

Evidence for the presence of makisterone A in *Drosophila* larvae and the secretion of 20-deoxymakisterone A by the ring gland

(ecdysteroid secretion/phytosterols)

CHRISTOPHER P. F. REDFERN*

Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, England

Communicated by John Maynard Smith, May 16, 1984

ABSTRACT Ring glands or brain–ring gland complexes from third-instar *Drosophila melanogaster* larvae secreted ecdysone [(22*R*)-2*β*,3*β*,14*α*,22,25-pentahydroxy-5*β*-cholest-7-en-6-one] and two less polar ecdysteroids (LP1 and LP2) *in vitro*. Radioimmunoassay with different antisera indicated that LP1 and LP2 are side-chain-modified analogues of ecdysone. In high-performance liquid chromatography, the retention time of LP2 was equivalent to that of a precursor of makisterone A [(20*R*,22*R*)-2*β*,3*β*,14*α*,20,22,25-hexahydroxy-24-methyl-5*β*-cholest-7-en-6-one] secreted by *Dysdercus fasciatus* prothoracic glands *in vitro*. LP2 was metabolized *in vitro* by the fat body of *Drosophila* larvae to a product with the characteristics of makisterone A when analyzed by gas chromatography/mass spectrometry (selected ion monitoring). Evidence was obtained for the presence of makisterone A in *Drosophila* larvae. These data suggest that LP2 is 20-deoxymakisterone A (24-methylecdysone) and that makisterone A could function as an additional moulting hormone in *Drosophila*, although 20-deoxymakisterone A production is apparently dependent on the sterol composition of the diet. LP1 has not been identified.

The insect steroid ecdysone, a C₂₇ ecdysteroid [(22*R*)-2*β*,3*β*,14*α*,22,25-pentahydroxy-5*β*-cholest-7-en-6-one], is secreted by the prothoracic glands (1) or, in cyclorrhaphous Diptera, by the ring gland (2). Ecdysone is hydroxylated at C-20 by monooxygenase enzyme systems in the fat body, gut, and Malpighian tubules to give the biologically active moulting hormone 20-hydroxyecdysone [(20*R*,22*R*)-2*β*,3*β*,14*α*,20,22,25-hexahydroxy-5*β*-cholest-7-en-6-one] (3). Recently, the moulting hormone in *Dysdercus fasciatus* (4) and some other species of phytophagous Hemiptera (5) has been identified as a C₂₈ ecdysteroid, makisterone A [(20*R*,22*R*)-2*β*,3*β*,14*α*,20,22,25-hexahydroxy-24-methyl-5*β*-cholest-7-en-6-one], which differs from 20-hydroxyecdysone in the presence of an additional methyl group at C-24 of the ecdysteroid side chain. By analogy with other insects (1), the prothoracic glands of these hemipterans would be expected to secrete the 24-methylated analogue of ecdysone, 20-deoxymakisterone A (24-methylecdysone).

In a previous study, it was shown that *Drosophila* ring glands secrete ecdysteroids *in vitro* and that the rate of secretion reaches a maximum towards the end of the last larval instar (6). *Sarcophaga* ring glands secrete ecdysone *in vitro* (2), and the experiments reported here were initially designed to ask whether or not this is also true of *Drosophila melanogaster* ring glands. However, in addition to ecdysone, explanted *Drosophila* ring glands secreted apparently significant quantities of two other ecdysteroids. Further analysis has indicated that one of these ecdysteroids is 20-deoxymakisterone A and that its production is apparently dependent on the sterol composition of the diet.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Animal Culture. Unless stated otherwise, the Oregon R strain of *D. melanogaster* was cultured on cornmeal/yeast medium: 118.54 g of whole cornmeal, 21.43 g of dried yeast (heat killed), 107.15 g of sucrose, and 6.43 g of agar per liter of water containing 0.003% Nipagin, added as a 10% solution in 95% ethanol. The medium was sprinkled lightly with live yeast before use. For culturing *Drosophila* axenically on defined media, the recipe and methods of Sang (7) were used. Yeast RNA was replaced with uridine and inosine, and lecithin was replaced with choline chloride (7). The medium constituents were purchased from Sigma [uridine, nicotinic acid, thiamine-HCl, calcium pantothenate, pyridoxine, biotin, and ergosterol (ergosta-5,7,22*E*-trien-3*β*-ol)], BDH (sucrose, choline chloride, inosine, fat- and vitamin-free casein, and folic acid), Koch-Light Laboratories (Bucks, England) [riboflavin and "sitosterol" (stigmasta-5-en-3*β*-ol)], Oxoid (Basingstoke, England; technical agar no. 3), and Steraloids (Wilton, NH) [stigmaterol (stigmasta-5,22*E*-dien-3*β*-ol)]. Dietary sterol was either cholesterol (cholest-5-en-3*β*-ol; 0.03%), ergosterol (0.03%), or a mixture of 0.015% stigmaterol, 0.01% ergosterol, and 0.02% sitosterol/campesterol [(24*R*)-24-methylcholest-5-en-3*β*-ol] (ca. 7:3, wt/wt; H. H. Rees, personal communication), according to the experiment.

Fourth-instar *D. fasciatus* were obtained from May & Baker and maintained as described (4).

Organ Culture. Ring glands or brain–ring gland complexes from third-instar *Drosophila* larvae with partially or fully bloated larval salivary glands were dissected and cultured (three to six ring glands or brain–ring gland complexes per culture) in Grace's medium (Flow Laboratories) for 4–8 hr as described (6). Prothoracic glands dissected from late fifth-instar *D. fasciatus* were cultured under similar conditions. After culture, the tissue was pelleted by centrifugation, and the supernatant was added to methanol to a final methanol concentration of 70% vol/vol.

Fat body from five late-wandering, third-instar *Drosophila* larvae (selected according to salivary gland morphology as above) were dissected in Grace's medium, washed by gentle agitation in three successive 0.5-ml aliquots of fresh medium (1–2 min per wash), and cultured in 20 μ l of Grace's medium for 3 hr at 28°C. After culture, the medium was withdrawn, and the tissue was washed with 20 μ l of Grace's medium. The combined supernatants, after pelleting the tissue, were added to 200 μ l of methanol and processed for analysis. The conversion of [23,24-³H]ecdysone to 20-hydroxy[23,24-³H]ecdysone was approximately 45% under these conditions.

Abbreviation: GC/MS(SIM), gas chromatography/mass spectrometry (selected ion monitoring).

*Present address: Department of Dermatology, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 4LP, England.

Sample Processing. Culture medium samples (stored at -20°C) were purified by reversed-phase chromatography using C_{18} Sep-Pak cartridges (Waters Associates) (8). Free ecdysteroids, eluted with 4 ml of 60% (vol/vol) methanol/water, were retained for analysis. Under these conditions, recovery of $[23,24\text{-}^3\text{H}]$ ecdysone is $>90\%$ (8).

Extraction of Ecdysteroids from *Drosophila* Larvae. Wandering, third-instar *Drosophila* larvae were washed, blotted dry, and stored at -20°C until extracted. The larvae (245 mg, wet weight) were extracted three times by homogenization in 4 ml of 70% (vol/vol) methanol/water (on ice) and finally with 4 ml of methanol. The extract, made up to 25 ml with 70% methanol/water, was partitioned against an equal volume of hexane. The aqueous phase was dried under vacuum, and the residue was redissolved in 5 ml of methanol and adsorbed onto Celite (Koch-Light Laboratories) (9). After silicic acid chromatography of the adsorbed extract (9), free ecdysteroids were eluted with methanol/chloroform, 30:70 (vol/vol), dried under nitrogen, redissolved in 2 ml of 10% (vol/vol) methanol/water, and further purified by using a C_{18} Sep-Pak cartridge as above; the 60% methanol/water fraction was retained for analysis by HPLC. In a control experiment, ca. 75% of 20-hydroxy $[^3\text{H}]$ ecdysone added to a batch of larvae at the initial homogenization was recovered (verified by reversed-phase HPLC) from the final Sep-Pak purification.

HPLC. Reversed-phase HPLC was carried out by using a Waters instrument (Waters Associates) (8) with either an Altex (Berkeley, CA) Ultrasphere ODS column (spherical C_{18} ; particle size, 5 μm ; column dimensions, 4.6 mm i.d. by 15 cm) or a Waters Associates Resolve column (spherical C_{18} ; 5 μm ; 3.9 mm i.d. by 15 cm) and by eluting with a linear gradient of methanol/water, increasing from 35:65 (vol/vol) to 80:20 (vol/vol) over 30 min at a flow rate of 1 $\text{ml}\cdot\text{min}^{-1}$. Except where stated, only fractions (0.5 or 1 min) that were eluted between 12 and 22 min after injection were assayed by RIA. A blank or control injection was performed before each analysis, and ecdysone and 20-hydroxyecdysone standards were run immediately after each group of samples. The susceptibility of unidentified ecdysteroid peaks to enzymic hydrolysis was tested by using esterase (Sigma), α -glucosidase (Boehringer Mannheim), and *Helix pomatia* "arylsulfatase" (Sigma; type H1, with phosphatase and β -glucuronidase activity) (8, 10), followed by HPLC/RIA analysis.

Gas Chromatography/Mass Spectrometry (Selected Ion Monitoring) [GC/MS(SIM)]. Culture medium samples (after C_{18} Sep-Pak chromatography) and selected HPLC fractions were dried under dry nitrogen and derivatized with trimethylsilylimidazole (8). Before derivatization, ponasterone A [5 ng; (20R,22R)-2 β ,3 β ,14 α ,20,22,-pentahydroxy-5 β -cholest-7-en-6-one] was added to all samples and standards as an internal standard for the GC/MS(SIM) analysis. Subsequent sample preparation and GC/MS(SIM) analysis were as described by Mendis *et al.* (8). Fragment ions m/z 567 and m/z 561 were monitored.

RIA. HPLC fractions were assayed by using the Horn I2 (16 wk) antiserum (11), a gift of J. D. O'Connor, or the ICT-1 antiserum (12) supplied to H. H. Rees by K.-D. Spindler. The radioimmunoassay method was as described (6). At the antiserum dilutions used (1:300 and 1:3000, for the Horn I2 and ICT-1 antisera, respectively, unless stated otherwise) ca. 50% of the radiolabeled ecdysone was bound in the absence of competing ligand. Where necessary, background values (blank injection) have been subtracted.

Two further antisera were used for dose-response experiments: the DHS I-15 antiserum (13), a gift of J. D. O'Connor to H. H. Rees (used at a dilution of 1:500) and an antiserum (MAS 7) generated in mice by using as antigen 20-hydroxyecdysone conjugated through C_6 to bovine serum albumin (Sigma) (14, 15). Five mice (CBA/Ca strain) were each in-

jected i.p. with 200 μg of the antigen (hapten:carrier molar ratio, 3.3:1) emulsified in 0.5 ml of 1% Tween 80/0.45% NaCl/25% Freund's complete adjuvant. Booster injections (200 μg of antigen per mouse) were given 7 and 19 wk later. The resulting antiserum (pooled from five mice) was used at a dilution of 1:550 and had a K_d for ecdysone of 4.9×10^{-10} M; the affinity for 20-hydroxyecdysone was approximately half that for ecdysone.

RESULTS

HPLC Separation of Ecdysteroids Secreted by *Drosophila* Ring Glands. With the culture conditions used here, brain-ring gland complexes from *Drosophila* larvae, dissected from animals shortly before puparium formation, secreted ecdysteroids at a rate of about 90 pg ecdysone equivalents per hr per complex (6). To identify the ecdysteroids secreted, the culture media from 11 brain-ring gland cultures and 15 ring gland cultures were analyzed separately by reversed-phase HPLC. RIA activity corresponding to ecdysone represented 33–42% of the total for all fractions (Fig. 1). Less-polar cross-reacting material, eluted 1–4 min after ecdysone, represented 45–49% of the total RIA activity. The results with brain-ring gland complexes or ring glands alone were similar, indicating that the less-polar material did not result from ecdysteroid secretion or metabolism by the brain tissue of brain-ring gland complexes. In two subsequent HPLC experiments, the less-polar material (LP in Fig. 1) represented 52% and 67% of the total RIA activity that was eluted between 12 and 22 min after injection; ecdysone [confirmed by GC/MS(SIM)] comprised the remainder.

With fractionation every 0.25 or 0.5 min, the less-polar ecdysteroid material was clearly resolved into two components: these are referred to as LP1 and LP2 (e.g., see Fig. 3 Upper). LP2 was the major component, and in two analyses represented approximately 75% of the less-polar ecdysteroids and 30–50% of the RIA activity detected in ring gland incubations. The retention times of LP1 and LP2, relative to ecdysone, are given in Table 1.

Evidence that brain-ring gland complexes of *Calliphora erythrocephala* are capable of synthesizing esters and glycosides of hormonally active steroids has been reported (10). LP1 and LP2 were eluted in the free ecdysteroid fraction after C_{18} Sep-Pak chromatography and were unaffected by incubation with esterase, α -glucosidase, or *Helix pomatia* arylsulfatase; therefore, it seems unlikely that they are ecdysteroid conjugates. Furthermore, incubating ring glands with $[23,24\text{-}^3\text{H}]$ ecdysone did not result in any detectable conversion to less-polar ecdysteroids.

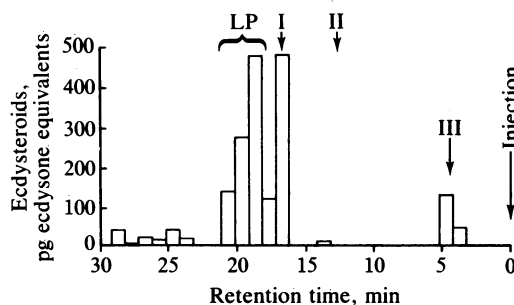


FIG. 1. HPLC/RIA analysis of ecdysteroids secreted by *Drosophila* ring glands cultured *in vitro* for 4 hr at 25°C . One third of the sample from 15 ring gland cultures (3 glands per culture) was injected onto the column (Ultrasphere ODS), and 0.25 ml of each 1-min fraction of the 30-min gradient was assayed by RIA [Horn I2 (16 wk) antiserum]. The positions of authentic ecdysone (I) and 20-hydroxyecdysone (II) are marked. LP, less-polar ecdysteroids. Peak III was not present in subsequent analyses and is assumed to be an artefact.

Table 1. HPLC retention times of ecdysteroids relative to ecdysone

Ecdysteroid	Relative retention time	
	Column: Resolve	Ultrasphere ODS
20-Hydroxyecdysone	0.71	0.74
Makisterone A	0.91	ND
LP1	1.22	1.21
LP2	1.15	1.12

ND, not determined.

Cross-Reaction of LP1 and LP2 with Ecdysteroid Antisera.

Structural information on unknown ecdysteroids may be obtained by using antisera differing in their specificity towards different parts of the ecdysteroid molecule. Four different antisera were used for dose-response studies with samples of LP1 and LP2, purified by two successive reversed-phase HPLC separations. Both the Horn I2 [16 wk; a batch related to the H21B antiserum (11)] and the ICT-1 antisera are ecdysone nucleus specific (16); the ecdysteroids used in their preparation were conjugated to the carrier via the ecdysteroid side chain (12, 16). Conversely, the DHS I-15 antiserum, prepared by conjugating through C₂ on the steroid nucleus, is relatively side chain specific (13). The MAS-7 antiserum has not been fully characterized but was prepared by conjugating through C₆ and, like the DLW antiserum (16) prepared similarly, should be side chain specific.

Dose-response curves with the Horn I2 (16 wk), DHS I-15, and MAS-7 antisera are shown in Fig. 2. By comparison with unlabeled ecdysone, LP2 competed relatively ineffectively with [23,24-³H]ecdysone for binding to the DHS I-15 and MAS-7 antisera (Fig. 2). A similar result with respect to the MAS-7 antiserum was obtained by using LP1 (DHS I-15 was not tested). The ICT-1 antiserum appeared to show a greater affinity towards LP2 than for ecdysone: 190 pg ecdysone equivalents of LP2 (as measured with the Horn I2 antiserum) or 370 pg of ecdysone were required to effect a 50% reduction in the binding of [23,24-³H]ecdysone to the ICT-1 antiserum (1:1000 dilution). With ecdysone as a reference, the marked difference in specificity for LP1 and LP2 be-

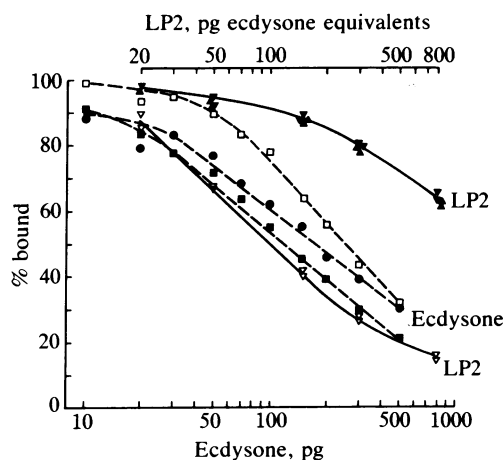


FIG. 2. RIA dose-response curves of ecdysone and LP2 with the Horn I2 (16 wk), DHS I-15, and MAS-7 antisera. LP2 is expressed as pg ecdysone equivalents as measured with the Horn I2 (16 wk) antiserum. Solid lines: LP2 was assayed in duplicate with the Horn I2 (16 wk) (■), MAS-7 (●), and DHS I-15 (▼) antisera. Lines for these latter two antisera were superimposed. Dashed lines: ecdysone was assayed with the Horn I2 (■), MAS-7 (●), and DHS I-15 (▼) antisera (□). "% bound" (ordinate) refers to the amount of [23,24-³H]ecdysone bound expressed as a percentage of label bound in the absence of competing ligand.

tween the nucleus-specific and side-chain-specific antisera suggests that LP1 and LP2 are ecdysteroids with modified side chains.

Ecdysteroid Secretion by *D. fasciatus* Prothoracic Glands *in Vitro*. In view of indications that LP1 and LP2 differ from ecdysone with respect to side-chain modifications, the possibility that LP1 and LP2 are methyl-substituted derivatives was investigated by HPLC analysis of ecdysteroids secreted by *D. fasciatus* prothoracic glands. The major ecdysteroid (presumably 20-deoxymakisterone A) secreted by *D. fasciatus* prothoracic glands *in vitro* had a retention time (reversed-phase HPLC) equivalent to LP2 (Fig. 3). This suggests that LP2 is 20-deoxymakisterone A (24-methylecdysone).

In dose-response RIA experiments with the ICT-1 antiserum (1:1000 dilution), 580 pg of makisterone A or ca. 1000 pg of 20-hydroxyecdysone were required to reduce [23,24-³H]ecdysone binding by 50%. Thus, the presence of a C-24 methyl group on the ecdysteroid side chain apparently confers greater affinity for this antiserum, perhaps by mimicking the chemical bridge of the original antigen. That ICT-1 also shows greater affinity towards LP2 than ecdysone is additional evidence supporting the identification of LP2 as a 24-methyl-substituted analogue of ecdysone.

20-Hydroxylation of LP2 *in Vitro*. If it is correct that LP2 is 20-deoxymakisterone A, makisterone A should be present in third-instar *Drosophila* larvae and should also result from the metabolism of LP2 by larval fat body *in vitro*. Fat bodies from five, late-wandering, third-instar larvae of *Drosophila* were incubated in Grace's medium (control) or Grace's medium containing 4 ng ecdysone equivalents of LP2. The culture media from the control and experimental incubations were analyzed by GS/MS(SIM), together with samples of LP1 and LP2.

After derivatization with trimethylsilylimidazole both LP1 (not shown) and LP2 (Fig. 4) gave peaks with ion m/z 567. This ion is characteristic of fully silylated ecdysone and ecdysteroids with an ecdysone nucleus and lacking a C-20 hydroxyl group (8, 17). Retention times relative to the ponasterone A internal standard are given in Table 2. In addition to the internal standard, four peaks resulted from the incubation of LP2 with larval fat body (Fig. 4): two of these represent unknown compounds that were eluted or secreted from the fat body and present in the control sample. The remaining two peaks have relative retention times corresponding to fully silylated makisterone A (peak C, m/z 561) and LP2

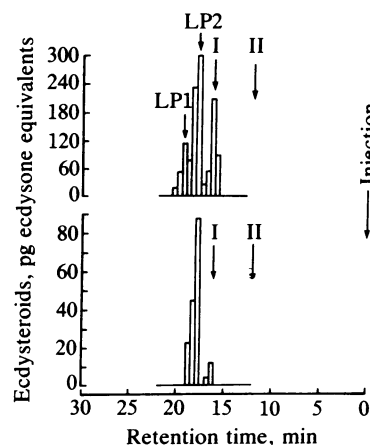


FIG. 3. HPLC/RIA analysis [Ultrasphere ODS column, Horn I2 (16 wk) antiserum] of ecdysteroids secreted by brain-ring gland complexes from *Drosophila* (Upper) or prothoracic glands from *D. fasciatus* (Lower) cultured *in vitro*. LP1 and LP2 and the positions of authentic ecdysone (I) and 20-hydroxyecdysone (II) are marked.

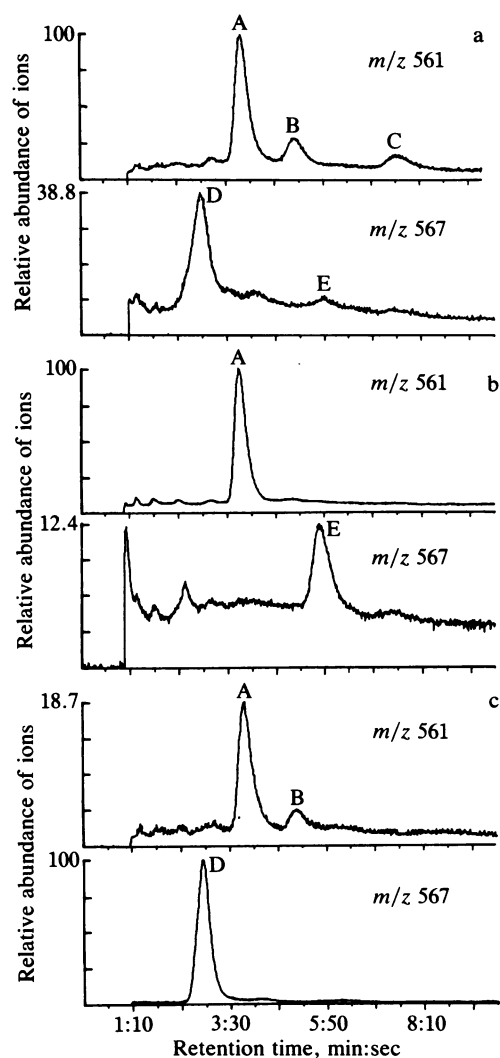


FIG. 4. GC/MS(SIM) analyses. (a) The product(s) of *in vitro* 20-hydroxylation of 4 ng ecdysone equivalents of LP2 by fat body from late-wandering *Drosophila* larvae. (b) Four nanogram ecdysone equivalents (Horn I2 antiserum) of purified LP2. (c) Control experiment: culture medium after incubation with fat body from late-wandering third-instar *Drosophila* larvae. Peak A (m/z 561) is the ponasterone A internal standard; peaks B [m/z 561; retention time relative to ponasterone A (RRT), 1.32] and D (m/z 567; RRT, 0.74) are unknown compounds present in the control; peak C (m/z 561; RRT, 1.97) in a has a retention time, relative to ponasterone A, which corresponds to fully silylated makisterone A (Table 2); peak E (m/z 567) corresponds to LP2. Ordinates: ion intensity relative to the most abundant ion. For this experiment, LP2 was purified from the culture medium of 49 brain-ring gland cultures by two successive HPLC separations.

(peak E, m/z 567), respectively. Ion m/z 561 is the major ion characteristic of ecdysteroids with a C-20 hydroxyl group (8, 17), and these data therefore indicate that LP2 was 20-hydroxylated *in vitro* to produce makisterone A.

Analysis of Ecdysteroids Extracted from Larvae. A reversed-phase HPLC analysis of ecdysteroids extracted from wandering, third-instar larvae of *Drosophila* revealed RIA activity with retention times equivalent to 20-hydroxyecdysone (20 pg ecdysone equivalents/mg of wet weight), makisterone A (8 pg ecdysone equivalents/mg of wet weight), and ecdysone (120 pg ecdysone equivalents/mg of wet weight). The makisterone A fraction was analyzed by GC/MS(SIM), and the result (Fig. 5) is further evidence that makisterone A is present in third-instar *Drosophila* larvae. LP1 and LP2

Table 2. GC/MC(SIM) retention times of fully silylated trimethyl-ecdysteroid ethers relative to the ponasterone A internal standard

Ecdysteroid	Relative retention time*	Major ion m/z
Ecdysone	1.28	567
20-Hydroxyecdysone	1.56	561
Makisterone A	1.98	561
LP1	1.56	567
LP2	1.51	567

*On 1% OV-1 column.

were not detected by either HPLC/RIA or GC/MS(SIM) in this extract.

Ecdysteroid Secretion by Ring Glands of *Drosophila* Larvae Grown on Defined Media. *D. fasciatus* does not dealkylate phytosterols and utilizes C_{28} phytosterols directly for moulting hormone production (4). Similarly, the production of 20-deoxymakisterone A (and LP1) by *Drosophila* ring glands cultured *in vitro* may be due to the direct utilization of C_{28} and C_{29} phytosterols, which account for a major proportion of the sterols in corn (18) and yeast (19). This is supported by the finding that brain-ring gland complexes from *Drosophila* larvae grown axenically on a defined medium containing cholesterol as the sole dietary sterol only secreted ecdysone *in vitro*. However, the same result was obtained when cholesterol was replaced by ergosterol (Fig. 6 Upper) a major sterol of yeast (19). The inclusion in the *Drosophila* food of sitosterol, campesterol, and stigmasterol together with ergosterol consistently (three experiments) resulted in the production of small amounts of LP2 as shown by HPLC/RIA analysis (Fig. 6 Lower).

DISCUSSION

Together, the GS/MS(SIM) data and RIA dose-response curves with different antisera are clear indications that LP1 and LP2 are side-chain-modified analogues of ecdysone. The HPLC retention time of LP2 was equivalent to that of a major ecdysteroid secreted *in vitro* by *D. fasciatus* prothoracic glands, and this suggests that LP2 is the 24-methyl-substituted analogue, 20-deoxymakisterone A, since in this species

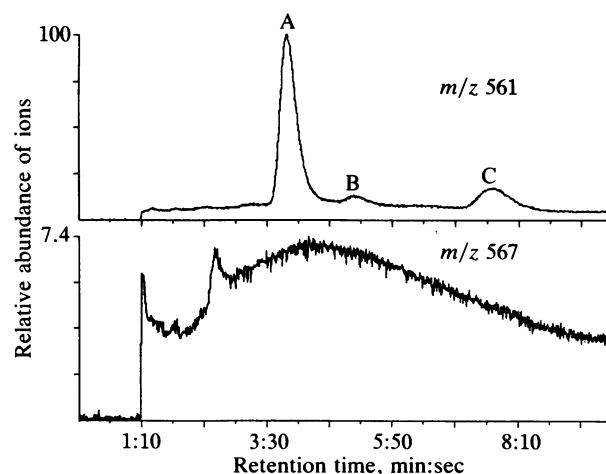


FIG. 5. Ecdysteroids extracted from *Drosophila* larvae: GC/MS(SIM) analysis of the HPLC fraction corresponding to makisterone A. Peak A is the internal standard, ponasterone A; peak C (m/z 561) has a relative retention time (1.98) corresponding to fully silylated makisterone A. The identity of peak B (m/z 561) is unknown. The sample used in this analysis contained *ca.* 2 ng makisterone A equivalents by RIA. The area of peak C represents approximately 1 ng of makisterone A.

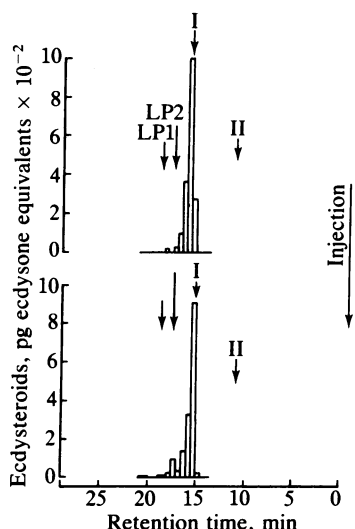


FIG. 6. HPLC/RIA analysis (Resolve column, ICT-1 antiserum) of ecdysteroids secreted *in vitro* by brain-ring glands from *Drosophila* larvae grown on defined media containing either ergosterol (*Upper*) or a mixture of sitosterol, campesterol, stigmasterol, and ergosterol (*Lower*). The positions of LP1, LP2, authentic ecdysone (I), and 20-hydroxyecdysone (II) are marked. A distinct peak of material corresponding to LP2 is visible (*Lower*).

the corresponding 20-hydroxylated ecdysteroid, makisterone A, is the major ecdysteroid at peak ecdysteroid titer (4). The apparent conversion of LP2 to makisterone A by 20-hydroxylation *in vitro* supports the identification of LP2 as 20-deoxymakisterone A. That makisterone A is detectable in *Drosophila* larvae is additional corroboration and is evidence that the ring gland secretes 20-deoxymakisterone A *in vivo*. LP1 is also likely to be a side-chain-modified ecdysone analogue, but its identity is at present unknown.

The C₂₈ phytosterol campesterol is likely to be the precursor of makisterone A in *Dysdercus fasciatus* (4). Dealkylation of the C₂₈ sterol, ergosterol, apparently occurs in *Drosophila*, as has been suggested (20), since only ecdysone is secreted by ring glands from larvae grown on an ergosterol-containing medium. The mechanism leading to the production of 20-deoxymakisterone A by ring glands from larvae grown on cornmeal/yeast medium or on a medium containing a mixture of C₂₈ and C₂₉ sterols is uncertain. Current evidence indicates that dealkylation of C₂₉ sterols by insects does not proceed via a stepwise reduction in carbon atoms of the C-24 ethyl group (21). Thus, C₂₉ sterols are unlikely to be precursors of 20-deoxymakisterone A. Campesterol (24-methylcholesterol) bears a greater structural similarity to cholesterol than does ergosterol and could possibly be an effective substrate for ecdysteroid biosynthesis without prior dealkylation.

The presence of both C₂₇ and C₂₈ ecdysteroids in an arthropod is not unprecedented: Faux *et al.* (22) have identified 20-hydroxyecdysone and makisterone A in the crustacean *Callinectes sapidus*. With respect to insects, makisterone A has been reported to be 10 times more active than 20-hydroxyecdysone in *Oncopeltus* bioassays (23) and at least as active as 20-hydroxyecdysone in the *Calliphora* bioassay

(24). Thus, this ecdysteroid may contribute to physiological moulting hormone levels in *Drosophila* grown on cornmeal/yeast media. Clearly, 20-hydroxyecdysone may not necessarily be the only functional moulting hormone in laboratory-grown *Drosophila*.

I am indebted to Dr. H. H. Rees for his interest and encouragement and for the many informative and stimulating discussions. I thank Prof. T. W. Goodwin and Dr. H. H. Rees at the Department of Biochemistry, University of Liverpool, for research facilities; Mr. M. Prescott for mass spectra; and Profs. J. H. Sang, L. I. Gilbert, and J. W. Fristrom and Drs. H. H. Rees, G. Richards, and D. S. King for their interest and comments on the manuscript. The MAS-7 antiserum was raised with the help of Dr. J. D. Ansell and a Medical Research Council grant to Dr. M. Bownes. Typing by Linda Davison and gifts of antiserum are gratefully acknowledged.

- King, D. S., Bollenbacher, W. E., Borst, D. W., Vedekis, W. V., O'Connor, J. D., Ittycheriah, P. I. & Gilbert, L. I. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 793-796.
- Bollenbacher, W. E., Goodman, W., Vedekis, W. V. & Gilbert, L. I. (1976) *Steroids* **27**, 309-324.
- Gilbert, L. I. & Goodman, W. (1981) in *Metamorphosis: A Problem in Developmental Biology*, eds. Gilbert, L. I. & Frieden, E. (Plenum, New York), pp. 139-175.
- Gibson, J. M., Majumder, M. S. I., Mendis, A. H. W. & Rees, H. H. (1983) *Arch. Insect Biochem. Physiol.* **1**, 105-120.
- Aldrich, J. R., Kelly, T. J. & Woods, C. W. (1982) *J. Insect Physiol.* **28**, 857-861.
- Redfern, C. P. F. (1983) *J. Insect Physiol.* **29**, 65-72.
- Sang, J. H. (1978) in *The Genetics and Biology of Drosophila*, ed. Ashburner, M. A. & Wright, T. R. F. (Academic, London), Vol. 2a, pp. 159-192.
- Mendis, A. H. W., Rose, M. E., Rees, H. H. & Goodwin, T. W. (1983) *Mol. Biochem. Parasitol.* **9**, 209-226.
- Dinan, L. N. & Rees, H. H. (1981) *J. Insect Physiol.* **27**, 51-58.
- Willig, A., Rees, H. H. & Goodwin, T. W. (1971) *J. Insect Physiol.* **17**, 2317-2326.
- Horn, D. H. S., Wilkie, J. S., Sage, B. A. & O'Connor, J. D. (1976) *J. Insect Physiol.* **22**, 901-905.
- Spindler, K.-D., Beckers, C., Groschel-Stewart, U. & Emmerich, H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1269-1275.
- Soumoff, C., Horn, D. H. S. & O'Connor, J. D. (1981) *J. Steroid Biochem.* **14**, 429-435.
- Porcheron, P., Foucrier, J., Gros, G., Pradelles, P., Cassier, P. & Dray, F. (1976) *FEBS Lett.* **61**, 159-162.
- Borst, D. W. & O'Connor, J. D. (1974) *Steroids* **24**, 637-656.
- Reum, L. & Koolman, J. (1979) *Insect Biochem.* **9**, 135-142.
- Lafont, R., Somme-Martin, G., Mauchamp, B., Maume, B. F. & Delbecq, J.-P. (1980) in *Progress in Ecdysone Research*, ed. Hoffmann, J. A. (Elsevier/North-Holland, Amsterdam), pp. 45-68.
- Kemp, R. J., Goad, L. J. & Mercer, E. I. (1967) *Phytochemistry* **6**, 1609-1615.
- Bos, M., Burnet, B., Farrow, R. & Woods, R. A. (1976) *Genet. Res.* **28**, 163-176.
- Cooke, J. & Sang, J. H. (1970) *J. Insect Physiol.* **16**, 801-812.
- Rees, H. H. (1983) in *Biosynthesis of Isoprenoid Compounds*, eds. Porter, J. W. & Spurgeon, S. L. (Wiley, New York), Vol. 2, pp. 463-505.
- Faux, A., Horn, D. H. S., Middleton, E. J., Fales, H. M. & Lowe, M. E. (1969) *Chem. Comm.* **1969**, 175-176.
- Kelly, T. J., Woods, C. W., Redfern, R. E. & Borkovec, A. B. (1981) *J. Exp. Zool.* **218**, 127-132.
- Imai, S., Fujioka, S., Murata, E., Sasaki, Y. & Nakanishi, K. (1968) *Tetrahedron Lett.*, 3887.