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Note

Semipreparative synthesis and purification of juvenile hormone acids by high-performance liquid chromatography

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The juvenile hormones (JHs) are central to postembryonic development in insects. According to the classical scheme of insect development, JH titers must be high during the larval stage but must then decline to permit the insect to undergo metamorphosis[1]. The hormone levels rise again during the adult stage and JH acts as a gonadotropin. Hormone titers are very tightly controlled through regulation of synthesis, hemolymph metabolism, tissue uptake and excretion. One of the primary pathways of hormone degradation is the enzymatic conversion of JH to JH acid, an inactive metabolite. JH acid is also an important starting compound for the generation of hapten-protein conjugates[2,3], and for the synthesis of affinity resins[4]. Given the importance of obtaining sufficient quantities of highly purified JH acid, we here report the development of methods for rapid synthesis and purification of the hormone metabolite in high yields.

EXPERIMENTAL

Reagents and chemicals

All solvents used in this study were UV grade (Burdick and Jackson). Radioinert JH standards JH I (methyl 2*E*,6*E*-3,11-dimethyl-7-ethyl-*cis*-10,11-epoxytrideca-2,6-dienoate), JH II (methyl 2*E*,6*E*-3,7,11-trimethyl-*cis*-10,11-epoxytrideca-2,6-dienoate) and JH III (methyl 2*E*,6*E*-3,7,11-trimethyl-10,11-epoxydoceca-2,6-dienoate) were obtained from Sigma. Radiolabeled JH I and JH III (10 Ci/mmol) were obtained from New England Nuclear. Deuterated chloroform (C^2HCl_3) and 1,2-propanediol were obtained from Aldrich.

Intrumentation

Chromatography was carried out isocratically at ambient temperature using an Altex Model 100 pump and an Altex Model 210 injector (20-μl loop). An Altex model 155-01 variable-wave length detector (8-μl flow cell) was used to monitor column effluent.

Proton magnetic resonance spectra of JH acid were obtained using a Bruker

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WH-270 spectrometer while ^{13}C nuclear magnetic resonance spectra were determined using a JEOL FX-200 spectrometer. Extinction coefficients were determined on a Perkin-Elmer Model 552 spectrophotometer. Radioassays were performed on a Packard Prias liquid scintillation counter.

Procedure

JH III (10–30 mg) was mixed with [^{3}H]JH III (3 μCi) in a 20-ml vial and the carrier solvent was evaporated under nitrogen. A volume of 6 ml of methanol–1 *M* sodium hydroxide (1:1, v/v) was added to the vial which was then wrapped in foil, placed in a shaking water bath and incubated 4 h at 40°C. After incubation, the reaction mixture was carefully titrated to pH 5.0 with 1 *M* hydrochloric acid and then extracted 5 times with an equal volume of chloroform–toluene (9:1, v/v). The pooled extract was then dried under nitrogen and stored at −20°C until purification was performed. All manipulations of JH acid were performed in semidarkness.

Chromatography

Semipreparative purification of JH III acid (2*E*,6*E*-3,7,11-trimethyl-10,11-epoxydodeca-2,6-dienoic acid) was performed using a LiChrosorb DIOL column (500 × 9.4 mm I.D.; 10 μm; Applied Sciences). Samples were dissolved in chloroform and approximately 2 mg of the crude JH III acid injected. Chloroform (preserved with 1% ethanol) was used as the mobile phase at a flow-rate of 3 ml/min. Analytical purification of JH III acid was performed using a LiChrosorb DIOL column (250 × 4.5 mm I.D.; 10 μm; Applied Sciences). In both the analytical and semipreparative procedures, column effluent was monitored at 244 nm.

Analytical separation of JH acid homologues was performed using a Spherosorb silica column (250 × 4.5 mm I.D.; 10 μm; Applied Sciences). The mobile phase, composed of hexane–diethyl ether–1,2-propanediol–acetic acid (475:25:1:0.5), was prepared in the following manner, diethyl ether was first passed through alumina (grade I, neutral alumina; BioRad) and then mixed with hexane. 1,2-Propanediol was added, the mixture was shaken, and the excess propanediol was removed. Acetic acid was added and the solvent mixture degassed with helium just prior to use. Up to 50 μg of homologue mixture was injected and eluted at a flow-rate of 0.5 ml/min. Column effluent was monitored at 220 nm.

Spectroscopy

The extinction coefficient of JH III acid was determined because a difference in the absorption of the acid and the methyl ester was expected. Absorbance of a solution (8.9 μg/ml) in acetonitrile was determined to have an absorbance of 0.42 at λ_{max} = 211 nm or ε = 11,840.

RESULTS AND DISCUSSION

Two methods have been employed to prepare JH acid from the methyl ester parent compound; enzymatic hydrolysis[5] and saponification[6]. Although enzymatic hydrolysis is useful for the generation of microgram quantities of JH acid, it is impractical for the synthesis of milligram amounts. The preparation of JH acid by saponification using slightly elevated temperatures is an efficient and rapid means of

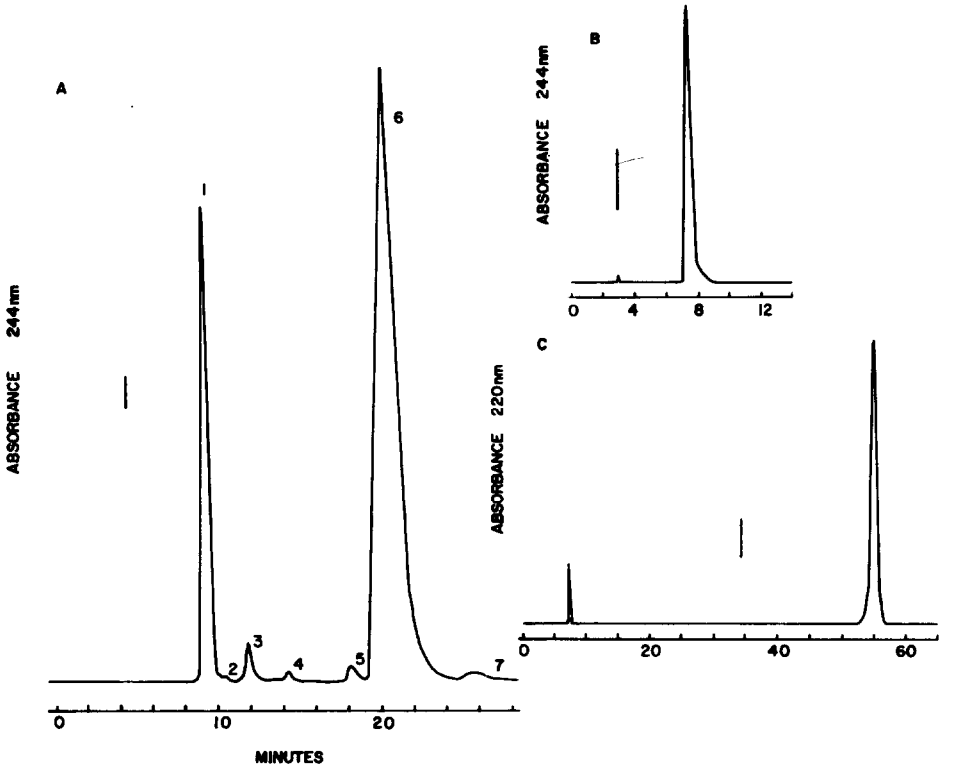

Fig. 1. HPLC of JH III acid. (A) Semipreparative separation of the saponification products of JH III. Hydrolysis products (2 mg) separated on a 500 × 9.4 mm I.D. (10-μm) LiChrosorb DIOL column using chloroform–ethanol (99:1) with a flow-rate of 3 ml/min. Peak 1 = JH III; 6 = JH III acid. Bar represents 0.1 a.u. (B) Peak 6 (22 μg) rechromatographed on a 250 × 4.5 mm I.D. (10 μm) LiChrosorb DIOL column using chloroform:ethanol (99:1) with a flow-rate of 1 ml/min. Bar represents 0.1 a.u. (C) Peak 6 (24 μg) rechromatographed onn a 250 × 4.5 mm I.D. (10 μm) Spherosorb silica column using hexane–diethyl ether–1,2-propanediol–acetic acid (475:25:1:0.5) with a flow-rate of 0.5 ml/min. The identities of peaks 2–5 and 7 are unknown. Bar represents 0.1 a.u.

generating preparative amounts of the metabolite. The use of elevated temperatures to drive the reaction reduces the time necessary to complete the hydrolysis. Time course studies revealed that at 40°C hydrolysis periods less than 4 h resulted in incomplete saponification of the ethyl ester while periods greater than 4 h resulted in the generation of products more polar than JH acid[4]. Four hours was therefore chosen as the optimal incubation time even though not all of the parent compound was hydrolyzed. The unhydrolyzed JH was recovered upon chromatography of the hydrolysis products and used in later preparations. The same procedures worked equally well for the JH I and JH II homologues.

Products of base hydrolysis were separated by semipreparative high-performance liquid chromatography (HPLC) using the hydrophilic-bonded normal-phase packing LiChrosorb DIOL. Fig. 1A demonstrates that base line resolution of JH III acid (peak 6) and other metabolites can easily be achieved using chloroform–ethanol (99:1, v/v) as the mobile phase. As determined by radioanalysis, approximately 85%

of the crude hydrolysate was recovered in peak 6. Considering the efficiency of the hydrolysis and extraction (85%) and the recovery from HPLC, an overall yield of 70–75% was realized. In contrast to the reversed-phase separation of JH acid by Connat[8], the present separation procedure has the advantage of being semipreparative and using isocratic elution. Chang[9], using a radially compressed reversed-phase column run in an isocratic mode has obtained reasonably good separation of JH acid from other metabolites in the submicrogram range.

Given the relative instability of JH acid[10] peak 6 was analyzed by proton magnetic resonance and ^{13}C nuclear magnetic resonance to verify the chemical structure. Proton magnetic resonance at 270 MHz (C^2HCl_3 solution at room temperature, 32K data points over 3000 Hz spectral width, 30° pulse angle) showed peak 6 to be a single compound (S/N = 375:1 for the tallest peak) with the following parameters (all chemical shifts reported relative to TMS); δ 5.69, br s, 1H (C2) (proton on carbon 2 is listed as C2); δ 5.14, br s, 1H (C6); δ 2.69, t, J = 6.1, 1H (epoxide); δ 2.19, d, J = 3.1, 3H (C7′); δ 2.16, d, J = 1.1, 3H (C3′); δ 2.11, t, J = 7.7, 2H (C4); δ 1.6, m, 6H; δ 1.29, δ 1.25, 2 × s, 2 × 3H (C12). The assignment of the two allylic methyls was made on the following basis: the proton on C2 should show essentially no coupling except to the C3′ so that the signal for C2 should not be more than 3 J wide. Since $\nu_{\frac{1}{2}}$ for C2 $\approx$ 4 Hz, it cannot be coupled to the methyl which shows a coupling of 3.1 Hz. Therefore the signal at δ 2.16, J = 1.1 must be the C3′ methyl. The width of the resonance, $\nu_{\frac{1}{2}}$ = 12–13 Hz, is what one would expect for an unresolved triplet of quartets with J = 5–6 and 3 Hz.

^{13}C nuclear magnetic resonance (50.1 MHz, 12 kHz spectral width, broad band ^1H decoupling performed in C^2HCl_3) also showed a single compound within the limits of the signal to noise ratio obtainable (S/N = 16:1 for protonated carbons). Shifts relative to TMS = 0.0 were δ 171.5 (CO_2H), δ 162.3 (C3), δ 135.5 (C7), δ 123.4 (C2), δ 115.3 (C6), δ 64.1 (C10), δ 58.3 (C11), 41.1, 36.4, 27.5, 26.0, 24.8, 19.1, 18.8, 16.0. The spectra were consistant with the premise that peak 6 is indeed JH III acid.

To establish the purity of the material identified as JH acid, peak 6 from the

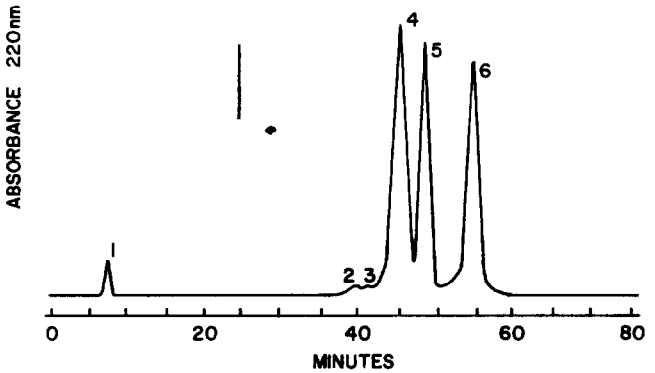

Fig. 2. HPLC of JH acid homologues. JH I acid (12 μg), JH II acid (11 μg) and JH III acid (11 μg) were chromatographed on a 250 × 4.5 mm I.D. (10 μm) Spherosorb silica column using hexane–diethyl ether–1,2-propanediol–acetic acid (475:25:1.0:0.5) with a flow-rate of 0.5 ml/min. Peak 1 represents the solvent front; 4 = JH I acid; 5 = JH II acid; 6 = JH III acid. The identities of peaks 2 and 3 are unknown. Bar represents 0.1 a.u.

semi-preparative purification was rechromatographed under two differing sets of conditions. An analytical LiChrosorb DIOL column indicated a single peak (Fig. 1B), as did a normal-phase silica column run in the liquid–liquid mode[7] (Fig. 1C). Both analytical procedures resulted in greater than 98% recovery of the injected radiolabel.

A particularly attractive aspect of chromatography on the Spherisorb silica column developed in the liquid–liquid mode is the reasonably good separation of the JH acid homologues (Fig. 2). This separation coupled with its excellent recovery may prove useful in the analysis of homologue metabolites from biological samples.

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