larvae/HPLC analysis) and purified on a C_{18} Sep-pak cartridge [14]: a polar fraction was first eluted with 5 mL 30% methanol and ecdysteroids were then eluted with 5 mL of absolute methanol. No immunoreactivity (see Section 2.4) was ever detected in the 30% fraction, which was not further analyzed. Culture media were purified by adsorption on a C_{18} Sep-pak cartridge, which was rinsed with water then eluted with pure methanol. Methanol eluates were evaporated and samples re-dissolved in HPLC mobile phase for analysis.

2.3. HPLC separations

Ecdysteroids were analyzed on a Beckman apparatus (System Gold), with UV detection at 245 nm and an isocratic normal-phase (NP) mode, at a flow rate of $1\,mL\,min^{-1}$. We used a Zorbax® Sil column (250 × 4.6 mm i.d.) and ecdysteroids were eluted with a mixture of dichloromethane/propan-2-ol/water (125:30:1.5, v/v/v). Collected fractions (0.7 mL each) were divided into a 150 μL aliquot (for nanoLC–MS/MS analyses), and the remaining part for ecdysteroid determination by enzyme immunoassay. All samples were then evaporated to dryness. Using the addition of tritium-labelled ecdysteroids, the recovery of this procedure was shown to lie in the range of 60% after the HPLC step.

2.4. Enzyme immunoassay detection of ecdysteroids

Ecdysteroids were detected with an enzyme immunoassay (EIA) adapted from the method described by [15], by using goat anti-rabbit IgG (Jackson Immunoresearch Lab) and 2-succinyl-20-hydroxyecdysone coupled to peroxidase as enzymatic tracer. The enzymatic activity was measured using *ortho*-phenylene-diamine (Sigma) as substrate. The polyclonal anti-ecdysone antiserum L2 (generous gift from Dr. M. De Reggi, Marseille) was used because of its great sensitivity towards several ecdysteroids. Its highest affinity was towards ecdysone (52 fmol giving 50% maximum binding (I_{50})). It displayed lower, but significant, affinities towards other ecdysteroids. The cross-reactivities for the different reference ecdysteroids (I_{50} test ecdysteroid/I_{50} ecdysone) were as follows: ecdysone, 1; 20E, 6.4; MaA, 50; 20dMaA, 22; 24epiMaA, 22.3. In routine experiments, calibration curves were generated with ecdysone (range 4–500 fmol) and results given as ecdysone equivalents. Dried samples were resuspended in EIA buffer solution. The HPLC-EIA allows us to detect immunoreactive peaks at precise retention times, corresponding eventually to known ecdysteroids. The quantification is accurate for ecdysone used in the calibration curve, it is underestimated for other ecdysteroids less recognized by antiserum L2. A better quantitative estimation is obtained after correction with the known cross-reactivity factors.

2.5. NanoLC ESI-MS/MS

NP-HPLC aliquots corresponding to immunoreactive peaks, as detected by EIA, were pooled for analysis by nanoLC–MS/MS. The LC system consisted of a Dionex-LC Packings Ultimate Plus integrated micro-, capillary and nano-HPLC system, including a helium degasser, a first micropump with a 650:1 split device, a WPS-3000 autosampler, and a Switchos switching device, including a second micropump used for the precolumn. A Dionex Acclaim Pepmap100 column (150 mm × 75 μm i.d., C18, 3 μm particle size, 100 Å pore size) was used for the separation, with a gradient of 0–60% B in 75 min (solvent A = water–acetonitrile, 98:2, v/v, with 0.1% formic acid, solvent B = acetonitrile–water, 98:2, v/v, with 0.1% formic acid) and a flow rate of $240\,nL\,min^{-1}$. Samples were trapped at a $30\,\mu L\,min^{-1}$ flow rate of solvent A on a Dionex – LC Packings μPrecolumn Acclaim PA (5 mm × 300 μm i.d., C18, 5 μm particle size), the switching unit connecting the precolumn to the nanoLC circuit after 15 min (allowing enough time for the different steps, i.e. loading the 20 μL injection loop at a rate of $3\,\mu L\,min^{-1}$, transferring and trapping the sample on the precolumn). The column output was connected to an Applied Biosystems Qtrap LC–MS/MS

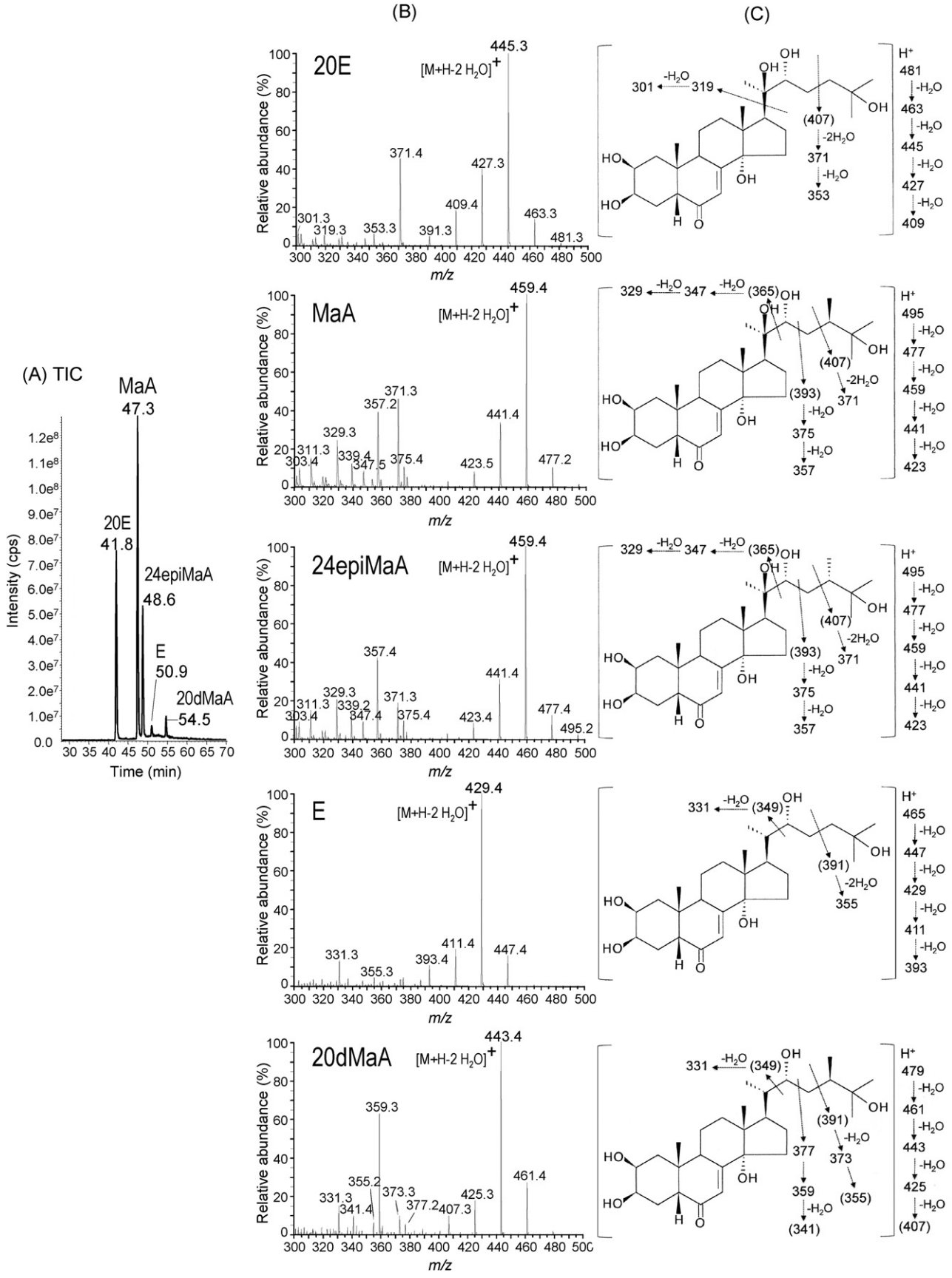

Fig. 2. NanoLC/MS/MS of ecdysteroid standards. (A) Total ion chromatogram. (B) Enhanced product ion scanning of the $[MH]^+$ of 20E (20-hydroxyecdysone, *m*/*z* 481.4), MaA (makisterone A, *m*/*z* 495.3), 24epiMaA (24-*epi*-makisterone A, *m*/*z* 495.3), E (ecdysone, *m*/*z* 465.4), and 20dMaA (20-deoxy-makisterone A, *m*/*z* 479.3) respectively from top to bottom. (C) Proposed MS/MS fragmentation of the $[MH]^+$ ions of ecdysteroids.

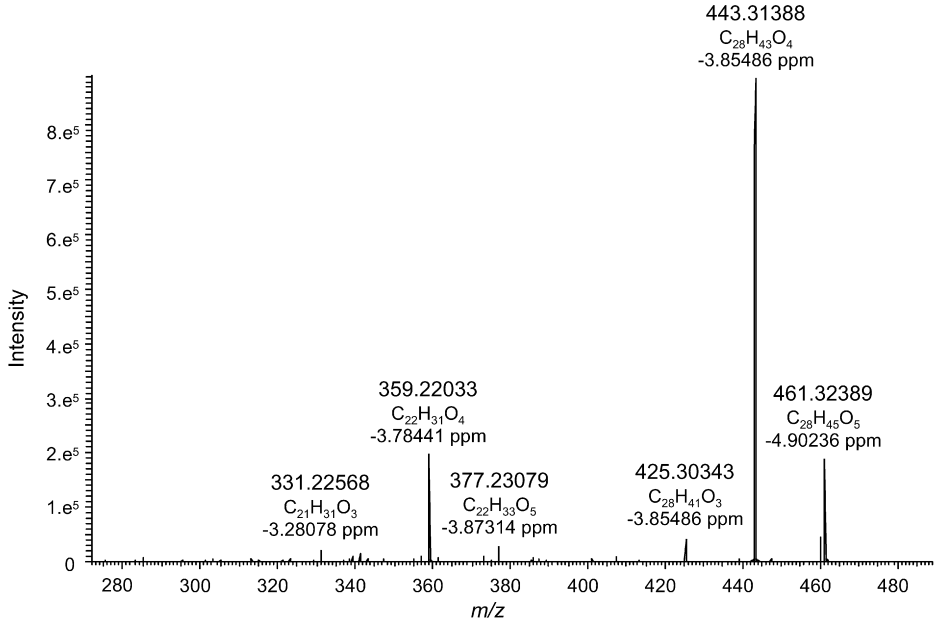

Fig. 3. High resolution p-ESI mass spectrum of 20dMaA. MS/MS of *m*/*z* 461.

system mass spectrometer through a NanoSpray ion-source interface (Spray voltage set to 2.4 kV, desolvating potential (DP) set to 40 V, collision energy (CE) set to 30 V). The LC–MS/MS system was controlled by Analyst 1.4.2 software (Applied Biosystems), allowing a 2.7 s cycle of 4 experiments for mass spectra acquisition: one full single MS by scanning the linear trap followed by three MS/MS experiments on the (pseudo-)molecular ions of the compounds.

MS/MS optimisation experiments on standard compounds were performed by direct injection on a 1 μL loop Rheodyne LC injection valve connected to a TurboIonspray source, at a 20 μL min^{-1} flow rate solvent A (Ionspray voltage set to 5.5 kV, DP = 40 V, CE = 30 V).

2.6. High resolution mass spectrometry

High resolution p-ESI mass spectrum of 20dMaA was acquired with a ultra-high resolution mass spectrometer, the hybrid linear ion trap LTQ-Orbitrap (Thermo Fisher Scientific, Les Ulis, France), in the Institut Parisien de Chimie Moléculaire (UMR 7201, UPMC, Paris). Direct introduction analysis was carried out using a syringe pump at a flow rate of 5 μL min^{-1} (10 ng/μL of sample in MeOH/H_2O containing 1% CH_3COOH). The electrospray voltage was set to 4.5 kV, the capillary voltage and the tube lens offset were set to 16 and 85 V, respectively. The sheath gas flow (nitrogen) was optimized at 12 (arbitrary units) and the drying gas temperature was set to 275 °C. The mass resolving power (full width at half maximum height) was set at 6×10^4 FWHM and at 3×10^4 FWHM for MS and CID experiments, respectively.

2.7. Chemicals

Some reference ecdysteroids were isolated in the laboratory from various plants: MaA, from *Ajuga iva* [16]; 20dMaA from the fern *Microsorum scolopendria* [17]. 24epiMaA was prepared by chemical reduction of 24,28-dehydromakisterone A [7]. 20E was a generous gift from Dr. Juraj Harmatha (Prague). Ecdysone was purchased from Northern Biochemical Company (Syktyvkar, Russia). Purity of these compounds was better than 98%.

Dichloromethane (Carlo Erba) and propan-2-ol (Prolabo) were of HPLC grade. Other chemicals were of analytical grade. Methanol was purchased from Merck, chloroform from Carlo Erba.

3. Results

3.1. Chromatography and mass spectrometric characteristics

Reference MS/MS spectra were obtained by direct injection of ecdysteroid standards, allowing further verification of daughter ion relative intensities in mass spectra from biological extracts. Collision energy was chosen to be average (30 eV); for these labile compounds, this resulted in a good structural recognition, with many fragment ions. Lowering the collision energy would enhance the signal/noise ratio for specific diagnostic ions. Both positive- and negative-modes were tested, and the positive-mode was chosen, because it provided a higher sensitivity and a more extensive fragmentation in the MS/MS mode.

Before identification of ecdysteroids in biological samples, we analyzed the reference molecules, 20E, MaA, 24epiMaA, E, 20dMaA, by nanoLC–MS/MS in order to check the efficiency of LC separation. When a mixture of these 5 standard ecdysteroids was injected, all references were well separated, including the 2 epimers of makisterone A (Fig. 2A). Experiments were reproduced several times, during the last two years, with different precolumns and columns. We found an intra-series relative standard error of 0.8% for retention times; furthermore, even if the elution times vary significantly by changing column or precolumn, the relative elution order was reproducible depending of the number and position of the hydroxyl groups, as seen for classical HPLC separation of ecdysteroids [ca. 18]. Enhanced products ion (EPI) spectra of the standards are shown in Fig. 2B. According to previous papers [9,19], there are several types of fragments: (I) consecutive losses of water molecules, (ii) partial loss of the side-chain, and (iii) cleavage of the skeleton bonds yielding low *m*/*z* fragments.

The EPI spectrum of *m*/*z* 481 of 20E is very similar to that previously reported [9,19], with side-chain cleavage taking place either between C23 and C24 or C17 and C20 (Fig. 2C); protonation on the 20,22-diol likely prevents the C20–C22 cleavage classically observed by chemical ionization/desorption MS.

The EPI spectra of *m*/*z* 495 for MaA and 24epiMaA are very similar (Fig. 2B) and result from consecutive water losses and side-chain cleavage between C23 and C24 as for 20E, but also from side-chain cleavage between C22 and C23 (Fig. 2C). Indeed, the HR-MS experiments made on 20dMaA (Fig. 3) showed that, on the MS/MS

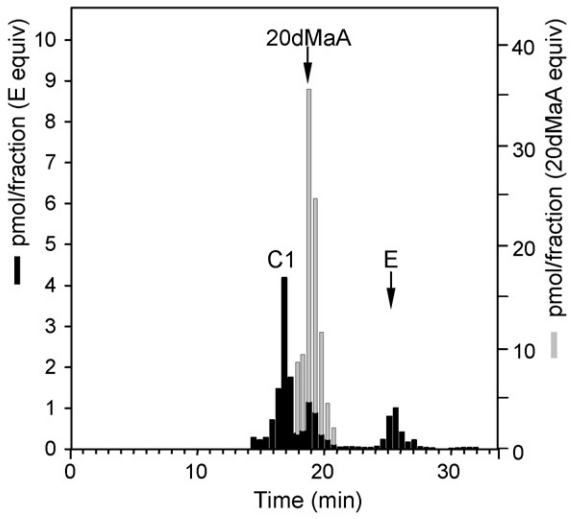

Fig. 4. NP-HPLC-EIA analysis of ecdysteroids secreted *in vitro* by the brain/ring-gland complexes of third-instar larvae. Silica column Zorbax Sil; solvent, dichloromethane/propan-2-ol/water (125:30:1.5, v/v/v); flow rate of 1 mL min^{-1}. Data are expressed in ecdysone (E) equivalents or 20-deoxymakisterone A (20dMaA) equivalents [only for the section between 18 and 21 min]. Arrows indicate retention times of ecdysteroid standards.

spectra of *m*/*z* 461, the ions at *m*/*z* 377.23079 and *m*/*z* 359.22033 corresponded to the formulae $C_{22}H_{33}O_5$ (−3.8 ppm) and $C_{22}H_{31}O_4$ (−3.8 ppm), respectively, and resulted from the cleavage of the C22–C23 bond. In MaA and 24epiMaA EPI 495 spectra, we can notice the corresponding ions at *m*/*z* 393 (very low), 375 and 357. Thus we have for these molecules two diagnostic ions at *m*/*z* 371 and 357, resulting from the cleavage of two C–C bonds in α and β position to the methylated C24. Not surprisingly, the fragmentation patterns of MaA and 24epiMA differ mainly by the relative abundance of these two fragments: the ratio $I_{357}/I_{371} \approx 1$ for MaA, ≈2 for 24epi-MaA (Fig. 2B, averaged spectrum from 10 single-scan spectra). The fragmentation patterns are reproducible at given DP and CE.

In the case of 20dMaA, the observed ions at *m*/*z* 377 (weak) and 359 result of the C22–C23 cleavage (and subsequent water loss), as assessed by HR-MS analysis (Fig. 3). By LC–MS/MS, C23–C24 cleavage also takes place, generating ions at *m*/*z* 373 and 355. The ratio of the relative intensities of the diagnostic ions, I_{359}/I_{373} is roughly 7 (Fig. 2B, averaged spectrum from 10 single-scan spectra). C20–C22 cleavage yielded a characteristic ion at *m*/*z* 331, also observed for E (Fig. 2B and C) [19].

The limit of detection lies in the low femtomole range for these whole spectrum experiments. Thus, when injecting 10 fmol 20E, the signal-to-noise ratio for the total ion current of the MS/MS experiment was ≥9 (data not shown). For a quantitative approach, Multiple Reaction Monitoring experiments, with parent and fragment pair ions (i.e. 495/357 and 495/371 to quantify MaA and its epimers) would be a better choice.

3.2. Secretory products of Drosophila ring-glands

Ecdysteroids secreted *in vitro* by ring-glands from last-instar larvae were analyzed by NP-HPLC followed by EIA. Three immunoreactive peaks were detected (Fig. 4), the two more polar ones co-migrating, respectively, with reference 20dMaA and ecdysone. The chemical identity of 20dMaA was confirmed by LC–MS/MS analysis (Fig. 5B), showing a base peak at *m*/*z* 443 $[M+H-2H_2O]^+$ and all characteristic fragments. The presence of 20dMaA among secreted ecdysteroids by Drosophila ring-glands was previously documented [6,20] and, in both studies, a third less polar unidentified immunoreactive compound was also observed,

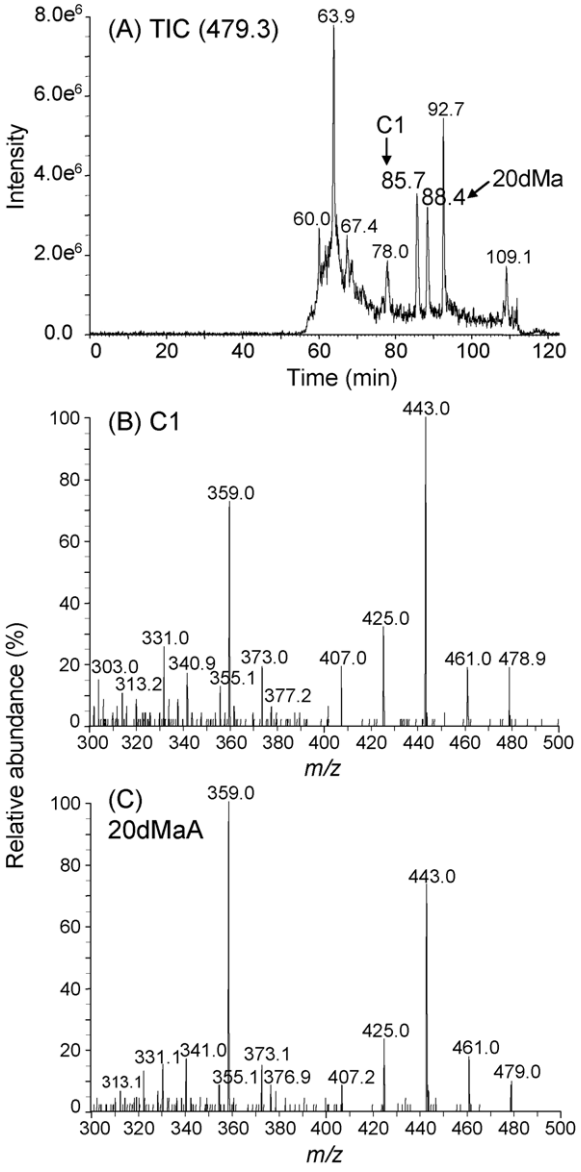

Fig. 5. NanoLC–MS/MS analysis of ecdysteroids secreted by the brain–ring-gland complexes after NP-HPLC/EIA (see Fig. 4). (A) Total ion chromatogram (TIC) of MS/MS fragments from *m*/*z* 479.3; (B) MS/MS spectrum at *m*/*z* 479 from 85.26 to 86.27 min; (C) MS/MS spectrum at *m*/*z* 479 from 88.07 to 89.15 min. 20dMaA, 20-deoxymakisterone A.

which probably corresponds to our compound C1 observed by NP-HPLC (Fig. 4). When peaks C1 and 20dMaA were analyzed by LC–MS/MS, at *m*/*z* 479, the two compounds were separated and gave very similar mass spectra (Fig. 5B and C). The diagnostic ions *m*/*z* 359 from the C22–C23 cleavage, and *m*/*z* 373, from the C23–C24 cleavage shown in the standard 20dMaA (Fig. 2) were present, with subsequent water losses. The ratio of the relative intensities of the diagnostic ions, I_{359}/I_{373} is about 3 for the spectrum of the first eluted compound (85.7 min, Fig. 5B) and about 7 for the second one (88.4 min, Fig. 5C), as seen for the standard 20dMaA spectrum (Fig. 2B), and this difference was repeatedly observed. It is proposed that this difference for the compounds C1 and 20dMaA with the same mass and slightly different MS/MS spectrum results from changes of the side-chain stereochemistry similar to those noticed for MaA epimers. This suggests that C1 is probably a 20dMaA epimer with a methyl group at R2 instead of R3 (Fig. 1), but unfortunately no reference 24epi-20dMaA was

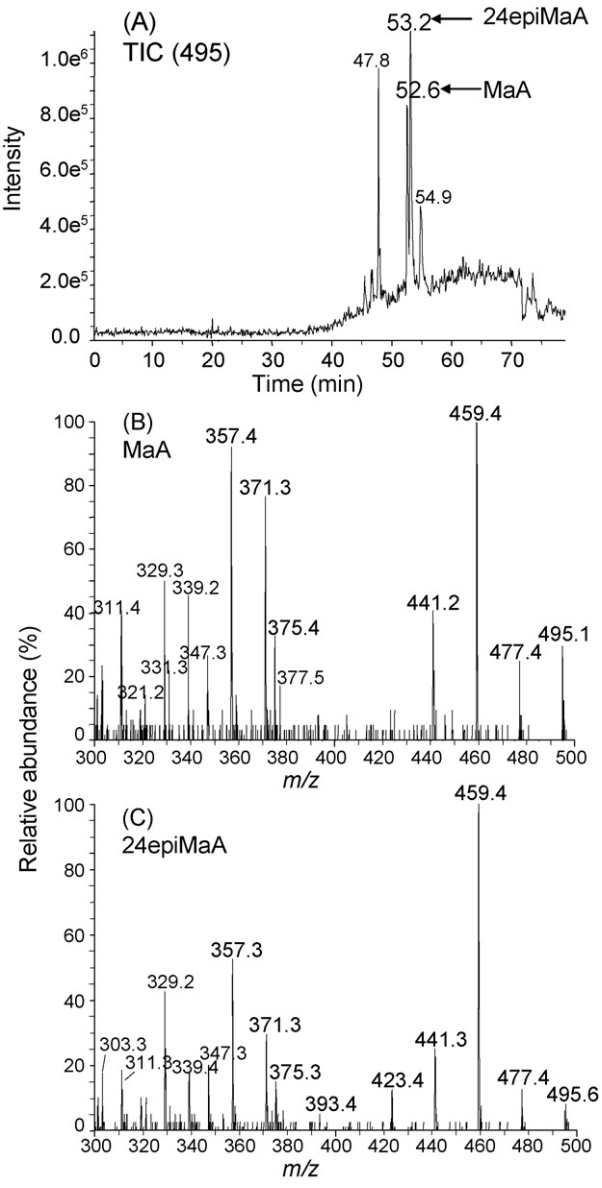

Fig. 6. NanoLC–MS/MS analysis of ecdysteroids secreted by ring-glands co-incubated with fat body from third-instar larvae. An immunoreactive region was selected first by NP-HPLC/EIA analysis. TIC of MS/MS fragments from *m*/*z* 495 (A) and MS/MS spectra of compounds eluting respectively at 52.6 min (MaA, makisterone A) (B) and at 53.2 min (24epiMaA, 24-*epi*-makisterone A) (C).

available to confirm this hypothesis. The identification of C1 was therefore indirectly assessed by nanoLC–MS/MS analysis of incubation media of brain/ring-gland complexes co-cultured with fat body. This tissue contains ecdysone 20-monooxygenase activity and can efficiently hydroxylate ecdysteroids at C-20 *in vitro* (see e.g. [20]). NP-HPLC separation of incubation media resulting from brain/ring-gland and fat body co-cultures followed by EIA quantification revealed a major immunoreactive peak co-migrating with reference 20E (data not shown), indicating that ecdysone produced by brain–ring-gland complexes had been 20-hydroxylated during incubation with fat body. Two small immunoreactive peaks at the retention times of standards 24epiMaA and MaA were also detected after NP-HPLC, and nanoLC–MS/MS analyses of the corresponding fractions showed indeed two peaks at *m*/*z* 495, corresponding respectively to the mass spectra of standards MaA and 24epiMaA (Fig. 6B and C, to be compared to Fig. 2B). These two ecdysteroids are logically expected to arise respectively from 20dMaA and 24epi-

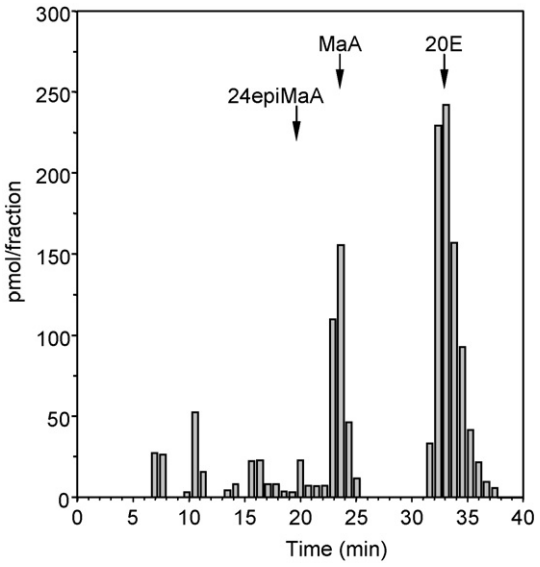

Fig. 7. NP-HPLC-EIA analysis of ecdysteroids extracted from wandering third-instar larvae. Same HPLC conditions as in Fig. 4. Data are expressed in ecdysone (E) equivalents. Arrows indicate retention times of references. 20E, 20-hydroxyecdysone; 24epiMaA, 24-*epi*-makisterone A.

20dMaA (i.e. compound C1). The two less polar secretory products of brain/ring-gland complexes are namely 28C-ecdysteroids, but their actual relative abundances as compared to ecdysone are strongly underestimated when measurements are expressed as ecdysone equivalents (Fig. 4).

3.3. Ecdysteroids from Drosophila larvae

Ecdysteroids extracted from wandering third-instar larvae were separated by NP-HPLC followed by EIA. Two major immunoreactive peaks were detected corresponding to the retention times of 20E, and of MaA/ecdysone (the latter being not fully resolved in our system) (Fig. 7). Given the widely different affinities of these ecdysteroids to antiserum L2, it is difficult to estimate accurately their relative abundance in larvae. A weak immunoreactivity was also present at the retention time of reference 24epiMaA. The fractions corresponding to these three immunoreactive peaks were further analyzed by LC–MS/MS. The different ion chromatograms (TIC) and mass spectra are presented in Fig. 7. LC conditions were the same as those described in the previous sections, with a new column, which explains longer retention times of ecdysteroids (compare with Fig. 6). The presence of 20E was confirmed by its mass spectrum (Fig. 8B) analyzed at *m*/*z* 481, similar to the standard spectrum (Fig. 2B). In the (MaA/E) HPLC fraction injected, two compounds were detected: MaA eluted at 61 min and *m*/*z* 495 (Fig. 8C) and E at 63.7 min and *m*/*z* 465.4 (Fig. 8E). Their identity was confirmed by comparison of their spectra (Fig. 8D and F) with those of the corresponding references (Fig. 2B). Their relative quantities in the biological sample were estimated as MaA being ca. 2.4-fold more abundant than ecdysone. The fourth, less polar, ecdysteroid was identified as 24epiMaA (Fig. 8G and H), thanks to its mass spectrum similar to the standard (Fig. 2B). This confirms that Drosophila larvae can use yeast 24β-methyl 28C-sterols (ergosterol, 24-methylcholesterol) present in their diet to synthesize the 28C-ecdysteroid 24epi-20dMaA, which is converted by peripheral tissues into circulating 24epiMaA.

When the standard diet was supplemented with cholesterol, wandering larvae exhibited a modified ecdysteroid pattern (data not shown). MaA and 24epiMaA were no longer detected, while 20E became the only significant ecdysteroid to be detected.

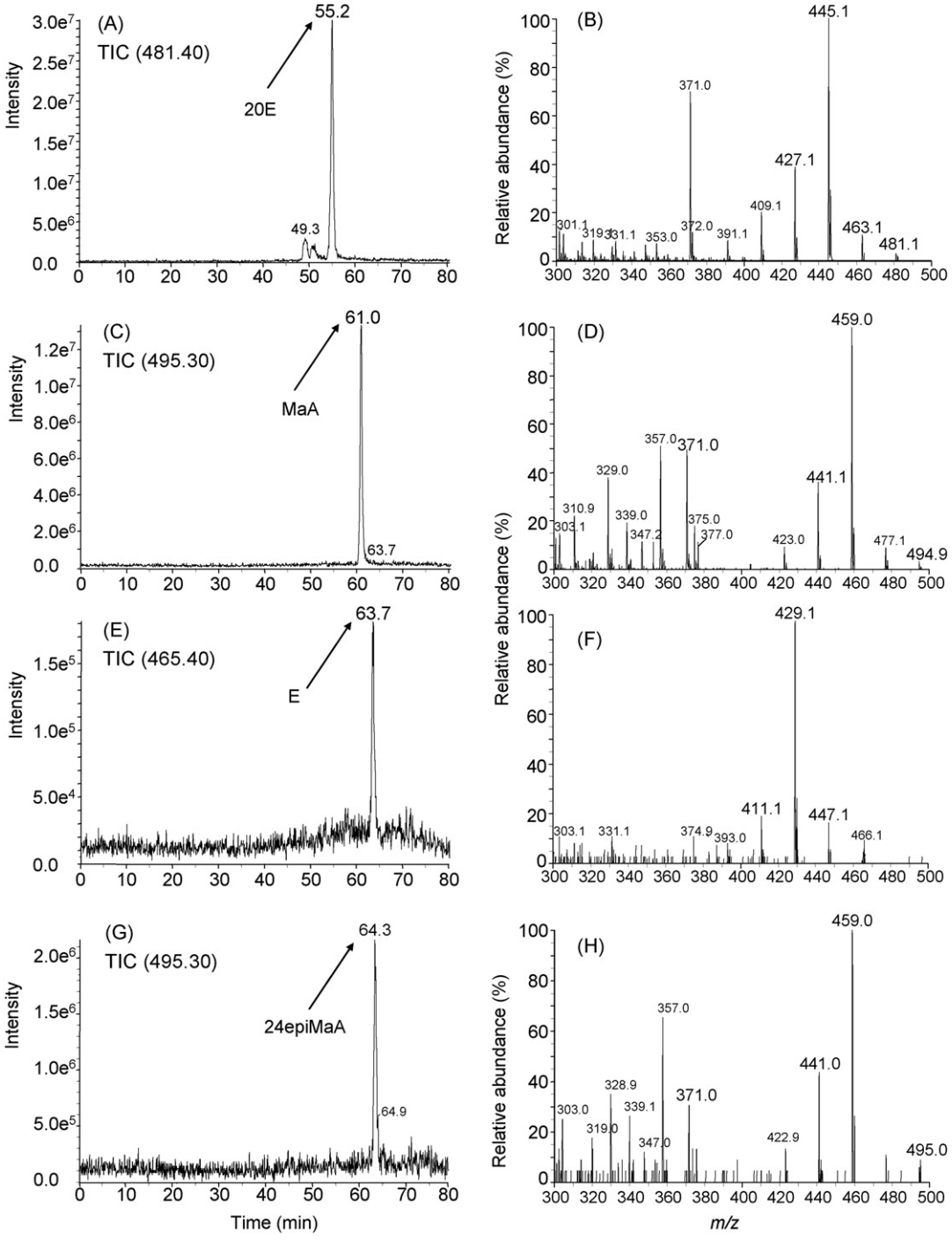

Fig. 8. NanoLC–MS/MS analysis of ecdysteroids in wandering third-instar larvae, after HPLC/EIA (see Fig. 7). TIC of MS/MS fragments (A, C, E, G) and the corresponding MS/MS spectra (B, D, F, H).

4. Discussion

In the present study, we have taken advantage of (1) the availability of a set of reference phytoecdysteroids, in particular 20dMaA and 24epiMaA, and (2) a particularly sensitive nanoLC–MS/MS methodology, to examine the diversity of steroid hormones (ecdysteroids) in Drosophila larvae. LC–MS techniques are increasingly used for ecdysteroid analyses [8–11,19,21,22] and their continuously improving performances allow detection in the low femtomolar range, making it possible to identify minor steroids even in such a small animal as Drosophila. The versatility of the mass spectrometer allows to use it either as a general purpose detector, as we did for identification of unknown species, or as a specific one, for example in MRM mode, to detect known compounds. Our aim was not to quantify Drosophila ecdysteroids with this technique. It could be feasible, if analyzing the whole sample with an added internal standard, e.g. polypodine B, a phytoecdysteroid never found in animals, and simplifying the purification steps. This has been applied for example for quantification of 20E in calf urine [11]. However, it is more time and money consuming than EIA quantifications.

In our experimental conditions, brain–ring-gland complexes secrete a mixture of three immunoreactive ecdysteroids: E, 20dMaA and a third compound, which we have identified as 24epi-

20dMaA. Our data confirm previous studies [5,6,20] evidencing the complexity of ecdysteroid pattern in Drosophila and establish the identity of the previously unknown compound. Campesterol, a typical phytosterol (from maize flour), is the likely precursor of (20d)MaA as previously hypothesized in Drosophila [4,5], and demonstrated in the honeybee [23]. By contrast, the synthesis of 24epi(20d)MaA requires a 24β-methylsterol precursor. It is well-established that yeast and, more generally, fungi contain 28C-sterols which differ from those of vascular plants by the stereochemistry of the methyl group attached to C-24 (see e.g. [24]). It is thus expected that 24epiMaA is produced from yeast sterols. The presence of 24epiMaA was previously demonstrated in the leaf-cutting ant, *Acromyrmex octospinosus*, which feeds on its symbiotic fungus [7], and, in a similar way, ecdysteroid-producing fungi also synthesize 24β-methyl (24*S*) "mycoecdysteroids" (e.g. [25]).

Sterols fulfil very different functions in insects: (i) as cell membrane components; (ii) as precursors to the moulting hormones, ecdysteroids; and (iii) as signalling molecules bound to the hedgehog group of proteins affecting development [26]. The bulk of them is present in cell membranes. For that structural function, it seems that phytosterols or fungal sterols may replace the cholesterol normally present, since some insect species seem almost devoid of cholesterol [26]. On the other hand, ecdysteroid production in insects represents at best 0.1–0.2% of the total sterol amount (e.g. [27]). The complexity of the ecdysteroid pattern found in Drosophila shows that this insect species, which cannot dealkylate sterols, can use a wide range of sterol substrates for ecdysteroid biosynthesis. However, as shown by our study and previous works, it appears that, when available, cholesterol is a preferred substrate for this purpose. Thus, when Drosophila diet contains even trace amounts of cholesterol (0.0005%), this sterol is preferentially used for 20E production [5]. When adding cholesterol (0.03%) to the diet, we did not detect 28C-ecdysteroids in larvae any more, as was previously noticed for ecdysteroids secreted by ring-glands [4] or for those present in Drosophila pupae [5]. Minor differences in the sterol composition of the culture medium might thus explain some discrepancies between the present or previous [6] data and some recent ones [28], where the authors did not detect any MaA in Drosophila wandering larvae (or at any time point during the third larval instar). Taken together, these results both indicate that the enzymes involved in ecdysteroid biosynthesis can use substrates bearing various side-chains and that they probably have a higher affinity for 27C-precursors. More extensive enzymatic studies are needed to confirm such hypothesis. As several biosynthetic enzymes have now been identified and cloned [29,30], studies using recombinant steroidogenic enzymes of Drosophila would now be possible, provided that the corresponding substrates are available.

The ecdysteroid pattern can thus adapt to large variations of dietary sterols, but we may wonder whether this is physiologically relevant or not. All three ecdysteroids (20E, MaA and 24epiMaA) are able to bind to the ecdysteroid receptor (DmEcR) and their EC_{50} activities are respectively, 7.5×10^{-9} M (20E), 1.3×10^{-8} M (MaA) and 2.2×10^{-7} M (24epiMaA) [31]. Therefore, in theory, all three molecules could be used as moulting hormones by Drosophila larvae, although 24epiMaA would be 30 times less potent than 20E and about 20 times less potent than MaA, thus probably requiring higher *in vivo* concentrations to sustain development. At present, it cannot be excluded that 24epiMaA may represent a physiologically active hormone in Drosophila larvae.

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