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Occurrence of 3-epi-22-deoxy-20-hydroxyecdysone and its phosphoric ester in diapause eggs of the silkworm, *Bombyx mori*

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Abstract. Ecdysteroids in diapause eggs of the silkworm, *Bombyx mori*, were analyzed using high-performance liquid chromatography (HPLC) combined with radioimmunoassay (RIA). A relatively large amount of an unidentified free ecdysteroid and its phosphoric ester (conjugated form) were detected. These two compounds were isolated by a combination of column chromatography on silicic acid, thin-layer chromatography (TLC), and HPLC using a reverse-phase (RP) column. The purified compounds were identified as 3-epi-22-deoxy-20-hydroxyecdysone (22d20E′) and 3-epi-22-deoxy-20-hydroxyecdysone 2-phosphate (22d20E′2P) by means of mass spectrometry and nuclear magnetic resonance spectroscopy. To our knowledge, this is the first report of 22d20E′ and 22d20E′2P.
Key words. Molting hormone; 3-epi-ecdysteroids; ecdysteroid conjugate; silkworm; *Bombyx mori*.

The occurrence of large amounts of ecdysteroids in the ovaries and eggs of insects including the silkworm, *Bombyx mori*, has been well documented[1–3]. Ovaries and eggs thus serve as convenient materials for the isolation and identification of new species of ecdysteroids. In the ovaries of the *Bombyx* silkworm, the Ohnishi group identified six free ecdysteroids: 20-hydroxyecdysone (20E), ecdysone (E), 2-deoxy-20-hydroxyecdysone (2d20E), 2-deoxyecdysone (2dE), 2,22-dideoxy-20-hydroxyecdysone (2,22d20E) and bombycosterol (B), and their phosphoric ester (conjugated form), 20E 22-phosphate (20E22P), E22P, 2d20E22P, 22d20E3P, 2dE22P, 2,22d20E3P and B3P[4–7]. We have recently isolated and identified six additional ecdysteroids in the ovaries and diapause eggs of the *Bombyx* silkworm, 22-deoxy-20-hydroxyecdysone (22d20E), 3-epi-ecdysone (E′) and 3-epi-2-deoxyecdysone (2dE′) and their phosphoric esters (22d20E3P, E′22P and 2dE′22P), and furthermore indicated the presence of several other unidentified ecdysteroids[8,9]. In the present communication we report the isolation and identification of two compounds detected in diapause eggs of the *Bombyx* silkworm, provisionally termed UKE (unknown ecdysteroids in the free eccysteroid fraction) and C-UKE (conjugated form of UKE).

Materials and methods

Insects. Hybrid strains (Tokai-Fuyou, Tokai-Asahi, Kinshuu-Shouwa and Shunrei-Shougetu) of the silkworm, *Bombyx mori*, were used as a source of diapause eggs. Diapause eggs which were maintained for 10 days at 25 °C after oviposition (embryos cease their development at the gastrula stage and enter diapause[10]) were used as the source of ecdysteroids for extraction. The eggs were stored at −80 °C until extraction.

Preparation of ecdysteroids for analytical high-performance liquid chromatography (HPLC). The procedure for extraction of ecdysteroids from diapause eggs was essentially the same as that for the preparation of ovarian ecdysteroids[8]. Briefly, 10 g (wet wt) of diapause eggs were homogenized in 23.5 ml of 70% aqueous methanol and the homogenate was centrifuged. The supernatant was shaken with an equal volume of hexane to remove lipids. The methanol layer was evaporated and dissolved in ethanol, and then stored at 4 °C overnight in order to precipitate gummy impurities. The insoluble matter was removed by centrifugation. The concentrated supernatant was dissolved in a small volume of 30% (v/v) methanol in chloroform and applied to a silicic acid column. The column was first developed with 30% (v/v) methanol in chloroform, followed by 100% (v/v) methanol. Free and conjugated ecdysteroids were eluted at 30% and 100% methanol, respectively. The free ecdysteroid fraction was analyzed by HPLC. The conjugated ecdysteroids were hydrolyzed to the free form using calf intestine alkaline phosphatase (Grade II, Boehringer, Mannheim) prior to HPLC analysis.

HPLC analysis of ecdysteroids. Free ecdysteroids (hydrolyzed products in the case of conjugated ecdysteroids) were analyzed by a Shimadzu (LC-9A system) or Pharmacia Biotech (LKB 2150 LC system) liquid chromatograph. A reverse-phase (RP) column (Wakosil

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$5C_{18}$, 4.6 by 250 mm; Wako) was eluted under isocratic conditions of 45% aqueous methanol at 1 ml/min flow rate. Fractions were collected every 0.5 min. Each fraction was quantified by radioimmunoassay (RIA) using antiserum S-3 (refs. 11, 12).

Large-scale preparation of UKE and C-UKE. Approximately 9 kg diapause eggs were homogenized with 35.1 liters of 80% aqueous ethanol. Subsequent extraction was performed as described above: homogenization was followed by partitioning between 70% aqueous methanol and hexane, and then by removing gummy impurities. Extracts were applied to a silicic acid column. The free ecdysteroid fraction isolated by the silicic acid column was subjected to preparative TLC on precoated silica gel plates ($60F_{254}$ 20 by 20 cm, 1.0 mm thick; Merck) with a solvent system of chloroform/96% ethanol (4:1, v/v). Ultraviolet (U.V.)-absorbing materials were visualized under a U.V. lamp. The silica area which contains UKE (an Rf-value of 0.25) was scraped off and extracted with a mixture of chloroform and methanol (2:1, v/v). The extract was concentrated under reduced pressure, and was applied to HPLC. Purification of conjugated ecdysteroids obtained from the silicic acid column was also accomplished by HPLC. Details of columns and separation conditions of the HPLC are given in the relevant portions of the text.

Mass spectrometry and nuclear magnetic resonance. [^{1}H]Nuclear magnetic resonance (NMR) spectra were recorded with a JEOL GSX-500 spectrometer in CD_3OD and referenced to tetramethylsilane at 0 ppm. Postitive- and negative-ion fast-atom bombardment mass (FAB-MS) spectra of C-UKE were obtained with a JEOL JMS-DX 300 spectrometer using a glycerol matrix.

Results

Analysis of ecdysteroids in diapause eggs. The composition of free ecdysteroids was analyzed by HPLC-RIA as described in 'Material and methods'. As shown in figure 1, the retention times of immunoreactive fraction Nos 18, 30, 36, 45, 53, 80, 83 and 92 on HPLC were identical to those of authentic 20E, E, E', 2d20E, 22d20E, 2dE, 2dE', and 2,22d20E, respectively. In addition to these eight ecdysteroids, a relatively large peak showing RIA activity (fraction No. 58–62) was observed. This peak, which had not been identified in the eggs of *Bombyx* silkworm, was provisionally termed UKE (fig. 1).

In order to see whether UKE also occurs in a conjugated form, the conjugated ecdysteroid fraction isolated by silicic acid chromatography was incubated with calf intestine alkaline phosphatase, and the hydrolyzed products were analyzed by HPLC-RIA. A large peak of UKE was also observed, as in the free ecdysteroid fraction (data not shown). This peak was provisionally

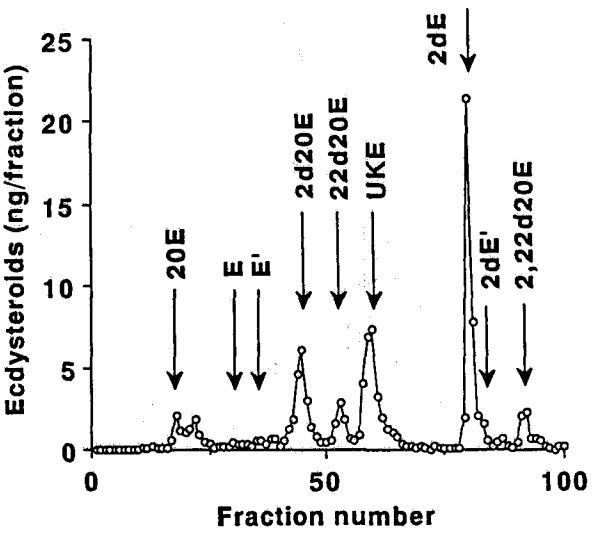

Figure 1. HPLC profile of free ecdysteroids from diapause eggs. Free ecdysteroid sample equivalent to 1 g eggs was subjected to RP-HPLC as described in the text. Each fraction was quantified by RIA. RIA activity was expressed in terms of ecdysone equivalents. Retention times of authentic 20-hydroxyecdysone (20E), ecdysone (E), 3-epi-ecdysone (E'), 2-deoxy-20-hydroxyecdysone (2d20E), 22-deoxy-20-hydroxyecdysone (22d20E), 2-deoxyecdysone (2dE), 3-epi-2-deoxyecdysone (2dE'), and 2,22-dideoxy-20-hydroxyecdysone (2,22d20E) are indicated by arrows. 'UKE' indicates the position of the unknown ecdysteroid.

termed C-UKE. These results indicate that UKE is also present as a phosphoric ester in the diapause eggs.

Purification of UKE. In order to purify UKE, a large amount of UKE extract prepared as described in 'Materials and methods' was separated into several groups. Each group was subjected to HPLC on an RP-column (Wakosil $5C_{18}$, 4.6 by 250 mm; Wako) using isocratic conditions of 45% aqueous methanol at a flow rate of 1 ml/min. The elution pattern is shown in figure 2a. A large amount of UKE was observed together with several U.V.-absorbing substances. Fractions corresponding to UKE obtained from several RP-HPLC runs were pooled and then applied to a normal-phase (NP) column (NUCLEOSIL 100-5, 4.6 by 150 mm; Chemco) using isocratic conditions of 7% methanol in dichloromethane at a flow rate of 1 ml/min. As shown in figure 2b, a single sharp peak was obtained in this step. Approximately 1 mg (estimated by U.V.-absorbance at 254 nm in ethanol) of UKE was isolated from about 9 kg of diapause eggs.

Purification of C-UKE. A large amount of the conjugated ecdysteroid fraction obtained from the silicic acid column was separated into several groups. Each group was subjected individually to HPLC on a semi-preparative RP-column (Wakosil $5C_{18}$, 10 by 250 mm; Wako) using a linear gradient (70 min) of methanol in 20 mM phosphate buffer (pH 5.56) changing from 1:9 to 7:3 (v/v) at a flow rate of 2 ml/min. The representative

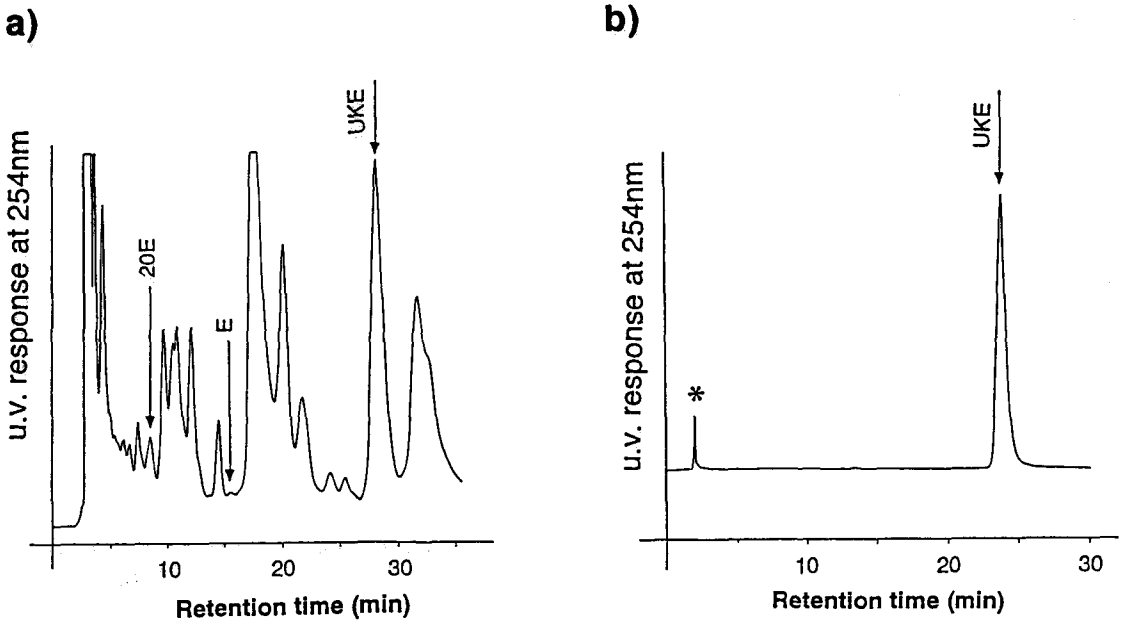

Figure 2. Purification of UKE by HPLC.
a) HPLC profile of partially purified UKE. The silica area with an Rf-value of 0.25 was scraped off the TLC plate, and extracted with a mixture of chloroform and methanol (2:1, v/v). The extract was concentrated under reduced pressure, and further purified on an RP-column as described in the text. The eluate was monitored at 254 nm. *Abbreviations of ecdysteroids are as in figure 1.*
b) Final step of purification of UKE. UKE orginating from RP-HPLC was finally purified on an NP-column as described in the text. The eluate was monitored at 254 nm. The peak marked * originated from the solvent.

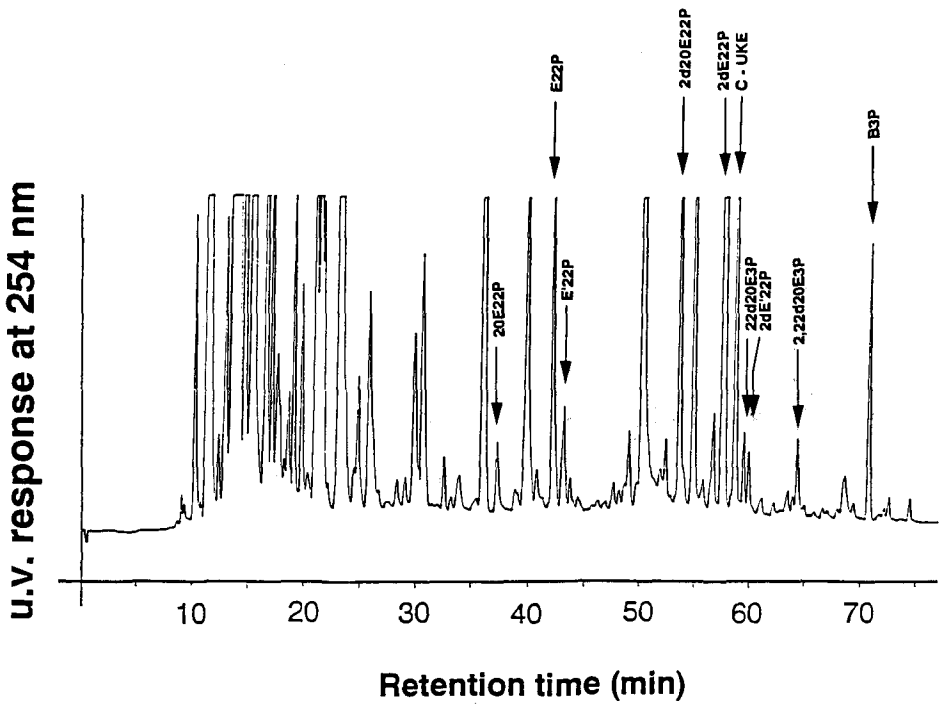

Figure 3. HPLC profile of conjugated ecdysteroids from diapause eggs. Conjugated ecdysteroid sample equivalent to 10 g eggs was subjected to RP-HPLC as described in the text. The eluate was monitored at 254 nm. Retention times of 20-hydroxyecdysone 22-phosphate (20E22P), ecdysone 22-phosphate (E22P), 3-epi-ecdysone 22-phosphate (E'22P), 2-deoxy-20-hydroxyecdysone 22-phosphate (2d20E22P), 2-deoxyecdysone 22-phosphate (2dE22P), 22-deoxy-20-hydroxyecdysone 3-phosphate (22d20E3P), 3-epi-2-deoxyecdysone 22-phosphate (2dE'22P), 2,22-dideoxy-20-hydroxyecdysone 3-phosphate (2,22d20E3P), bombycosterol 3-phosphate (B3P) and conjugated UKE (C-UKE) are indicated by arrows.

elution pattern of conjugated ecdysteroids is shown in figure 3. In order to locate C-UKE, the major U.V.-absorbing peaks were hydrolyzed with calf intestine alkaline phosphatase and the hydrolyzed products were analyzed by RP-HPLC as described in 'Materials and methods'. A compound derived from the fraction with a retention time of 58.5 min (fig. 3) was eluted as a sharp peak at a point corresponding to the retention time of UKE in the RP-HPLC (fig. 1). Co-chromatography of the compound with the UKE reference also yielded a single sharp peak on RP-HPLC. These results indicate that C-UKE is present in the fraction having a retention time of 58.5 min in the HPLC on a semi-preparative RP-column (fig. 3).

A large amount of C-UKE fraction was collected and then subjected to the HPLC using a RP-column (Wakosil $5C_{18}$, 10 by 250 mm; Wako) and eluted with a linear gradient (60 min) of methanol in 20 mM phosphate buffer (pH 5.56), changing from 3:7 to 1:1 (v/v) at a flow rate of 2 ml/min. In this system, C-UKE was eluted as a single sharp peak (data not shown). This C-UKE fraction was further purified by HPLC on an anion exchange column (CHEMCOSORB 7 SAX, 4.6 by 250 mm; Chemco) isocratically with 0.1 M ammonium acetate at a flow rate of 2 ml/min. A single sharp peak was obtained in this system as well (fig. 4). Approximately 4 mg (estimated by U.V.-absorbance at 254 nm in ethanol) of C-UKE were isolated from about 9 kg of diapause eggs.

Characterization of isolated UKE and C-UKE. The [^{1}H]NMR spectral data of UKE and C-UKE are summarized in the table. The positive FAB-MS spectrum of UKE showed an ion peak at m/z 487 $(M+Na)^+$, therefore establishing the molecular weight as 464. The chemical shifts for the steroid nucleus part and side chain of UKE were in good agreement with those of 3-epi-poststerone[13] and 22d20E[8], respectively. The epimerization at the C-3 position was also confirmed by selective proton decoupling experimentation (data not shown). From these results, UKE was established to be 3-epi-22-deoxy-20-hydroxyecdysone (22d20E', fig. 5(**A**)). The fact that upon incubation with calf intestinal phosphatase, 22d20E' was released from C-UKE as stated above, suggests that C-UKE is a phosphoric ester of UKE. The 2-H axial signal was shifted downfield by +0.61 ppm in comparison with that of 22d20E' (table). These results indicate that the phosphate group was located at the C-2 position. The molecular weight of C-UKE was determined as 544 on the basis of the negative-ion FAB-MS spectrum, which showed ion peaks at m/z 565 $[M-H+Na]^-$ and 543 $[M-H]^-$. The spectrum also exhibited intensive ion peaks at m/z 79 $[PO_3]^-$ and 97 $[H_2PO_4]^-$, which corroborated the occurrence of a phosphate group. From these results, C-UKE was identified as 3-epi-22-deoxy-20-hydroxyecdysone 2-phosphate (22d20E'2P, fig. 5(**B**)).

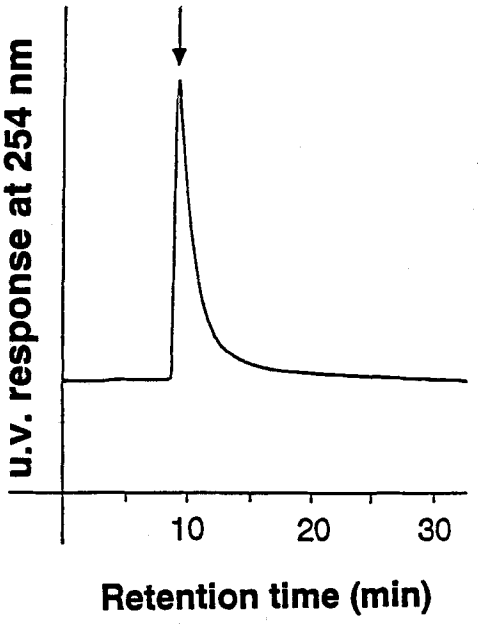

Figure 4. Final HPLC profile of purified C-UKE. C-UKE obtained from the second RP-HPLC was purified by HPLC using an anion exchange column as described in the text. Retention time of C-UKE is indicated by the arrow.

Discussion

Various 3-epi-ecdysteroids have previously been identified in several insect species[14,15]. 22d20E' and 22d20E'2P have been newly identified in the present study. Epimerization at the C-3 hydroxyl group has been demonstrated to proceed enzymatically via 3-dehydro compounds which serve as intermediates[16]. Therefore, 22d20E' is expected to be derived from 22d20E via 3-dehydro-22-deoxy-20-hydroxyecdysone (3D22d20E). Although 22d20E has been identified in the *Bombyx* silkworm[8], 3D22d20E has not yet been found to occur in the eggs of *Bombyx*. 3-Epiecdysone (E') and 3-epi-2-deoxyecdysone (2dE') have also been isolated from silkworm eggs, but the expected intermediates deriving from E and 2dE, i.e. 3-dehydro-ecdysone and 3-dehydro-2-deoxyecdysone, have not yet been found. These facts suggest that 3α-reduction of 3-dehydro-ecdysteroids in the silkworm eggs may proceed rapidly. In fact, in the tobacco hornworm, *Manduca sexta*, 3α-reduction of 3-dehydroecdysone was found to be 13 to 100 times faster than the oxidation of ecdysone[17].

The bulk of the ecdysteroids which accumulate in the ovaries and eggs of insects has been shown to be the conjugated forms[1-3,14,15]. In the ovaries and eggs of *Bombyx* silkworm, the chemical structures of all of the conjugated ecdysteroids have so far been shown to be phosphoric esters in which the phosphate is bound at the C-22 or C-3 position[6,8,9]. In the present study, we

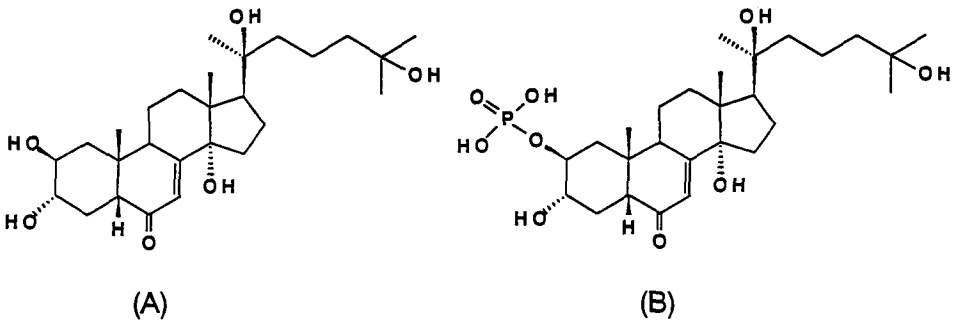

Figure 5. Structures of 3-epi-22-deoxy-20-hydroxyecdysone (**A**) and 3-epi-22-deoxy-20-hydroxyecdysone 2-phosphate (**B**) isolated and identified from diapause eggs of *Bombyx* silkworm.

Table. [^{1}H] NMR data (500 MHz, CD_3OD) for UKE and C-UKE

	18-Me	19-Me	21-Me	26-Me	27-Me	2-Hax	3-Hax	7-H	9-Hax
UKE	0.85 (s)	0.95 (s)	1.27 (s)	1.19 (s)	1.19 (s)	3.63 (m)	3.34 (m)	5.81 (brs)	3.18 (m)
C-UKE	0.85 (s)	0.95 (s)	1.27 (s)	1.19 (s)	1.19 (s)	4.24 (m)	3.56 (m)	5.81 (brs)	3.21 (m)

Chemical shifts ($-\delta$ in ppm) from TMS (0 ppm) as standard; multiplicity of signals is indicated as (s) singlet, (brs) broad singlet and (m) multiplet; Hax = H axial.

found that 22d20E′ was phosphorylated at the C-2 position (fig. 5(**B**)). The amounts of 22d20E′ and 22d20E′2P increase gradually while the amount of 22d20E decreased during embryogenesis in diapause eggs of the silkworm (Sonobe and Masumoto, unpubl. data). Consequently, the amount of 22d20E′2P is about 4-fold higher than that of 22d20E′ in the diapause eggs. These facts suggest that 22d20E′2P might be formed as an end product of 22d20E metabolism during embryogenesis.

Although it has been proposed that 22d20E might be derived from 2,22d20E, the pathway of biosynthesis of 22d20E has not been precisely established[8]. 22d20E has as yet not been found in the ovaries and eggs of other insects besides *Bombyx* silkworm (ref. 8 and fig. 1). Therefore, the ovaries and eggs of the silkworm may serve as a convenient source of material for clarification of the pathways of synthesis and metabolism of 22-deoxy-ecdysteroids.

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