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Identification of in vitro release products of corpora allata in female and male loreyi leafworms, *Leucania loreyi*

H. Y. Ho, M. P. Tu, C. Y. Chang, C. M. Yin[a] and R. Kou*

Institute of Zoology, Academia Sinica, Nankang, 11529, Taipei, Taiwan (Republic of China) and [a]Department of Entomology, University of Massachusetts, Amherst (MA 01003, USA)
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Abstract. The in vitro release of juvenile hormones (JH) by female, and of JH acids (JHA) by male corpora allata (CA) of *Leucania loreyi* was identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Separation and quantification were accomplished by HPLC and GC, respectively. JH II and JH III were the major components released by CA of females. Four JHA analogues were identified as the release products of male CA, i.e. JHA III, Iso-JHA II, JHA II and JHA I. JHA III and Iso-JHA II were reported for the first time as the major release products of CA of adult male Lepidoptera. Iso-JHA II is a new member of the insect juvenile hormone analogue family.
Key words. Juvenile hormone; JH acid; corpora allata; gas chromatography; gas chromatography-mass spectrometry; *Leucania loreyi*; Iso-JHA II.

Juvenile hormones (JHs), which possess morphogenetic and gonadotropic activity, are structurally related sesquiterpenoids secreated by the corpora allata (CA) of insects. To date, a total of six different JHs have been identified, i.e. JH 0, Iso-JH 0, JH I, JH II, JH III and JHB_3. It appears that only the lepidopteran CA has the ability of synthesize ethyl branched JH 0, Iso-JH 0, JH I and JH II[1]. In adult female Lepidoptera, the major release products for *Manduca sexa* were JH II and JH III[2] and those for *Pseudaletia unipuncta* were JH I, II and III[3]. But in adult male Lepidoptera, JH acid (JHA) I and II were reported as the major release products in *Hyalophora cecropia*[4] and JHA I, II and homo-farnesoic acid (FA) in *Pseudaletia unipuncta*[5].

In our previous study of sexual dimorphism in the morphology of the CA in adult *Leucania loreyi*, we reported that the male moth's hypertropic CA is more than 20 times larger than the female's[6]. In this study we undertook an analysis of the in vitro release products of CA from both sexes, as the starting point of future research on how these different JH analogues function in different sexes.

Materials and methods

Larvae of *L. loreyi* were reared on a modified artificial diet[7] and the sexes separated at the pupal stage. Larvae and pupae were maintained under a 16L:8D regime at 26–28 °C. Emerged moths were fed with 8% sucrose solution.

Standards of JH III and JH I were obtained from the Sigma Chemical Co. JH II was obtained from the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic. Diazomethane ethereal solution was prepared from N-methyl-N-nitroso-p-toluenesulfonamide[8]. N-methyl-N-nitroso-p-toluenesulfonamide, trimethylchlorosilane and hexamethyldisilazane were obtained from Merck Chemical Co.

Both female and male CA were dissected at the age of 4 days and put directly into medium TC199 (Gibco). Thirty pairs of both female and male glands were incubated separately in 1 ml TC199 for 24 h at 28 °C with shaking. At the end of the incubation period, glands were removed and the released products extracted from the medium with isooctane (female) or chloroform (males).[9,10]

The isooctane extract from females was concentrated with a flow of N_2 to a concentration such that an aliquot of 1 µl contained 2.5 female equivalents, and 2 µl of the extract was injected into a gas chromatograph (GC) for JH analysis. Gas chromatography of the extract was performed on a Shimadzu 14-A GC equipped with a flame ionization detector. Three different capillary columns were used; a 30 m × 0.315 mm (ID) fused silica capillary column of DB-23 phase, a 30 m × 0.25 mm fused silica capillary column of DB-1701 phase, and a 30 m × 0.25 mm fused silica capillary column of DB-1 phase.

Chromatographic conditions: nitrogen carrier 0.5 kg/cm^2; column temperature isothermal at 170 °C, 250 °C and 250 °C for the above three columns, respectively. Aliquots of 10 µl of the female isooctane extract were analyzed by GC-MS in the electron impact mode (70 eV) using a Finnigan MAT INCOS 50 instrument. Samples were analyzed on two GC columns of different

* Corresponding author.

polarity, i.e. DB-23 and DB-5, and the chromatographic conditions were programmed from 50 °C to 250 °C at the rate of 5 °C/min for the DB-23 column and from 50 °C to 280 °C at the rate of 5 °C/min for the DB-5 column. The JHs released by female CA were identified by comparing their mass spectra with those of authentic standards. Subsequently, the identification was verified by comparison of the GC retention times of the natural products to those of the authentic standards using the three capillary columns. The quantification of released JH II and JH III was achieved by the external standard method of quantitative analysis.

The chloroform extract from males was concentrated with a flow of N_2 to a concentration such that an aliquot of 10 µl contained 1 male equivalent, and then the male chloroform extract was separated and analyzed by HPLC using a Spheri-5 µm silica S5W column (25 cm × 4.6 mm) monitored at 245 nm. The solvent system was 25% water-saturated ether in hexane at a flow rate of 1 ml/min. Components collected from HPLC were concentrated with a flow of N_2 and then 10 µl of ethereal diazomethane was added, this diazomethane-treated male extract was then subjected to GC and GC-MS analysis under the same conditions as for females.

For the identification of the release products of male CA, the following processes were conducted: JHs were converted to the respective methoxy-d_3-hydrin derivatives by stirring in 0.08M d_4-methanolic trifluoroacetic acid (1.5 h, 20 °C), followed by quenching with 10 µl of 2M aqueous potassium hydrogen carbonate[11]. JH diols were prepared by hydrolyzing JH acids (release products of male CA) with 0.01N H_2SO_4 in 5:3 tetrahydrofuran:water[12] and the resulting JH acid diols were then

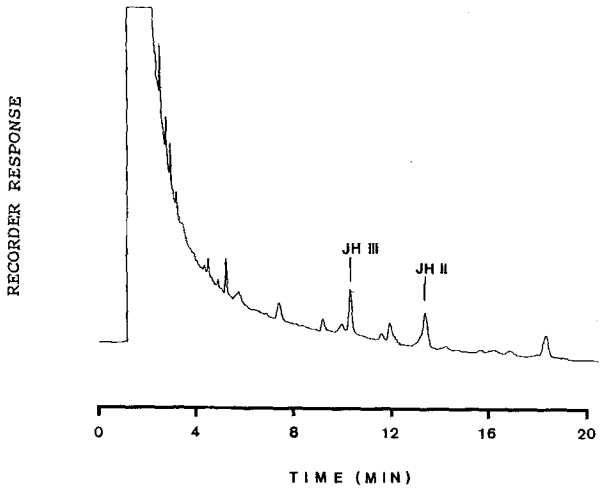


Figure 1. Gas chromatogram of the release products of CA from female *L. loreyi*. The gas chromatography was performed on a 30 m × 0.315 mm DB-23 column. Temperature was held at 170 °C isothermally. Five female equivalents were injected.

Table 1. Retention times of JHs released from CA of female *L. loreyi* and methyl esters of the release products from CA of male *L. loreyi*.

Column type	Retention times				
	JH I	JH II	JH III	female extract	male extract*
DB-23	15.73	13.84	10.39	10.375	10.35
					12.12
				13.437	13.42
					15.67
DB-1701	11.66	9.91	8.94	8.94	8.94
				9.83	9.91
					10.44
					11.66
DB-1	8.75	8.08	7.22	7.21	7.22
					7.77
				8.05	8.05
					8.76

*Treated with diazomethane.

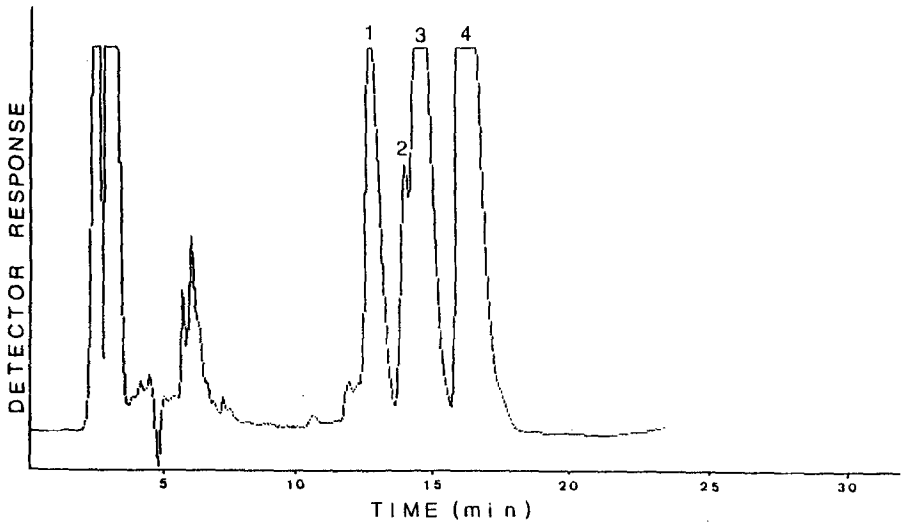


Figure 2. HPLC chromatogram of the release products of CA from male *L. loreyi*. Samples were chromatographed at a speed of 1 ml/min with a solvent system of 25% water-saturated ether in hexane. Fractions were collected and absorbance was followed at 245 nm. One male equivalent was injected.

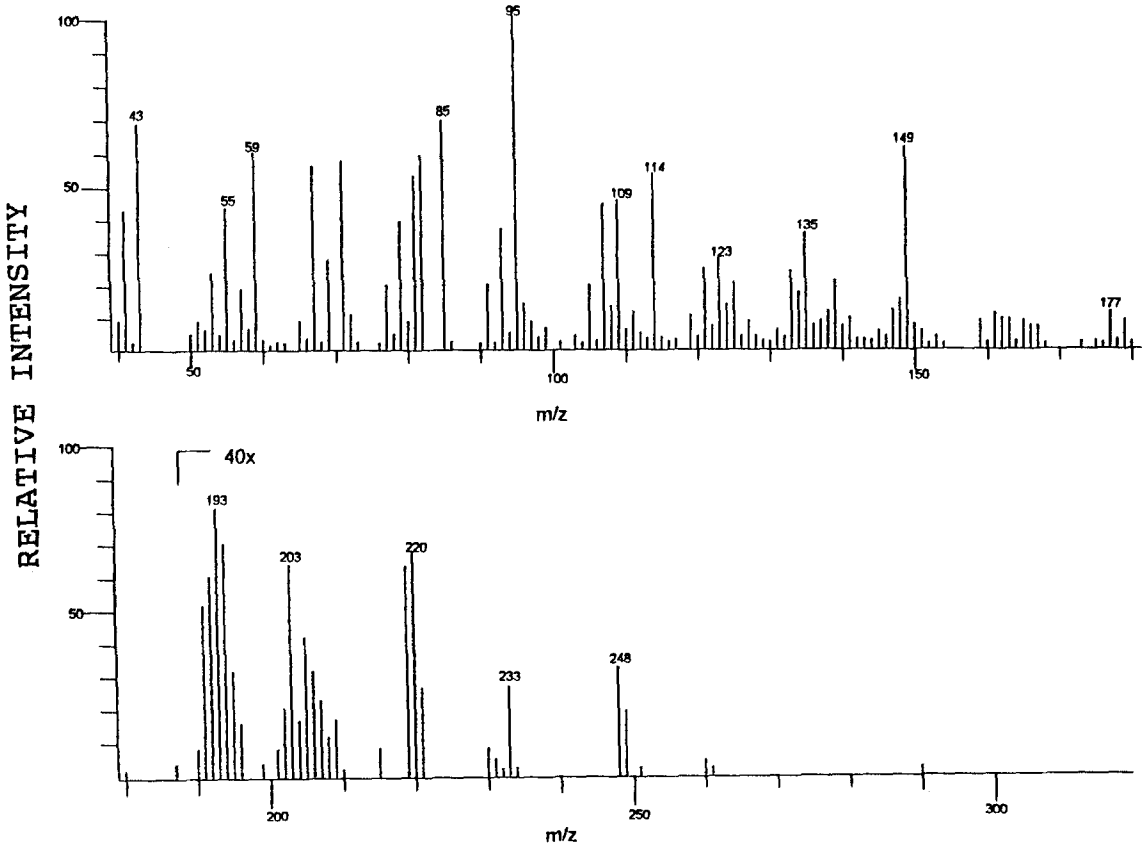


Figure 3. Mass spectrum of the diazomethane treated unknown component 3 collected from HPLC.

treated with ethereal diazomethane solution. JH diol trimethylsilyl ethers (TMS derivatives) were prepared by addition of 35 µl of pyridine followed by 50 µl of trimethylchlorosilane and 50 µl of hexamethyldisilazane to the JH diol solution. This mixture was kept at room temperature for 2 h and then concentrated with N_2 flow. The residue was dissolved in hexane (25 µl) and injected into the GC-MS for analysis. The GC quantification of diazomethane-treated releasing products of male CA was also achieved by the external standard method of quantitative analysis.

Results

The major components of the products released in vitro by CA of female *L. loreyi* are JH III and JH II (fig. 1). Retention times on three columns of different polarity are shown in table 1. The amounts of JH III and JH II released were approximately 8.43 ng and 6.47 ng/female, respectively.

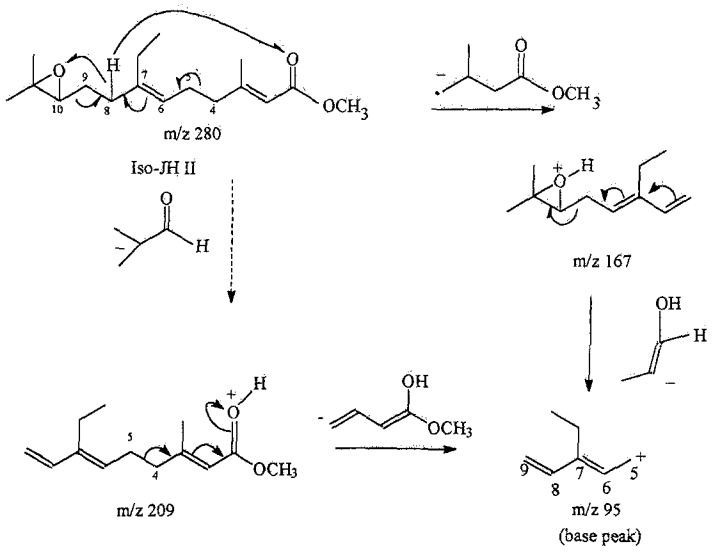
HPLC analysis of products released in vitro by CA of male *L. loreyi* showed that there were four major peaks labeled as 1, 2, 3 and 4 in the chromatogram (fig. 2). These four components were collected separately and concentrated with N_2 flow. They were treated with ethereal diazomethane to give four new derivatives 1′, 2′, 3′ and 4′, among which 1′, 2′ and 4′ were identified by GC and GC-MS to be JH I, JH II and JH III respectively, by comparing the retention times and mass spectra with those of the standards.

The fourth product, derivative 3′ (resulting from component 3), has a mass spectrum (fig. 3) virtually identical to that of JH II, with a slight difference in the relative abundance of certain diagnositc ions distinguishing them (table 2). The mass spectral data suggested

Table 2. Mass spectral data of the diazomethane treated four components which were released by the CA of the male *L. loreyi*.

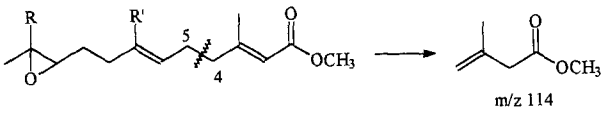
Diazomethane-treated component*	Mass spectral data
1	43(40), 57(70), 67(50), 81(58), 95(85), 99(100), 109(58), 114(55), 135(43), 149(35), 163(25), 217(1), 234(1.1), 247(0.5), 262(0.5)
2	43(30), 57(65), 81(90), 95(55), 99(100), 114(40), 135(35), 149(25), 167(12), 192(1.6), 203(1), 220(1.5), 248(0.5)
3	43(68), 59(60), 85(70), 95(100), 109(48), 114(55), 135(35), 149(65), 167(8), 193(2), 203(1.5), 209(0.4), 220(1.5), 248(0.6)
4	43(70), 59(60), 71(58), 81(100), 85(95), 95(53), 107(32), 114(43), 121(40), 135(63), 163(12), 192(1.6), 206(2), 234(0.9)

*Components, 1, 2 and 4 were converted to JH I, JH II and JH III, respectively, after being treated with diazomethane.



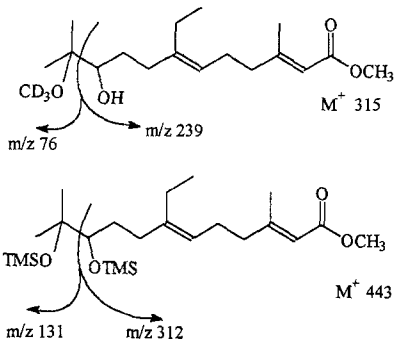
Scheme 1.

that derivative 3′ is a homologue of JH II. The base peak in the mass spectrum of derivative 3′ appears at m/z 95, which is one methylene unit (CH_2) more than that of JH III. The ion of the base peak (m/z 81) of JH III derived from a fragment that contains carbon atoms 5, 6, 7, 8 and 9. According to the typical branching pattern in the biosynthesis pathway of JH analogues, the only plausible way to accommodate the extra methylene unit in this fragment is to have the methyl group on C-7 of JH III replaced by an ethyl group in derivative 3′. The formation of the base peak ion and other major fragmentation ions of derivative 3′ is proposed in scheme 1[13]. An ion peak at m/z 114 found in the mass spectrum of derivative 3′, and the same ion peak was also seen for JH I, JH II and JH III, suggesting that there is a 'methyl' group on C-3 for all these four components, originating from allylic cleavage between C-4 and C-5 with concurrent proton migration as shown in scheme 2[14].



Scheme 2.

For JH III, JH II and JH I, base peaks in the mass spectra of the methoxy-d_3-hydrin derivatives are at m/z 76, 90 and 90, respectively. For derivative 3′, the base peak of the methoxy-d_3-hydrin derivative appears at m/z 76. Base peaks of the mass spectra of the diol TMS derivatives of JH III, JH II and JH I are at m/z 131, 145 and 145 respectively. For derivative 3′, the base peak of the diol TMS derivative appears at m/z 131 (table 3 and scheme 3). From these data it is inferred that derivative 3′ has the same structural feature on C-11 as JH III does, i.e. a methyl group on C-11.



Scheme 3.

Comparison of the mass spectrum of the methoxy-d_3-hydrin derivative of derivative 3′ with that of the methoxy-d_3-hydrin derivative of the internal standard, ethyl-10,11-epoxy-3,11-dimethyl-7-ethyl-2,6-dodecadienoate, used by Bergot et al.[11], reveals that the ion at m/z 253 of the internal standard has shifted to 239 for derivative 3′, whereas other mass fragmentation ions are virtually the same. This data further supported the proposition that derivative 3′ is the methyl ester isomer of ethyl-10,11-epoxy-3,11-dimethyl-7-ethyl-2,6-dodecadienoate. From the data above, derivative 3′ can be identified as methyl-10,11-epoxy-3,11-dimethyl-7-ethyl-2,6-dodecadienoate, named Iso-JH II by Bergot et al.[11]. Thus the products released by the CA of male *L. loreyi* are JHA I, JHA II, Iso-JHA II and JHA III. The gas chromatogram of the diazomethane-treated release products of male CA was shown in figure 4. The amounts of JH I, JH II, Iso-JH II and JH III produced were found to be 0.07, 0.04, 0.21 and 0.58 µg/male, respectively.

Discussion

In the present study, we have shown the sexual dimorphism of the release products of CA in adult *L. loreyi*.

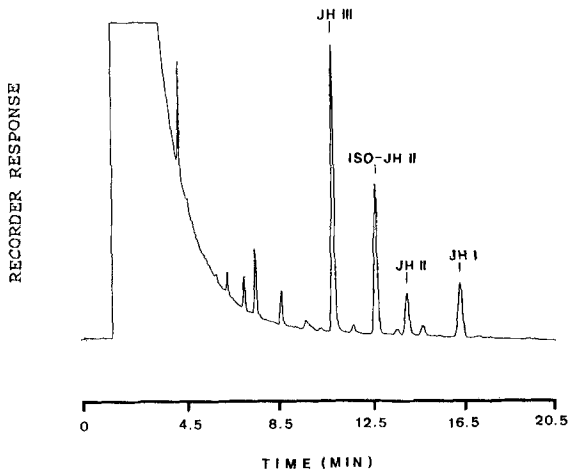


Figure 4. Gas chromatogram of the diazomethane-treated release products of CA from male *L. loreyi*. The gas chromatography was performed on a 30 m × 0.315 mm DB-23 column. Temperature was held at 170 °C isothermally. 0.33 male equivalent was injected.

Table 3. Mass spectral data of methoxy-d_3-hydrin and diol TMS derivatives of the methyl esters of the four components released from the CA of male *L. loreyi*

Diazomethane-component*	Mass spectral data of the derivatives	
	methoxy-d_3-hydrin derivatives	diol TMS derivatives
1	90(100), 149(5), 207(20), 239(20)	145(100%)
2	90(100), 135(5), 193(20), 225(20)	145(100%)
3	76(100), 149(18), 207(45), 239(30)	131(100%)
4	76(100), 135(15), 193(45), 225(50)	131(100%)

*Components 1, 2 and 4 were converted to JH I, JH II and JH III, respectively, after being treated with diazomethane.

The major release products of *L. loreyi* female CA are JH II and III, with JH III as the dominant component. In *M. sexta*, the CA of the adult female also releases JH II and III[2]. JH III was further reported to possess higher gonadotropic function in *M. sexta*[15] and *Helicoverpa zea*[16]. But in *Danaus plexippus*[17] and *P. unipuncta*[5], JH I and II were suggested as the gonadotropins. Since JH II and JH III are released in large quantities in *L. loreyi* (8.43 ng JH III and 6.47 ng JH II/female, 24 h incubation), their endocrine function, and whether this reflects exactly the endocrinologically relevant haemolymph titer, should be further investigated.

The relase products of *L. loreyi* male CA are JHA III, Iso-JHA II, JHA II and JHA I, with JHA III and Iso-JHA II as the dominant components. This is the first time that JHA III and Iso-JHA II have been reported as the major release products of male CA in Lepidoptera, and Iso-JHA II is a new member of the insect juvenile hormone analogue family. To date, the release products of CA in male adult moths have been reported only for two species, *Hyalophora cecropia* and *P. unipuncta*, and in both species JHA I and JHA II were the dominant components[4,5]. However, in male and female prepupae, the acids of all three JH homologues were released[18]. Because the CA of these males lack the JH acid methyl transferase, the released precursor acids are converted to JH in the accessory sex glands[4,18]. The amounts of the four JHA components released by CA of *L. loreyi* male moths are very large (0.58, 0.21, 0.07, 0.04 μg JH III, Iso-JH II, JH I and JH II/male, respectively, 24 h incubation, after esterification of the corresponding JHA). Whether these JHAs are metabolized by the same biosynthetic pathways as in other Lepidoptera male moths, or whether they are hormones by themselves, still has to be determined.

In Lepidoptera, the synthesis of ethyl branched JHs (EBJHs) was reported to be determined by the existence of transaminase[19], and it was hypothesized that variation in the activity or concentration of the branched-chain amino acid transaminase will change the composition of JHs[5], so the biosynthetic pathway of Iso-JH II and its relation to transaminase should also be examined.

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