*THE TWO JUVENILE HORMONES FROM THE CECROPIA SILK MOTH**

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Some years ago we reviewed the status of the insect juvenile hormone (JH) and its isolation.[1] Since then two noteworthy contributions toward purification of the hormone have appeared.[2, 3] In all these purification attempts adult male Cecropia silk moths[4] proved to be an invaluable source material. The first nearly pure hormone preparation was isolated in 1965.[1] We obtained it from Cecropia oil (second batch) and characterized it by means of a gas-liquid chromatographic (GLC) analysis in conjunction with an ultrasensitive argon ionization system.[5] The hormonal activity was located exclusively in the two major peaks B and E. Both these substances had a specific activity of the same magnitude as the original preparation. They were retained on neopentyl glycol adipate (NGA) columns at ratios of 1.60 and 1.29 relative to methyl stearate. Only one contamination, peak H, was apparent, which amounted to less than 10 per cent of the total peak area. Thus our 3×10^5-fold enriched preparation was at least 90 per cent pure.

A host of subsidiary gas chromatographic peaks (A,D, etc.) were artifacts that resulted from reactions of the active compounds in the analytical system. These substances were produced even though the hormonal preparations had been injected with a Hamilton syringe directly onto the packed glass columns, but they were more plentiful in the fractions that had been previously submitted to a GLC separation and collected after passage through the brass detector cell. Since the same pyrolytic products were formed whether they were derived from compound B or compound E, the two hormonally active compounds had to be closely related structurally.

We have now processed a new (third) batch of Cecropia oil by a five-step purification sequence similar to that applied to the second batch. These steps were: (I) molecular distillation at $\sim$1 mTorr and 100°C; (II) separation from acidic products formed; (III) leaching with cold aqueous methanol; (IV) SiO_2 chromatography; and (V) Al_2O_3 chromatography. One modification had been made in step IV in which an improved silica gel system was substituted for the zinc carbonate system[6] previously used. This resulted in eliminating compound H. In the *Galleria* wax test, fractions of the final preparation again exhibited a potency of some 3×10^5 JH units per milligram (Table 1).

Our GLC unit was perfected by construction of a metal-free injector and a glass effluent splitter. As a result, the hormone preparation could be processed through the system without contacting any metal. From then on, much simplified chromatograms have been recorded (Fig. 1). The GLC analysis of the biologically most active fractions of the pure preparation revealed that they were composed of only two substances: compounds B and E (Table 1). When the

TABLE 1. *Third batch of Cecropia oil: Purification step V.*

Fraction no.	Eluent composition (%)	Residue weight (μg)	Estimated bioactivity (k-unit)	Specific activity (k-unit/gm 10^{-5})	Peak Areas in GLC Analysis Peak B (% of total)	Peak E (% of total)
1–10	5 → 22	1350	0	...	...	...
11	23	38	0	...	0	0
12	23	48	+	...	70	0.3
13	24	210	++	...	97	3
14	24	381	114	3.0	93	7
15	25	219	79	3.6	82	18
16	26	120	++	...	60	40
17	27	35	+	...	19	29
18	28	49	0	...	0	3
19–28	29 → 100	1660	0	...	...	...
Σ 13/6	...	930	...	...	87	13

Purified Cecropia oil (12.30 mg) from step IV was chromatographed at 22 ± 1°C on 5.5 gm of neutral alumina with an activity grade nearly III. An eluent fraction of 15.0 ml of heptane containing increasing volumes of benzene percolated under slight nitrogen pressure through the 0.6 × 19.0-cm column in about 50 min.

combined fractions 15–17 from purification step V were submitted to a separation by preparative GLC, about 65 μg of compound E and 185 μg of compound B were collected. On rechromatography of samples of these effluents on the analytical column, the retention times of these compounds were unchanged (Fig. 1). All the subsidiary peaks were now absent; plainly they were conversion products of thermal metal catalysis.

Establishing the Hormonal Nature of the Active Compounds.—Prior evidence[1] did not permit us to decide whether the two active compounds represented true hormone molecules or conversion products of a single hormone. After all, the appearance of a symmetrical peak does not assure *a priori* that a substance, whose structure is unknown, may not have been modified by a rapid, thermally aided reaction (e.g., see below, compound IIIb). However, the following new data allow us to conclude that both active substances are indeed juvenile hormones. Table 1 shows that the relative proportions of the more volatile compound E increase steadily with increasing polarities of the fractions of the liquid-solid chromatogram. This strongly suggests that two hormones are present in the preparation and that the active compounds are not conversion products of a single juvenile hormone. In subsequent proton magnetic resonance (PMR) measurements, a sample of essentially pure compound B (fraction 14) that had *not* been submitted to GLC was compared with a sample of compound B from GLC. The spectra were practically identical, demonstrating that compound B is not a conversion product of the GLC.

It should be noted that both hormones were present in all concentrates prepared from Cecropia oil that we have examined, but their proportions varied from batch to batch. In the *second batch*[1] the B-to-E ratio was 4:1, whereas in the *third batch* it was about 7:1 (Table 1). It appears unlikely that any fractionation or differential destruction of the two hormones occurred during purification or handling. Hence, these differences represent true variations in the juvenile hormone content of the oils.

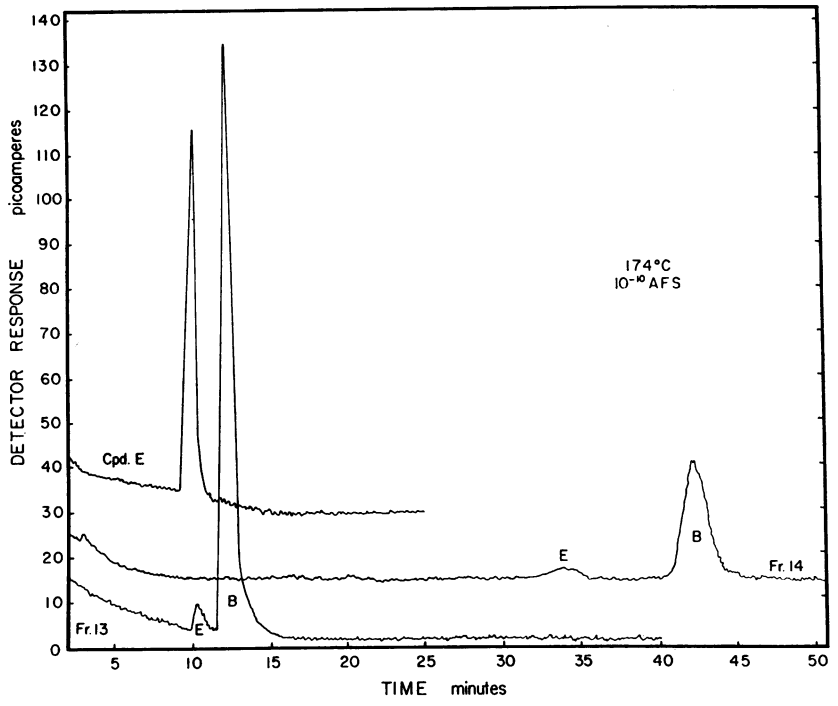


FIG. 1.—Tracings of three analytical gas chromatograms of juvenile hormone fractions after purification step V. Dry sample introduced by a specially designed metal-free injector directly onto packed glass columns (0.3 × 180 cm). Argon carrier flow of 60 ml/min entered a small argon detector[5] through the anode; E_{bb} = 1020 V, I_b = 15.5 nA, I_o = 5.4 nA. Chromatograms: 72 ng of *fraction 13* on Gas-Chrom Q coated with 3% neutral Carbowax 1500; t_R of methyl stearate 7.25 min. 81 ng of *fraction 14* on Gas-Chrom P coated with 1% polyvinylpyrrolidon and then with 8% NGA;[1] t_R of methyl stearate 26.85 min. Rechromatography of some 44 ng of *compound E* on the above Carbowax column; the combined fractions 15/17 had been processed first through a Carbowax 4000 column and peaks E and B individually collected.

The recognition of two juvenile hormones in Cecropia oil was presaged by an earlier observation. When a highly purified juvenile hormone preparation of a *first batch* of oil (1.60 mg with an activity of 4.5 × 10^4 JH units per milligram) was subjected to a 300-transfer countercurrent distribution, the higher activity of the most polar segment of the band comprising the hormone indicated the presence of a second, slightly more polar compound.[7] At that time we drew the tentative conclusion that "if the juvenile hormone should not be a single compound, a possible active companion substance would have very similar physicochemical properties." The B-to-E ratio of this preparation can be appraised from the abundance of the relevant molecular ions in the mass spectrum and was estimated to be minimally 4:1.

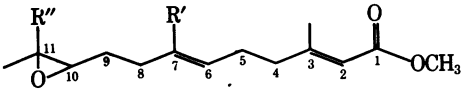
Some Chemical Properties.—Williams and Law[2] have commented on the remarkable resistance of juvenile hormone toward alkaline saponification. We also noted this resistance when hormone preparations were exposed at room temperature to 1 or 2 N ethanolic alkaline potassium hydroxide, but occasionally

the preparations behaved erratically; therefore, a saponification step was not incorporated into our purification sequence. The hormones are very sensitive toward acidity; under slightly acidic conditions the main conversion product of compound B has been identified as compound A. Furthermore, the importance of the quality of solvents used in handling minute quantities of juvenile hormone cannot be overemphasized. Even small residues of insufficiently purified solvents react at 10°C with the hormones to form as yet unknown products. These interactions, which until recently were more of an impervious affliction than a quantitatively assessable occurrence, caused us a lot of difficulties and may have been a source of trouble in aborted biosynthetic studies in various laboratories.

Spectrometric Evidence.—In the spring of 1967 we had just begun the structural elucidation of the biologically active compounds of the third batch when an announcement by Röller *et al.* was published[8] in which the juvenile hormone from Cecropia oil was reported to be a methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate. The identification was carried out with less than 300 μg of material and provides a fine example of what can be achieved with the help of modern methodology, especially when dealing with thermostable compounds. A second juvenile hormone was not noticed by this group of researchers. Although the structural deductions presented in their preliminary publication were not complete, the proposed constitution seemed reasonable to us, especially because the reported values of the mass spectrum corresponded to those we had obtained from a highly purified preparation of the first batch of Cecropia oil (see above). Regrettably, this hormone sample had been contaminated with butyl phthalate and other solvent residues of lower molecular weight, undoubtedly stemming from the various solvents used in that particular purification sequence (unpublished data). The hormone structure as proposed was remarkable in more than one way: (1) The molecule has a carbon skeleton that is novel among natural products. It differs from sesquiterpenoids in having two extra carbon atoms which lengthen the methyl groups attached to the branching points at C-7 and C-11 to ethyl groups. (2) The apparent ease with which the epoxy function was eliminated by catalytic hydrogenolysis[8] was surprising. (3) Hormones with an epoxy group are known only among the gibberellins, and the presence of the group in this series of plant hormones modifies their effects on particular tissues.[9]

With these considerations in mind, we decided to proceed with our structural investigation of compound B (III) and compound E(II) and to include in our spectroscopic comparisons methyl 10,11-epoxy-2,6-*trans,trans*-farnesate (I), a known compound and a lower homologue of the proposed structure of juvenile hormone. The latter (I) had been first synthesized by van Tamelen *et al.* in connection with fundamental cyclization studies.[10] Following a proposal by Dr. E. E. van Tamelen (private communication), we will designate this compound henceforth as methyl 2,6-*trans,trans*-juvenate or, for short, juvenate (I). In 1965 Bowers *et al.* discovered, in their assay on the *Tenebrio* beetle, that I has considerable juvenile hormone activity.[11] In our *Galleria* wax test its potency is three orders of magnitude less than that of the pure juvenile hormone

preparation. No methyl juvenate was, however, found in the Cecropia extract.



I Juvenate: $R' = R'' = CH_3$; $M^+ = C_{16}H_{28}O_3^+$
II Compound E: $R' = CH_3$; $R'' = C_2H_5$; $M^+ = C_{17}H_{28}O_3^+$
III Compound B: $R' = R'' = C_2H_5$: $M^+ = C_{18}H_{30}O_3^+$

Mass, PMR, and infrared (IR) spectra of compound E (II) are presented in Figures 2–4. Space limitations do not permit a discussion of the spectra at this time, except to say that all the data are consistent with the conclusion that II is the 12-homo analogue of I as well as the 14-nor analogue of III. Key spectral data, including their assignments, are summarized in Table 2.

Hydrogenation Experiment.—An ethanolic solution of compound B containing 7 per cent of compound E (57 μg of fraction 14 in 0.3 ml) was hydrogenated at room temperature over a 1 per cent palladium catalyst deposited on calcium carbonate (5 mg). Three reaction products (IIIa, IIIb, and IIa) were detected upon GLC analysis at 160°C on the Carbowax 1500 column specified in the legend to Figure 1, and their retentions relative to compound B (R_B) provided a preliminary characterization. The compounds were then isolated by a GLC separation and the collected effluents analyzed by high-resolution mass spectrometry. The main product (78%) with R_B 0.54 has been identified by its molecular ion as a tetrahydro derivative of compound B (IIIa). The fragments formed upon cleavage of the C-8,9 bond have a composition $C_{12}H_{23}O_2^+$ and $C_{12}H_{22}O_2^+$ which suggests that both double bonds have been reduced, but not the epoxide ring. Moreover, the absence of any C_9 or $C_{10}H_mO_2^+$ ions demonstrates that an ethyl side chain is attached at C-7. The mass spectral data at hand do not allow us to decide whether or not the epoxide ring has been rearranged to a C-10 ketone.

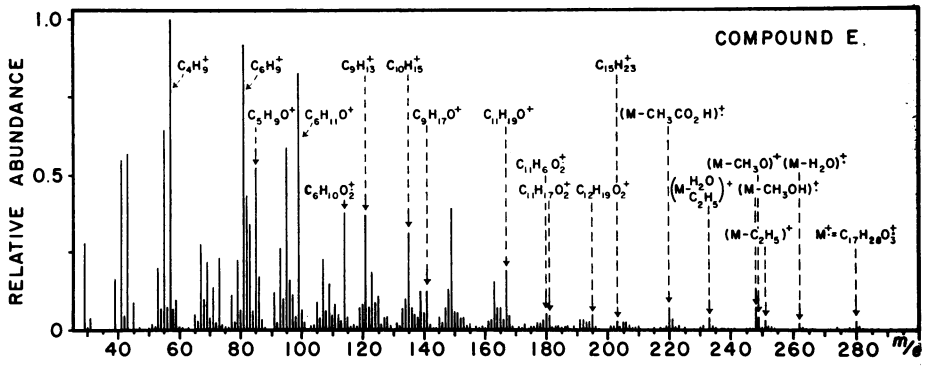


FIG. 2.—Low-resolution mass spectrum of compound E recorded on a Hitachi Perkin-Elmer RMU 6-D instrument. The sample was introduced directly into the ion source. Elemental compositions were determined by high-resolution mass spectrometry on a CEC 21-110 instrument[12] and have been indicated in the diagram for a number of selected ions. The abundance of these ions amounts to 80% or more of that represented by the particular bar. Some of the most abundant fragments have lost all heteroatoms, and their compositions do not yield much structural information about the particular compound in question. The mass spectra and their interpretation were graciously provided by Dr. K. Biemann and his associates.

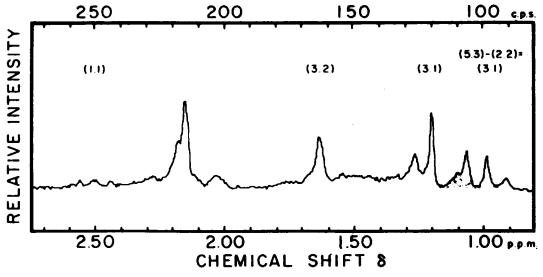


FIG. 3.—Photographic reproduction of high-field segment of PMR spectrum. About 55 μg of compound E were dissolved in carbon tetrachloride to yield an approximately 0.008 *M* solution. Benzene (2%) was added to the solution to provide a firm internal lock. The solution was then transferred to a microcell,[13] placed into the magnet pole gap, and cooled to 14°C. The spectrum was recorded by Clarence Gust on a Varian HA-100 instrument. The 146 field-sweep scans at a sweep rate of 1 Hz sec^{-1} were fed into the 1024 channels of a time-averaging computer. The values in parentheses are relative peak areas determined with an Amsler polar disc planimeter. The stippled zone is due to solvent impurities and its correct relative area was determined on a separate solvent run.

The more polar compound with R_B 0.98 appears to be a hexahydro derivative of compound B (IIIb). Its retention ratio is drastically reduced (R_B 0.76) when the GLC is carried out at 177°C; this indicates that at the higher temperature the compound has suffered thermal dehydration. Likewise, in the mass spectrum there is no sign of the molecular ion, but the elemental composition of IIIb can be deduced from its $(M - H_2O)^+$ ion. The relatively abundant fragment ion $C_{14}H_{28}O_2^+$ strongly suggests that the less substituted bond of the oxirane ring has been hydrogenolyzed[14] to yield 16 per cent of the tertiary alcohol. Finally a small amount (6%) of a more volatile substance with R_B 0.42 or R_E 0.51 has the structure of a tetrahydro derivative of compound E (IIa). Hence, under our experimental conditions, hydrogenolytic elimination of the epoxide has not occurred. This is in marked contrast to the previously reported observation that the desoxyhexahydro derivative has been the main hydrogenation product of III when palladium black was the catalyst.[8] The unusual proportions of reactants in hydrogenations on a microscale may have contributed to this uncommon result,

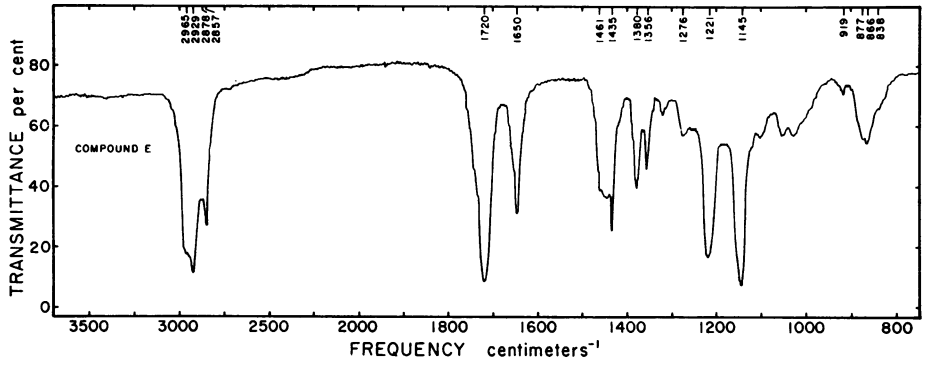
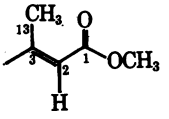
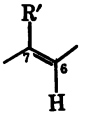
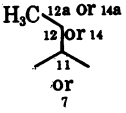
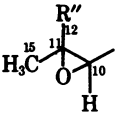
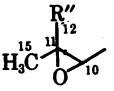
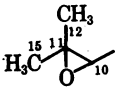


FIG. 4.—Tracing of the IR spectrum of compound E. Sample recovered from PMR determination was transferred to a 0.5-mm pathlength ultramicro cavity cell and dissolved in carbon tetrachloride to yield an approximately 0.07 *M* solution. The spectrum was recorded on a Perkin-Elmer 421 instrument fitted with a Limit 4× beam condenser. Solvent absorptions were compensated by means of an ordinary variable pathlength cell.

TABLE 2. *Synopsis of spectral data.*

PMR (100 MHz) (cps from TMS)	IR (cm^{-1})	Mass (*m/e*)	Partial Structures	Found in
361 (s, 3H) 559 (s, 1H) 216 (3H; *trans*)	1720 (C=O) 1221, 1145 (C—O; *trans*) 1650 (C=C) 1435 (ester; *trans*) 1356 (C-13; *trans*)	$(M - OCH_3)^+$ $(M - HOCH_3)^+$ $C_3H_5O_2^+$ etc.		I, II + III
512 (m, 1H)				I, II + III
163 (s, 3H; *trans*)				I + II
99 (t, 3 or 6H) J 7.7.	2965, 2878 (C-12a; 14a)			II or III
250 (t, 1H) J6		$(M - H_2O)^+$		I, II + III
120 (s, 3H; *cis*)	877 (epoxy; *cis*) 1380 (C-15)	$(M - C_2H_5)^+$ $C_4H_9^+$ $C_5H_9O^+$		II + III
121, 124 (6H)	1382, 1377 (C-12 + 15) 2952 (C-12)	$C_3H_7^+$ $C_4H_7O^+$		I

As far as applicable, assignments have been facilitated by the standard compilations of Jackman (1959), Elvidge (1967); Jones (1956), Rao (1963), Colthup (1964); Biemann (1962), McLafferty (1966), and Budzikiewicz *et al.* (1967).

possibly by a mechanism similar to that operating in high-temperature hydrogenations of saturated alcohols or more likely by facilitating double-bond migration.

Conclusions.—We have demonstrated that compound B is identical with the juvenile hormone (III) isolated by Röller *et al.*[8] Some additional physical characteristics of this compound in form of high-resolution mass and IR spectra are presented. The recent synthesis by Dahm *et al.*[15] of methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6,10-*trans*, *trans*, *cis*-2,6-tridecadienoate or

(±)-12,14-dihomo-10-*cis*-juvenate has established the stereochemistry of the hormone molecule III. The PMR values of our compound B (Table 2) are in excellent agreement with the reported[15] resonances of the synthesized hormone. The second juvenile hormone, compound E, is a new substance present in Cecropia oil at 1/4 to 1/7 the amount of III. Its structure has been determined to be methyl 10,11-epoxy-3,7,11-trimethyl-2,6,10-*trans*, *trans*, *cis*-2,6-tridecadienoate or 12-homo-10-*cis*-juvenate (II). Spectral data summarized in Table 2 show that both juvenile hormones have the same stereochemistry. Thus compounds B and E exhibit identical epoxide absorptions in the IR as well as identical chemical shifts of the relevant peaks in their PMR spectra.

From a study of the relation between structure and juvenile hormone activity in the *Galleria* wax moth, a chain length of 12 carbon atoms had been associated with optimal juvenile hormone activity,[16] and the formal designation of the hormones as tridecanoates (rather than dodecanoates) should not obscure this fact. This is another reason why, in this context, a juvenate notation is convenient. At this juncture it is not known whether the elaboration of two juvenile hormones of very similar structures by the Cecropia silk moth has any special physiological or phylogenetic significance or whether one of the hormones is merely an intermediate in some metabolic pathway of the other.

We thank Prof. E. E. van Tamelen for a sample of methyl juvenate and Dr. R. E. Lundin for bringing his very handy PMR microtechnique to our attention before publication. The mass spectra were obtained at the NIH-sponsored Mass Spectrometry Facility for Biomedical Research at MIT through the courtesy of its director, Prof. K. Biemann (NIH grant FR 00317). We are much indebted to Dr. C. Cone, Mr. R. Murphy, and especially to Dr. G. P. Arsenault for determining the spectra and for the congenial hospitality in their laboratories.

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