THE ECDYSTEROIDS FROM THE TOBACCO HORNWORM DURING PUPAL-ADULT DEVELOPMENT FIVE DAYS AFTER PEAK TITER OF MOLTING HORMONE ACTIVITY

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ABSTRACT

Six naturally occurring C_{27} ecdysteroids were isolated and identified from the tobacco hornworm during pupal-adult development five days after peak titer of molting hormone activity. In order of decreasing quantities the hormones were: 20,26-dihydroxyecdysone, 3-epi-20-hydroxyecdysone, 20-hydroxyecdysone, 3-epi-20,26-dihydroxyecdysone, 3-epi-ecdysone, and ecdysone. 20-Hydroxyecdysone, in an earlier study, was the major molting hormone present at peak titer during pupal-adult development. The major ecdysteroid present during embryonic development in this insect, 26-hydroxyecdysone, was not detected. The copresence of all six of these ecdysteroids from a single developmental stage of an insect provides information on the metabolic interrelationships that exist among these steroids and on their possible function(s) in insects. The 3α-ecdysteroids were far less active than the 3β-epimers in the house fly assay. The significance of epimerization is discussed.

INTRODUCTION

In previous communications (1, 2), we reported the presence of ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone in the tobacco hornworm Manduca sexta (L.) during pupal-adult development at the time of peak titer of molting hormone activity. During this developmental stage in post-embryonic development, 20-hydroxyecdysone was the major molting hormone (1,2). However, studies of the molting hormones during embryonic development with two different age groups of eggs revealed that 26-hydroxyecdysone was the predominant ecdysteroid accounting for more than 80% of the total unconjugated molting hormones (3, 4); ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone accounted for the remainder.
Subsequently another ecdysteroid, 3-epi-20-hydroxyecdysone, that differed from that of the known insect molting hormones in orientation of the hydroxyl group at the C-3 position, was isolated and identified from the meconium of the tobacco hornworm (5). Concurrently, the 3α-epimer of ecdysone was also isolated from the incubation of ecdysone with an enzyme preparation from the midgut of prepupae from this insect (6).

In our continuing effort to gain a better understanding of the molting hormone profile in different ages and/or stages in insects, we examined the free ecdysteroids present in the hornworm during pupal-adult development five days after peak titer of molting hormone activity.

**EXPERIMENTAL METHODS AND RESULTS**

**Instrumentation.** An International Model PR-2 portable refrigerated centrifuge (7) was used to centrifuge the extracts. A manually operated Craig-Post 60 tube counter-current distribution (CCD) apparatus with 10 ml volumes each for the upper and lower phase was used to separate and/or to purify the molting hormones (MHs). Precoated TLC Silica Gel
60 F-254 plates were used for the separation, purification, and analyses of the ecdysteroids. A chromato-vue instrument box allowed direct visualization of the spots or zones under short wavelength ultraviolet light. Spots were also detected by spraying the plate with 50% sulfuric acid and heating at 100°. Ultraviolet spectra were taken in methanol with a Bausch and Lomb Spectronic 505. Mass spectra were obtained with an LKB Model 9000 mass spectrometer equipped with a Varian Spectra System 100 MS data system. The samples were introduced directly into the ionization chamber (ionization energy was 70 ev). NMR spectra were recorded at 60 MHz with a Varian A-60A NMR Spectrometer equipped with a Varian C-1024 time averaging computer. Deuterated pyridine was used as the solvent and TMS as the internal standard.

Biological Material. Tobacco hornworms were reared in the laboratory on an artificial diet (8). Migrating 5th-instar larvae (early prepupae) were collected daily and placed in individual wooden cells (9). Pupae of mixed sex that were in the process of shedding their larval skin or had aged it within two hours were removed from the cells and held at 25 ± 1.5° until 12 days old. On the 12th day, the insects were weighed and then frozen at -20° and held until 3000 (10.5 kg wet weight) were accumulated for extraction.

Molting Hormone Assay. The house fly assay (10) was used for determining the total MH activity in crude extracts of fractions obtained during the course of purification and for determining the specific activity of the purified ecdysteroids; the results are expressed in house fly units (HFU). For assessing total MH activity, a house fly unit is defined as that quantity of ecdysteroid(s) required to give a 50-60% response (puparium formation) in the treated insects.

Microcolumns and Thin-Layer Chromatography (TLC). During the course of purification and/or separation of the ecdysteroids, two adsorption microcolumns were employed. One column consisted of 1.13 g (1.1 x 2.5 cm) of benzene-washed silicic acid capped off with 1 g of sand. After introduction of the material, the column was eluted and the following fractions were collected: [1] 75 ml of benzene-methanol (95:5); [2] 25 ml of benzene-methanol (95:5); [3] 50 ml of benzene-methanol (90:10); [4] 15 ml of benzene-methanol (75:25); and [5] 15 ml of methanol. The other microcolumn was 3 g (1.1 cm x 2.5 cm) of Woelm neutral alumina (activity Grade 1 + 20% water). The column was dry packed in a minimum amount of methanol without permitting any of the solvent to pass through. After introduction of the material directly on the adsorbent with methanol, the column was eluted first with 50 ml of methanol and then with 50 ml of 75% methanol.

Pre-coated TLC Plates Silica Gel 60F-254 were used for detecting, purifying and separating the free ecdysteroids. The plates were developed at ambient temperature in the solvent system of chloroform-ethanol (65:35) with wick (2).

Isolation and Identification of the Molting Hormones from the Tobacco Hornworm. After groups of 600 to 800 hornworms had thawed overnight in the blender in the presence of 0.5 ml methanol per gram of tissue, the
insects were homogenized at low speed for 5 min. The homogenate was transferred to 250-ml glass cups and centrifuged at 2000-2500 RPM for 10 min. The supernatants were decanted off and filtered through medium pore, fritted disc glass funnels. The residues in the tubes were combined and rehomogenized twice with 75% methanol (0.25 ml/g) and treated as described previously. Procedures used in handling solids that precipitated out during concentration and the steps necessary for extracting and isolating the active crude extracts are described in detail elsewhere (1).

The 70% methanol phases from 3000 hornworms (10.5 kg) yielded 3.7 g of residue with a total activity of $7.0 \times 10^3$ HFU. The residue was dissolved in 25 ml of methanol and introduced on a column of 50 g (4.4 cm ID x 2.5 cm) of Woelm neutral alumina (activity Grade I + 20% water) packed in a minimum amount of methanol. The column was eluted with 800 ml of methanol followed by 800 ml of 75% methanol. The methanol eluate (1.5 g) possessed 93% of the biological activity. The eluate was dissolved in 2.5 ml of methanol, adjusted with benzene to give a final concentration of benzene-methanol (95:5), and subjected to a 4.4 cm (I.D.) x 2.5 cm column of benzene-washed silicic acid (18 g). After 3 additional rinses (5 ml volumes) as described, to ensure transfer of the active material, the column was eluted with increasing concentrations of methanol in benzene. The elution pattern and distribution of mass and biological activity are presented in Table 1.

### Table 1
**Distribution of mass and biological activity following column chromatography on silicic acid of the methanol eluate from alumina.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Mass (mg)</th>
<th>Total Biological Activity (HFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi$-MeOH(95:5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1200</td>
<td>438.6</td>
<td>$-1.2 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>21.7</td>
<td>$6.0 \times 10^5$</td>
</tr>
<tr>
<td>$\phi$-MeOH(90:10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>800</td>
<td>177.0</td>
<td>$7.0 \times 10^4$</td>
</tr>
<tr>
<td>$\phi$-MeOH(75:25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>400</td>
<td>206.5</td>
<td>$1.0 \times 10^4$</td>
</tr>
<tr>
<td>MeOH</td>
<td>400</td>
<td>534.2</td>
<td></td>
</tr>
</tbody>
</table>

*a/ Column 4.4 cm (I.D.) x 2.5 cm, 18 g of silicic acid.

Fraction 3, which possessed about 13% of the mass and 87% of the biological activity and is known to elute ecdysone, 20-hydroxyecdysone, and 3-epi-20-hydroxyecdysone (11), was subjected to 50 transfers in a countercurrent distribution system of cyclohexane, butanol, water (5:5:10). The combined upper and lower phases of each tube were then
collected, reduced to dryness, weighed, and adjusted with a known volume of methanol for TLC analysis. An aliquot of 5-10 µg from each tube was spotted on a TLC plate along with ecdysteroid standards, and the plate was developed in a chloroform-ethanol solvent system. Tubes 10 to 25 gave spots with an $R_f$ value for 20-hydroxyecdysone (0.40) and for 3-epi-20-hydroxyecdysone (0.44). Tubes 11 through 23 were combined (17.5 mg) and subjected again to CCD for further purification. After monitoring each of the tubes by TLC, tubes 11 through 22 (10.4 mg) were combined and the sample was streaked across a single full size TLC plate. The lower zone coinciding with the $R_f$ value of 20-hydroxyecdysone, and the upper zone, whose $R_f$ value was that of 3-epi-20-hydroxyecdysone, were scraped from the plate and extracted with methanol. The extracted materials from the lower (1.42 mg) and upper (1.46 mg) zones were separately chromatographed on a microcolumn of silicic acid. Column fraction 3 of the lower zone gave 0.9 mg of residue that contained 20-hydroxyecdysone, $\lambda_{max}^{245}$ 245 nm in methanol, $\epsilon$ 8,200 (65% pure), Table 2. Column fraction 3 of the upper zone gave 1.2 mg of material that contained the 3-epi-20-hydroxyecdysone of 81% purity (Table 2).

The CCD tubes 30 to 44 when analyzed individually by TLC gave two major spots, a lower spot with an $R_f$ of 0.47, that of ecdysone, and an upper spot with an $R_f$ of 0.52, that of 3-epi-ecdysone. Tubes 30 to 44 when pooled (18.5 mg) and worked up as in the purification of 20-hydroxyecdysone and 3-epi-20-hydroxyecdysone yielded ecdysone and 3-epi-ecdysone (Table 2).

Column fraction 4 (206.5 mg, 7 x $10^4$ HFU, Table 1), known to elute 26-hydroxyecdysone and 20,26-dihydroxyecdysone (11), was also subjected to CCD. Tubes 1 to 6 were pooled (84.1 mg, 5.0 x $10^4$ HFU); likewise, tubes 9-24 were combined (21.7 mg, 1.0 x $10^4$ HFU) as were tubes 30-47 (72.6 mg). Since the material from tubes 30-47 did not exhibit any biological activity at a sensitivity level of 3000 HFU total (calculated for ecdysone) they were set aside. The active material from tubes 1-6 was further purified on a microcolumn of alumina. The methanol eluate (18.1 mg, 5.0 x $10^4$ HFU) was divided equally and streaked among three full-size TLC plates. When the developed plates were examined by UV, two major zones were detected, a lower zone with an $R_f$ value of 0.22 and an upper zone with an $R_f$ value of 0.27. The methanol extracts of the lower zones from the three plates were pooled (2.9 mg) as were the extracts from the upper zones (0.9 mg). The extracts were purified further on a microcolumn of silicic acid. Column fraction 4 for the lower and upper zones yielded 1.9 mg of residue that contained 20,26-dihydroxyecdysone, and 0.6 mg of residue that contained 3-epi-20,26-dihydroxyecdysone (Table 2), respectively.

The active material from CCD tubes 9-24 (21.7 mg, 1.0 x $10^4$ HFU) when further purified, yielded UV absorbing zones with $R_f$ values of 0.27, 0.32, 0.40 ($R_f$ of 20-hydroxyecdysone) and 0.44 ($R_f$ of 3-epi-20-hydroxyecdysone). However, only the material from the zones with an $R_f$ value of 0.40 and 0.44 were biologically active in the house fly assay, an indication that 26-hydroxyecdysone ($R_f$ 0.30) is not present during this period of insect development.
Table 2
Comparison of the physical properties of six ecdysteroids isolated from the tobacco hornworm during pupal-adult development five days after peak titer of molting hormone activity

<table>
<thead>
<tr>
<th>Ecdysteroids</th>
<th>$R_f$</th>
<th>$R_{Max}$</th>
<th>Purity</th>
<th>Weight Calculated</th>
<th>Nuclear Magnetic Resonances</th>
<th>Reaction with Acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Methyl resonances</td>
<td>Predominant acetonide formed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\delta$ 18-H 19-H 21-H 26-and/ or 27-H M$^+$$\cdot$18</td>
<td></td>
</tr>
<tr>
<td>Ecdysone</td>
<td>0.47</td>
<td>2000</td>
<td>16</td>
<td>0.048</td>
<td>0.75 1.07 1.23 1.32 1.40</td>
<td>none</td>
</tr>
<tr>
<td>3-Epi-ecdysone</td>
<td>0.52</td>
<td>9000</td>
<td>73</td>
<td>0.117</td>
<td>0.75 1.07 1.23 1.32 1.40</td>
<td>diacetonide(0.62)</td>
</tr>
<tr>
<td>20-Hydroxyecdysone</td>
<td>0.40</td>
<td>8200</td>
<td>65</td>
<td>0.59</td>
<td>1.22 1.08 1.58 1.38</td>
<td>monoacetonide(0.11)</td>
</tr>
<tr>
<td>3-Epi-20-hydroxyecdysone</td>
<td>0.44</td>
<td>10000</td>
<td>81</td>
<td>0.97</td>
<td>1.22 1.07 1.58 1.38</td>
<td>triacetonide(0.77)</td>
</tr>
<tr>
<td>20,26-Dihydroxyecdysone</td>
<td>0.22</td>
<td>8100</td>
<td>65</td>
<td>1.24</td>
<td>1.22 1.08 1.58 1.47</td>
<td>diacetonide(0.30)</td>
</tr>
<tr>
<td>3-Epi-20,26-Dihydroxyecdysone</td>
<td>0.27</td>
<td>6500</td>
<td>52</td>
<td>0.30</td>
<td>1.22 1.07 1.58 1.48</td>
<td></td>
</tr>
</tbody>
</table>

* TLC plate developed in chamber with wick in the solvent systems of chloroform-ethanol(65:35).

** Quantitated by UV spectroscopy.

† Figure in parenthesis is the $R_f$ value obtained from TLC plate developed twice in chamber with wick in the solvent system of chloroform-ethanol(9:1).
DISCUSSION

Recovery of biological activity following column chromatography and CCD fractionation was between 90 and 100%. Although TLC served to purify and separate the 3α-ecdysteroids from the 3β-epimers, the recoveries from TLC plates were not quantitative. The microcolumns used after TLC removed additional impurities from the samples as well as impurities extracted by the methanol from the TLC plates. They also served to verify the elution behavior of the ecdysteroids in the final steps of isolation. Because of the relatively small amounts of the ecdysteroids present no attempt was made to crystallize any of them. Since all of the molting hormones with the exception of ecdysone gave sharp λmax at 245 nm in methanol, the actual amounts of ecdysteroids isolated from this group of insects were quantitated by UV analyses (Table 2).

As a result of this study, six ecdysteroids (Fig. 1), ecdysone, 20-hydroxyecdysone, 20,26-dihydroxyecdysone, 3-epi-ecdysone, 3-epi-20-hydroxyecdysone, and 3-epi-20,26-dihydroxyecdysone were isolated and identified. Ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone had Rf values, NMR and mass spectral data identical with those of authentic compounds. Though the molecular ion peaks of all six ecdysteroids were less than 1% of base peak, the M+ -18 peaks were clearly discernible (Table 2). The 3α-ecdysteroids also exhibited methyl resonances and mass spectral data that were almost indistinguishable from those of their corresponding 3β-epimers. However, the compounds designated 3α-ecdysteroids were more apolar than the corresponding 3β-epimers and when allowed to react with acetone yielded
one less acetonide group than the corresponding 3β-epimers (Table 2).

3-Epi-ecdysone, unlike ecdysone, does not react with acetone, an indication that this compound does not contain a cis-diol at the 2,3 positions but has a trans 2β,3α-diol system. This conclusion is also applicable to the other two 3α-ecdysteroids. Further support for the assigned 2β,3α-diol structure is the following: There was no noticeable down-field chemical shift in the C-19 methyl from that of the 3β-epimer which would have been expected if the compound contained a 2α-hydroxyl group. The recent report of the synthesis of 3-epi-ecdysone and 3-epi-20-hydroxyecdysone (12) show that these compounds are less polar than their 3β-epimers, exhibit similar NMR spectra and thus are in agreement with our findings. For these reasons and because of the arguments and conclusions presented previously (5,6), we conclude that the respective faster moving ecdysteroids differ from the corresponding slower moving ecdysteroids only in the orientation of the hydroxyl group at the C-3 position and that these compounds have the assigned 2β,3α-diol structure.

3-Epi-ecdysone and 3-epi-20,26-dihydroxyecdysone can now be added to the list of known naturally occurring MEs of insects, which brings the overall total of C27 MEs to eight (13,14,15,16). However, 3 epi-ecdysone was first reported as a metabolite of ecdysone from in vitro studies with this insect (6). Also, the TLC analyses, NMR and mass spectral data indicate that the unidentified compound that was isolated from the tobacco hornworm along with 20,26-dihydroxyecdysone at peak titer during pupal-adult development was most probably 3-epi-20,26-dihydroxyecdysone (2). The other four ecdysteroids, ecdysone, 20-
hydroxyecdysone, 3-epi-20-hydroxyecdysone, and 20,26-dihydroxyecdysone, were previously isolated and identified from the hornworm during post-embryonic development (1,2,5).

The first naturally occurring 3α-ecdysteroid, 3-epi-20-hydroxyecdysone, was isolated from the meconium of the tobacco hornworm (5). 3-Epi-ecdysone, first isolated and identified as a metabolic product of ecdysone in vitro from Manduca (6), was also detected from meconium; however, the impure nature of the material did not permit conclusive identification. All available evidence indicate that the unidentified ecdysteroid isolated from the tobacco hornworm at peak titer of MH activity (2) was 3-epi-20,26-dihydroxyecdysone, and it was present in our current study five days after peak titer. We also have evidence that both 3-epi-ecdysone and 3-epi-20-hydroxyecdysone are present at peak titer in undetermined amounts. Two of several ecdysteroids were recently isolated and tentatively identified as 3-epi-ecdysone and 3-epi-20-hydroxyecdysone from the eggs of the greater wax moth, Galleria mellonella (L.) (17). If these ecdysteroids from the eggs of the greater wax moth are the 3α-epimers of ecdysone and 20-hydroxyecdysone, then the occurrence of 3α-ecdysteroids may be as common in insects during both embryonic and post-embryonic development as are their 3β-epimers.

During pupal-adult development at peak titer of MH activity, 20-hydroxyecdysone was the major molting hormone followed by ecdysone and then 20,26-dihydroxyecdysone (1,2). However, five days after peak titer, 20,26-dihydroxyecdysone quantitatively exceeded the other two 3β-ecdysteroids. Thus, in these older hornworms, the titer of both
ecdysone and 20-hydroxyecdysone declined drastically as expected, whereas the titer of 20,26-dihydroxyecdysone increased. When the values are scaled upwards 3.8 fold to compensate for the differences in mass between this group of hornworms and those at peak titer (1), the increase in 20,26-dihydroxyecdysone was about 2.0 to 2.5 fold. This increase could well indicate that this ecdysteroid possesses a specific physiological role in insect development (2). This assumption is further supported by the fact that this ecdysteroid, like ecdysone and 20-hydroxyecdysone, is epimerized to a biologically less active ecdysteroid.

Five days after peak titer, 3-epi-20-hydroxyecdysone was the predominant 3α-ecdysteroid followed, in order, by 3-epi-20,26-dihydroxyecdysone and 3-epi-ecdysone. Interestingly, the 3α-compounds with the exception of 3-epi-20,26-dihydroxyecdysone quantitatively surpassed their 3β-isomers.

The 3α-ecdysteroids, like certain of their 3β-epimers, differ in their biological activity in the house fly assay. The 3-epi-ecdysone and 3-epi-20-hydroxyecdysone are approximately 1/10 as active as ecdysone or 20-hydroxyecdysone; the latter two are about equally active in the house fly assay (3-3.5 μg = 1 HFU) (11). The least active of the isolated ecdysteroids, 3-epi-20,26-dihydroxyecdysone, is 1/20 as active as 20,26-dihydroxyecdysone and 1/300 as active as ecdysone. Thus, the biological activity of the 3α-ecdysteroids, which are all less active than their 3β-isomers, strongly points to epimerization as a means of inactivation of the more active 3β-forms.
The following proposed scheme expresses the metabolic interrelationships that exist among the 3β-ecdysteroids and their 3α-epimers based on the known conversions and isolated metabolites.

\[
\begin{align*}
\text{ecdysone} & \xrightarrow{\text{I}} 3\text{-epi-ecdysone} \\
20\text{-hydroxyecdysone} & \xrightarrow{\text{I}} 3\text{-epi-20-hydroxyecdysone} \\
20,26\text{-dihydroxyecdysone} & \xrightarrow{\text{I}} 3\text{-epi-20,26-dihydroxyecdysone}
\end{align*}
\]

The conversion of the 3β-isomers to their 3α-epimers may proceed through their respective 3-dehydroecdysteroids. Labeled ecdysone and 20-hydroxyecdysone were converted to 3-dehydroecdysone and 3-dehydro-20-hydroxyecdysone, respectively, in both the blowfly, Calliphora vicina Robeneau-Desvoidy (18,19) and the locust Locusta migratoria (L.) (20). However, the dehydroecdysteroids have not yet been found to occur in the hornworm. Perhaps, like the candidate labeled precursor, 22,25-dideoxyecdysone, which is efficiently utilized in hormone biosynthesis (21), the dehydro compounds may be ephemeral in nature since none of the three has as yet been isolated as a naturally occurring ecdysteroid from insects.

The major molting hormone present during embryonic development, 26-hydroxyecdysone, was not detected from the hornworm in these studies. Its absence lends support to the premise that quantitative and qualitative differences of the MHs may well exist in either the different stages and/or ages of certain species of insects (3). The recent synthesis and availability of 3-epi-ecdysone and 3-epi-20-hydroxyecdysone (12), as well as the 3-dehydro derivatives of ecdysone
and 20-hydroxyecdysone (12, 22), will now permit studies both in vivo and in vitro to determine both the metabolic interactions and the possible role(s) of the different ecdysteroids in insect development.

REFERENCES

7. Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the U. S. Department of Agriculture.
TRIVIAL AND IUPAC EQUIVALENT NAMES

Ecdysone = 2β,3β,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one

20-Hydroxyecdysone = 2β,3β,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one

26-Hydroxyecdysone = 2β,3β,14α,22R,25,26-Hexahydroxy-5β-cholest-7-en-6-one

20,26-Dihydroxyecdysone = 2β,3β,14α,20R,22R,25,26-Heptahydroxy-5β-cholest-7-en-6-one

3-Epi-ecdysone = 2β,3α,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one

3-Epi-20-hydroxyecdysone = 2β,3α,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one

3-Epi-20,26-dihydroxyecdysone = 2β,3α,14α,20R,22R,25,26-Heptahydroxy-5β-cholest-7-en-6-one

3-Dehydroecdysone = 2β,14α,22R,25-Tetrahydroxy-5β-cholest-7-ene-3,6-dione

3-Dehydro-20-hydroxyecdysone = 2β,14α,20R,22R,25-Penta hydroxy-5β-cholest-7-ene-3,6-dione