Girard derivatization for LC-MS/MS profiling of endogenous ecdysteroids in Drosophila

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Abstract  Ecdysteroids are potent developmental regulators that control molting, reproduction, and stress response in arthropods. In developing larvae, picogram quantities of individual ecdysteroids and their conjugated forms are present along with milligrams of structural and energy storage lipids. To enhance the specificity and sensitivity of ecdysteroid detection, we targeted the 6-ketone group, which is common to all ecdysteroids, with Girard reagents. Unlike other ketosteroids, during the reaction, Girard hydrazones of ecdysteroids eliminated the C14-hydroxyl group, creating an additional C14-C15 double bond. Dehydrated hydrazones of endogenous ecdysteroids were detected by LC-MS/MS in the multiple reaction monitoring (MRM) mode using two mass transitions: one relied upon neutral loss of a quaternary amine from the Girard T moiety; another complementary transition followed neutral loss of the hydrocarbon chain upon C20-C27 cleavage. We further demonstrated that a combination of Girard derivatization and LC-MS/MS enabled unequivocal detection of three major endogenous hormones at the picogram level in an extract from a single Drosophila pupa.—Lavrynenko, O., R. Nedielkov, H. M. Möller, and A. Shevchenko. Girard derivatization for LC-MS/MS profiling of endogenous ecdysteroids in Drosophila. J. Lipid Res. 2013. 54: 2265–2272.

Supplementary key words  ecdysone • Drosophila melanogaster • Girard reagent

Ecdysteroids are a large family of structurally diverse polyhydroxylated sterols found in plants and animals of the Arthropoda phylum (reviewed in Ref. 1). In insects, ecdysteroids control life-cycle progression, including molting, reproduction, stress response, and lifespan, via conserved regulatory pathways (reviewed in Ref. 2). The fruit fly Drosophila melanogaster is an established model organism in developmental biology and now is becoming an important system for studying lipid metabolism and its hormonal regulation (3–6). Drosophila is a sterol auxotroph, and its steroidogenesis relies entirely upon dietary sources of sterols. However, the exact molecular mechanisms by which the availability of dietary sterols alters the ecdysteroids profile and affects fly development remain poorly understood (7).

Ecdysone (E), 20-hydroxyecdysone (20H), and makisterone A (Fig. 1 and supplementary Fig. I) are major ecdysteroids in Drosophila. Their content in individual animals depends on both the developmental stage and the availability of dietary sterols (8, 9). Apart from these three major hormones, several low abundant molecules, including 20-deoxy-makisterone A (9), 24-epi-20-deoxy-makisterone A (8), and 20-hydroxyecdysone-22-acetate (10) have been characterized; the existence of other ecdysteroid-related structures has been inferred from indirect biological evidence (11). Furthermore, previous observations indicated that ecdysteroids may be present in a variety of conjugates with phosphate, sulfate, fatty acid, and carbohydrate moieties serving as storage or transport forms of active hormones (12–16). Depending on the stage of development, a single Drosophila larva might contain from 50 to 400 pg/mg of ecdysteroids in total (17, 18), along with over one billion-fold molar excess of structural (glycerophospholipid, ceramide, and steroid) and energy storage (di- and triacylglycerol) lipids. Radioimmunoassay (RIA) has historically been most common way of quantifying ecdysteroids in insects having small body size (e.g., Drosophila). However, RIA offers limited dynamic range and, not being able to distinguish individual ecdysteroids, may only report the total content of all ecdysone-related molecules. Methods have been developed for detecting individual endogenous insect ecdysteroids by GC-MS (19) and...
an ecdysteroid may comprise up to six hydroxyl groups. Second, they possess the 6-ketone group conjugated with the C7-C8 double bond along with the vinyl hydroxyl at C14. Therefore, it is conceivable that derivatizing the 6-ketone group with an appropriate reagent might enhance the ionization capacity and increase the analysis specificity of the entire ecdysteroid complement.

LMS/MS (8, 20); however, they appeared to be far less sensitive compared with RIA.

Ecdysteroids are detected by electrospray ionization mass spectrometry as protonated molecular cations (8, 20). However, their ionization capacity is low compared with major glycerophospholipids, such as phosphatidylcholines and phosphatidylethanolamines. MS/MS spectra of ecdysteroids are dominated by abundant peaks of water-loss fragments (21, 22), which is common for small relatively hydrophilic molecules. Therefore, unequivocal identification of known and discovery of novel endogenous ecdysteroids require alternative approaches relying on molecular forms having more specific fragmentation pathways.

Despite their structural heterogeneity, ecdysteroids share a few common features that distinguish them from the majority of oxysterols (Fig. 1). First, they are polyhydroxylated: an ecdysteroid may comprise up to six hydroxyl groups. Second, they possess the 6-ketone group conjugated with the C7-C8 double bond along with the vinyl hydroxyl at C14. Therefore, it is conceivable that derivatizing the 6-ketone group with an appropriate reagent might enhance the ionization capacity and increase the analysis specificity of the entire ecdysteroid complement.

Girard reagents (23), a family of quaternary ammonium hydrazides (24) typically comprising pyridine or trimethylamine (TMA) moieties, were employed for the derivatization of 3-ketone groups in mammalian oxysterols produced by their enzymatic oxidation (25–28): converting charge-neutral oxysterols to strongly positively charged hydrazones enhanced their solubility and ionization capacity (an approach recently termed “charge-tagging”) (29). If subjected to MS/MS, Girard hydrazones undergo
facile neutral loss of pyridine or TMA. Net positive charge is then transferred to the sterol backbone, and upon collision-induced dissociation (CID), several structure-specific informative fragments are produced. These oxidized oxysterols, however, are different from ecdysteroids; their ketone group is located at the outer A-ring, which might ease constraints imposed by sterical hindrance (24); additionally, there is no vinyl hydroxyl group next to the double bond within the B-ring. Oxysterols have fewer hydroxyl moieties and are generally less prone to water losses.

Here we demonstrate that a combination of Girard derivatization and LC-MS/MS enabled simultaneous detection of major ecdysteroids at the endogenous (picogram) level in the extract of a single Drosophila pupa. It paves the way toward the rapid comprehensive characterization of the ecdysteroidome - a full complement of ecdysteroids, their precursors, intermediate conjugates, and metabolites present in a developing fly.

MATERIALS AND METHODS

Materials and ecdysteroid standards

Standards of 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, and polyepoline B were purchased from Sigma-Aldrich Chemie (Munich, Germany); ecdysone from A.G. Scientific (Göttingen, Germany); muristerone A from Merck Biosciences (Bad Soden, Germany); and makisterone A and ponasterone A from Enzo Life Sciences (Lörrach, Germany). All solvents were LC-MS grade. Girard T [(carboxymethyl)trimethylammonium chloride hydradizide] and Girard C [potassium hydrazinecarbonyl acetate] were purchased from Sigma-Aldrich Chemie; and Girard P [1-(carboxymethyl)pyridinium chloride hydradizide] from TCI Europe (Eschborn, Germany).

MS and LC-MS/MS of native and derivatized ecdysones

HPLC was performed on Agilent 1200 system equipped with a trap column (OPTI-PAK, 1 µl, C18) from Dichrom GmbH (Marl, Germany) that was mounted in-line to a 0.5 mm × 150 mm analytical column packed with Zorbax SB-C18 (5 µm) from Waters (Eschborn, Germany), dried down in a vacuum centrifuge, and redisolved in isopropanol/methanol/chloroform 4:2:1 (v/v/v) containing 7.5 mM ammonium acetate. MS and MS/MS spectra were acquired on a Q Exactive tandem mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca, NY). Gas backpressure was 1.25 psi, and ionization voltage was 0.95 kV. Spectra were acquired in MS mode with the target mass resolution of Rm/z = 140,000 under automated gain control (AGC) target value of 1,000,000 and maximum injection time of 1 s. Fragmentation was performed in the HCD cell (30) at the normalized collision energy (nCE) of 20% for native ecdysteroids and 40% for their Girard derivatives.

Ecdysone derivatization with Girard reagents

Aliquots (10 µl) of the stock solution of ecdysone (5 mM in neat methanol) were spiked into 1 ml of 70% methanol (reaction temperature 50°C), 70% ethanol (70°C), and 70% isopropanol (85°C) in water. Then 50 µl of glacial acetic acid and 50 µg of the Girard reagent were added, and the mixture was incubated at the specified temperature. After certain time intervals, 5 µl aliquots were withdrawn and neutralized with 95 µl of methanol with 1% NH4OH. Then 10 µl aliquot of the neutralized sample was further diluted with 85 µl of water, and 5 µl of 0.5 µM muristerone A (internal standard) was spiked in. Of this mixture, 5 µl was injected into LC-MS system. Kinetics of the ecdysone derivatization was monitored by LC-MS/MS on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled with Agilent 1200 LC system and operated with 60 min gradient elution program as described above. TSQ Vantage was equipped with a H-ESI ion source operated under spray voltage of 2.7 kV, sheath gas pressure of 5 psi, auxiliary gas set at 2 arbitrary units, vaporizer temperature at 50°C, and transfer capillary temperature at 200°C. The sample was loaded, concentrated, and cleaned up at the trap column during first 10 min and then eluted into the analytical column. All samples were analyzed in duplicates.

Dh-G(p)E was quantified in MRM mode by monitoring pyridine neutral loss 580.4→501.4 (collision energy of 31 eV, S-lens at 215 V). Muristerone A (2.5 nM) spiked into samples as the internal standard was detected by monitoring the mass transition 497.4→425.4 (collision energy of 16 eV, S-lens at 145 V). Quantification was performed by the QuaLBrowser program from Xcalibur 2.2 software; chromatographic peaks were integrated using Genesis algorithm with five-point Gaussian smoothing.

NMR spectroscopy

Preparative quantities of the product of ecdysone derivatization with Girard P were purified using two-step cleanup. First, Oasis MCX (6 cc/150 mg) cartridge from Waters was washed with 3 ml of methanol and then 3 ml of water. The reaction mixture was loaded in 15% aqueous methanol, and the cartridge was washed with 5 ml of 0.1 M aqueous hydrochloric acid. The derivatization product was eluted with 2 ml of 5% NH4OH in methanol and dried in a vacuum centrifuge. Then the sample was redissolved 5% of B during first 10 min until the sample is loaded and concentrated on the trap column; ramping from 15% to 30% of B between 11 min to 30 min; increasing up to 100% of B in 1 min and holding for 9 min; stepping down to 5% of B in 1 min and holding for 19 min to equilibrate the column to starting conditions. The flow rate was 10 µl/min; injection volumes are specified for each experiment.

For direct infusion experiments, the reaction mixture was cleaned up by solid phase extraction on Oasis HLB (6 cc/150 mg) cartridges from Waters (Eschborn, Germany), dried down in a vacuum centrifuge, and redisolved in isopropanol/methanol/chloroform 4:2:1 (v/v/v) containing 7.5 mM ammonium acetate. MS and MS/MS spectra were acquired on a Q Exactive tandem mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca, NY). Gas backpressure was 1.25 psi, and ionization voltage was 0.95 kV. Spectra were acquired in MS mode with the target mass resolution of Rm/z = 140,000 under automated gain control (AGC) target value of 1,000,000 and maximum injection time of 1 s. Fragmentation was performed in the HCD cell (30) at the normalized collision energy (nCE) of 20% for native ecdysteroids and 40% for their Girard derivatives.
in 15% methanol, loaded on prewashed Oasis HLB (6 cc/150 mg) cartridge, washed with water, eluted with 2 ml of neat methanol, and vacuum dried.

For NMR analysis, 2 mg of ecdysone and its Girard P derivative (purified as described above) were dissolved in DMSO-d6 at the concentration of 14 mM and 5.5 mM, respectively, and then the samples were transferred into 5 mm NMR tubes. 1H, 13C, 1H-13C-HSQC, 1H-13C-HMBC, 2D-COSY, and 2D-NOESY NMR spectra were recorded at 300 K on a Bruker AVANCE III 600 MHz spectrometer equipped with a cryogenic 5 mm TCI-H/C/N triple-resonance probe with actively shielded z-gradient. Spectra were referenced to the residual DMSO signal at 2.50 ppm and were processed and analyzed with TopSpin v.2.1 software (Bruker).

**Detecting endogenous ecdysteroids in *Drosophila* pupae**

*Drosophila melanogaster* WT (Oregon-K) embryos were collected for 2 h on apple juice/agar plates, rinsed in PBS containing 0.05% Triton X-100, bleached by 50% sodium hypochlorite for 30 s, and rinsed with distilled water. Then, embryos were transferred to plates containing standard lab food consisting of malt, soy, cornmeal, and yeast (7). Animals at the pupae stage were collected. Frozen animals were homogenized with a plastic pestle and extracted overnight with 1 ml of methanol at 4°C. After 5 min centrifuging at 14,500 rpm on a Mini-Spin centrifuge from Eppendorf (Hamburg, Germany), the supernatant was collected and further dried in a vacuum centrifuge. The pellet was twice reextracted with 1 ml of methanol for 30 min, which was combined and cleaned up by solid-phase extraction on a HLB Oasis cartridge as described (20). The collected eluate was dried down and redissolved in 100 µl of 15% methanol.

Endogenous ecdysteroids were detected by LC-MS/MS as described above; however, the injection volume was increased to 30 µl. MRM transitions and detection settings are provided in supplementary Table IV.
RESULTS AND DISCUSSION

Derivatization of ecdysone with Girard reagents

Preliminary experiments performed under conventional reaction conditions (25) (overnight incubation in 5% acetic acid in methanol; ambient temperature; starting reagent concentration of 0.3 mM) showed that the 6-ketone group of ecdysone was much less reactive toward hydrazides compared with 3-ketosterols. The Girard hydrazone was only produced at elevated reaction temperature; however we noticed that it was undergoing a rapid loss of water (Fig. 1). Therefore, we first investigated whether water loss occurred via eliminating a hydroxyl group from one or in parallel from several positions at the ecdysone backbone.

We purified about 2 mg of dh-G(p)E by solid phase cleanup of the reaction mixture and analyzed it by NMR, which suggested that the specific loss of the 14-hydroxyl group yielded a double bond between C14-C15 (supplementary Fig. II), while other hydroxyl groups remained intact. Site-specific loss of the hydroxyl group corroborated LC-MS/MS analyses that revealed no alternative products that might differ by their retention times (see below). The control experiment performed without adding G(p) demonstrated that even at 85°C, ecdysone largely remained intact, suggesting that water loss occurred from or in parallel with the formation of G(p)E, rather than from ecdysone itself.

Mass spectrum acquired by direct infusion of the reaction mixture purified by solid-phase extraction was dominated by the abundant peak of dh-G(p)E, while peaks corresponding to native E and G(p)E were minor (Fig. 2A). Therefore, we ventured to determine whether the yield of dh-G(p)E could be improved by varying the reaction time and temperature. Aliquots withdrawn from the reaction mixture at specified time points were cleaned up on a SPE cartridge and, after adding muristerone A (internal standard), were analyzed by LC-MS/MS. While at 50°C (conventional temperature for Girard derivatization) and 70°C the product yield was steadily increasing with time, at 85°C the abundance of dh-G(p)E peaked at the time point...
of around 4 h and then decreased, presumably due to progressing water losses and/or product decomposition (Fig. 2B). A separate MRM-based quantification experiment showed that only about 40% of ecdysone reacted by this time (data not shown); however, the abundance of dh-G(p)E exceeded the total abundance of all forms related to ecdysone (including molecular adducts) by more than 5-fold. These reaction conditions were subsequently used for detecting endogenous ecdysteroids.

### MS/MS fragmentation of Girard derivatives

Next we derivatized ecdysone with a series of Girard reagents: G(p), G(t), and G(c). “Classic” Girard reagents G(p) and G(t) bear positively charged end groups (24), whereas G(c) is better suited for negative mode analyses, which we thought might provide the analytical advantage of having alternative fragmentation pathways.

Even at the low (<10 eV) collision energy, protonated molecular ions ([M+H]+) of ecdysteroids underwent successive loss of multiple (up to five, in the case of muristerone A) water molecules (21) (Figs. 3 and 4A). These relatively common fragmentation pathways did not contribute to higher detection specificity and compromised sensitivity of the analyses. At higher collision energy, we detected fragments produced by cleaving the bonds between C20-C22 (in E), C22-C23 (only observed in sterols with vicinal hydroxyls at C20 and C22, such as MA and 20H), and C17-C20 (8). In turn, these fragments also underwent successive water losses, yielding a series of relatively low abundant products (Fig. 3). In contrast, dh-G(p)E underwent rapid intramolecular cyclization accompanied by neutral loss of pyridine, either alone ([M+H]+ = 79.0; product m/z 501.3316) or together with concomitant loss of CO ([M+H]+ = 107.0; m/z 473.3371) (Fig. 4), similar to Girard hydrazones of 3-ketosterols (31). CID MS2 experiments performed at the linear ion trap of a LTQ Orbitrap tandem mass spectrometer suggested that these were parallel (rather than successive) pathways because they produced nonoverlapping series of fragment ions (data not shown). Each of these two major fragments underwent up to two successive losses of hydroxyl groups from their hydrocarbon chains. Independently, the entire chain was split off by cleaving the C17-C20 bond (Fig. 5). At the same time, vicinal hydroxyls at the A-ring (positions C2 and C3) remained intact.

Fragmentation of dh-G(t)E proceeded in the similar way. As expected, TMA neutral loss was a major pathway that produced the same fragments as dh-G(p)E (Fig. 4). We therefore reasoned that ecdysteroids could be detected by monitoring highly specific and abundant products of neutral loss of pyridine or TMA from their dehydrated precursors. In parallel, it should also be possible to monitor the fragments specific for the dehydroxylated at C14 position, but otherwise intact, sterol ring backbone. At the same time, we observed that no sterol core cleavage fragments or fragments of the side chain were sufficiently abundant for designing sensitive MRMs.
For comparison, we derivatized E with G(c) and checked how dh-G(c)E was fragmenting in negative ion mode. Similar to G(p) and G(t) hydrazones in positive mode, the MS/MS spectrum was dominated by neutral loss of the end (here, carboxyl) group of Girard reagent accompanied by creating intramolecular cycle; however, there was no parallel pathway with further loss of CO (Fig. 6), and the fragments abundances were low. Altogether, derivatization with G(c) did not increase the detection sensitivity compared with native ecdysone, and it was not employed in further work.

We then applied G(p) derivatization to seven individual ecdysteroids and observed both similar reaction efficiency and similar pathways of MS/MS fragmentation (supplementary Fig. II and supplementary Table I). Overall, the derivatization efficiency was similar for G(p) and G(t). However, G(t) derivatives were eluting about 30 s earlier than G(p) derivatives and offered slightly (on average, less than 2-fold) better sensitivity of MRM detection. Therefore, in our further work with endogenous hormones, we employed G(t).

**Endogenous ecdysteroids in *Drosophila* pupae**

We next applied Girard derivatization for detecting endogenous ecdysteroids in *Drosophila* pupae. Ecdysteroids were enriched by extracting homogenized pupae with cold methanol. The total extract was treated with G(t) for 4 h at 85°C, cleaned up on the SPE cartridge, and analyzed by LC-MS/MS (Fig. 7).

Dehydrated hydrazones of each expected ecdysteroid were monitored using MRM transitions relying upon the two major fragmentation pathways (Fig. 5): neutral loss of TMA was common to all G(t) derivatives; another complementary transition followed up a subsequent neutral loss of the hydrocarbon chains upon C17-C20 cleavage. By monitoring six transitions in parallel, we achieved confident detection of three major *Drosophila* ecdysteroids: E, 20H, and makisterone A at the endogenous level. In general, we were able to detect as low as 10 pg of ecdysteroids because of higher ionization capacity of corresponding dh-G(t) derivatives and reduced chemical noise in the monitored MRM transitions.

Ecdysteroids are asymmetrical ketones and, upon treatment with Girard reagents, produced a mixture of E/Z hydrazones that were detected as pairs of chromatographically resolved peaks, which further supported their identification. Note that endogenous makisterone A (Fig. 7E, F) has an epimer, which may be detected as an additional chromatographic peak.

Taken together, we demonstrated that Girard derivatization enhanced both the sensitivity and specificity of ecdysteroids detection by the method of MRM and that the endogenous ecdysteroids could be determined in the extract from a single *Drosophila* pupa or from 5–10 animals collected at the late larva stage.

**CONCLUSIONS AND PERSPECTIVES**

Girard derivatization is an established method for quantitative LC-MS profiling of mammalian oxysterols that have been enzymatically converted into 3-ketosterols. Our work further extended this “charge-tagging” approach to ecdysteroids. In contrast to 3-ketosterols, derivatization of the 6-ketone group in ecdysteroids was accompanied by facile loss of the 14-hydroxyl group, yielding an “additional” C14-C15 double bond. During MS/MS fragmentation, dh-G(t) and dh-G(p) ecdysteroids expectantly lost TMA and pyridine moieties, respectively, and a hydrocarbon side chain, while the steroid core remained intact. Together, these structure-specific neutral losses allowed us to design two complementary MRM transitions for each ecdysteroid and to recognize endogenous molecules in crude, lipid-rich extracts from larvae and pupae.

Derivatization of a broader selection of ecdysteroids with Girard reagents proceeded in the same way as the derivatization of ecdysone. Since the 6-ketone group is common to all ecdysteroid structures and the 14-hydroxyl group is not directly involved in making conjugates (25), Girard derivatization lends itself for unbiased compositional screening for yet unknown development stage-specific components of the ecdysteroidome.

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**REFERENCES**

1. Gilbert, L. I. 2012. Insect Endocrinology. 1st edition. Elsevier/Academic Press, London; Waltham, MA.
2. Schwedes, C. C., and G. E. Carney. 2012. Ecdysone signaling in adult Drosophila melanogaster. J. Insect Physiol. 58: 293–302.
3. Baker, K. D., and C. S. Thummel. 2007. Diabetic larvae and obese flies—emerging studies of metabolism in Drosophila. Cell Metab. 6: 257–266.
4. Broglio, W., H. Stocker, T. Ikeya, F. Rintelen, R. Fernandez, and E. Hafen. 2001. An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr. Biol. 11: 213–221.
5. Spindler, K. D., C. Honl, C. Tremmel, S. Braun, H. Ruff, and M. Spindler-Barth. 2009. Ecdysteroid hormone action. Cell. Mol. Life Sci. 66: 3857–3858.
6. Gutierrez, E., D. Wiggins, B. Fielding, and A. P. Gould. 2007. Specialized hepatocyte-like cells regulate Drosophila lipid metabolism. Nature. 445: 275–280.
7. Carvalho, M., D. Schwudke, J. L. Sampaio, W. Palm, I. Riezman, G. Dey, G. D. Gupta, S. Mayor, H. Riezman, A. Shevchenko, et al. 2010. Survival strategies of a sterile auxotroph. Development. 137: 3675–3685.
8. Blais, C., T. Blasco, A. Maria, C. Dauphin-Villemant, and R. Lafont. 2010. Characterization of ecdysteroids in Drosophila melanogaster by enzyme immunoassay and nano-liquid chromatography-tandem mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 878: 925–932.
9. Redfern, C. P. 1984. Evidence for the presence of makisterone A in Drosophila larvae and the secretion of 20-deoxymakisterone A by the ring gland. Proc. Natl. Acad. Sci. USA. 81: 5643–5647.
10. Maroy, P., G. Kaufmann, and A. Dubendorfer. 1988. Embryonic ecdysteroids of Drosophila melanogaster. J. Insect Physiol. 34: 633–637.
11. Guittard, E., C. Blais, A. Maria, J. P. Parvy, S. Pasricha, C. Lumb, R. Lafont, P. J. Dalborn, and C. Dauphin-Villemant. 2011. CYP18A1, a key enzyme of Drosophila steroid hormone inactivation, is essential for metamorphosis. Dev. Biol. 349: 35–45.

Endogenous ecdysteroids in *Drosophila* by LC-MS/MS 2271
12. Dubendorfer, A., and P. Maroy. 1986. Ecdysteroid conjugation by tissues of adult females of Drosophila melanogaster. Insect Biochem. 16: 109–113.

13. Wigglesworth, K. P., D. Lewis, and H. H. Rees. 1985. Ecdysteroid titer and metabolism to novel apolar derivatives in adult female Boophilus microplus (Ixodidae). Arch. Insect Biochem. Physiol. 2: 39–54.

14. Sonobe, H., and Y. Ito. 2009. Phosphoconjugation and dephosphorylation reactions of steroid hormone in insects. Mol. Cell. Endocrinol. 307: 25–35.

15. Isaac, R. E., H. P. Desmond, and H. H. Rees. 1984. Isolation and identification of 3-acetylecysone 2-phosphate, a metabolite of ecdysone, from developing eggs of Schistocera gregaria. Biochem. J. 217: 239–243.

16. Thompson, M. J., M. F. Feldlaufer, R. Lozano, H. H. Rees, W. R. Lusby, J. A. Svoboda, and K. R. Wilzer. 1987. Metabolism of 26-[C-14]hydroxyecdysone 26-phosphate in the tobacco hornworm, Manduca sexta L., to a new ecdysteroid conjugate - 26-[C-14]hydroxyecdysone 26-phosphate in the tobacco hornworm, Manduca sexta L., to a new ecdysteroid conjugate.

17. Kozlova, T., and C. S. Thummel. 2000. Steroid regulation of postembryonic development and reproduction in Drosophila. Trends Endocrinol. Metab. 11: 276–280.

18. Parry, J. P., C. Blais, F. Bernard, J. T. Warren, A. Petryk, L. I. Gilbert, M. B. O’Connor, and C. Dauphin-Villemant. 2005. A role for betaFTZ-F1 in regulating ecdysteroid titers during postembryonic development in Drosophila melanogaster. Dev. Biol. 282: 84–94.

19. Le Bizec, B., J-P. Antignac, F. Monteau, and F. Andre. 2002. Ecdysteroids: one potential new anabolic family in breeding animals. Anal. Chem. Acta. 473: 89–97.

20. Li, Y., J. T. Warren, G. Boysen, L. I. Gilbert, A. Gold, R. Sangiah, L. M. Ball, and J. A. Swenberg. 2006. Profiling of ecdysteroids in complex biological samples using liquid chromatography/ion trap mass spectrometry. Rapid Commun. Mass Spectrom. 20: 185–192.

21. Wang, Y. H., B. Avula, A. N. Jadhav, T. J. Smillie, and I. A. Khan. 2008. Structural characterization and identification of ecdysteroids from Sida rhombifolia L. in positive electrospray ionization by tandem mass spectrometry. Rapid Commun. Mass Spectrom. 22: 2413–2422.

22. Stevens, J. F., R. L. Reed, and J. T. Morre. 2008. Characterization of phytoecdysteroid glycosides in Meadowfoam (Limnanthes alba) seed meal by positive and negative ion LC-MS/MS. J. Agric. Food Chem. 56: 3945–3952.

23. Girard, A., and G. Sandulescu. 1936. On a new series of reactants of the carbonyl group, their use for the extraction of ketonic substances and for the microchemical characterisation of aldehydes and ketones. Helv. Chim. Acta. 19: 1096–1107.

24. Wheeler, O. H. 1968. The Girard reagents. J. Chem. Educ. 45: 435–437.

25. Karu, K., M. Hornshaw, G. Woffendin, K. Bodin, M. Hamberg, G. Alvelius, J. Sjovall, J. Turton, Y. Wang, and W. J. Griffiths. 2007. Liquid chromatography-mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols. J. Lipid Res. 48: 976–987.

26. Wang, Y., K. M. Sousa, K. Bodin, S. Theofilopoulos, P. Sacchetti, M. Hornshaw, G. Woffendin, K. Karu, J. Sjovall, E. Arenas, et al. 2009. Targeted lipidomic analysis of oxysterols in the embryonic central nervous system. Mol. Biosyst. 5: 529–541.

27. Griffiths, W. J., Y. Wang, G. Alvelius, S. Liu, K. Bodin, and J. Sjovall. 2006. Analysis of oxysterols by electrospray tandem mass spectrometry. J. Am. Soc. Mass Spectrom. 17: 341–362.

28. Shackleton, C. H., H. Chuang, J. Kim, X. de la Torre, and J. Segura. 1997. Electrospray mass spectrometry of testosterone esters: potential for use in doping control. Steroids. 62: 525–529.

29. Meljon, A., S. Theofilopoulos, C. H. Shackleton, G. L. Watson, N. B. Javitt, H. J. Knolker, R. Saini, E. Arenas, Y. Wang, and W. J. Griffiths. 2012. Analysis of bioactive oxysterols in newborn mouse brain by LC/MS. J. Lipid Res. 53: 2469–2483.

30. Schuhmann, K., R. Herzog, D. Schwudke, W. Metelmann-Strupat, S. R. Bornstein, and A. Shevchenko. 2011. Bottom-up shotgun lipidomics by higher energy collisional dissociation on LTQ Orbitrap mass spectrometers. Anal. Chem. 83: 5480–5487.

31. Griffiths, W. J. 2003. Tandem mass spectrometry in the study of fatty acids, bile acids, and steroids. Mass Spectrom. Rev. 22: 81–152.