Identification of 2-deoxyecdysone from the mite, *Tyrophagus putrescentiae*

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Abstract. 2-Deoxyecdysone was purified from the mite *Tyrophagus putrescentiae* and monitored by a radioimmunoassay. The compound was identified by GC-MS and LC-MS. This is the first report of an endogenous ecdysteroid from a mite.
Key words. Mite; ecdysteroid; 2-deoxyecdysone; *Tyrophagus putrescentiae*; LC-MS.

Chemical control of mites is very important both for agriculture and for sanitation. Many synthetic mitecides have been developed, but mites rapidly gain resistance to such exogenous compounds. Hormone-like substances may be able to control the life cycle of the mites, but little is known about any endogenous factors which regulate the metamorphosis of mites. In our study of endogenous substances which might regulate the mite life cycle, we investigated molting and juvenile hormone-like substances, and we report here on the ecdysteroids.

Materials and methods

We tried to establish an in vivo bioassay system with 20-OH-ecdysone (20-OH-Ecd) purchased from Rhoto Pharmacology Co. (Osaka) as the ecdysteroid and *Tyrophagus putrescentiae* as the mite. 20-OH-Ecd was applied to the mites using three different methods: it was sprayed as an acetone solution, coated on the inside of a small culture tube as a thin film, or adsorbed to the mite feed. The mites treated with 20-OH-Ecd were microscopically observed during ecdysis throughout the life cycle; however, even high doses of 20-OH-Ecd, e.g. 1000 ppm solution by the spray method had no effect on *T. putrescentiae*. In addition, Ecdysone (Ecd) and 2-deoxyecdysone (2-dEcd), obtained from Sigma chemical Co. (St. Louis, MO), did not show any activity in these bioassays. Probably exogenous ecdysteroids applied to the mouth or body surface could not reach the target organs of the mite. We decided to use a radioimmunoassay (RIA) to detect ecdysteroids according to K. Kadono-Okuda et al.[1]. The rabbit antiserum was kindly provided by Dr S. Sakurai and (23,24-^{3}H)-ecdysone(58.8 Ci/mmol) was purchased from New England Nuclear Co. (Boston, MA). The binding curve for Ecd was log linear between 0.05 and 1.0 ng, that for 20-OH-Ecd between 0.1 and 2.5 ng and that for 2-dEcd between 0.05 and 5.0 ng. *T. putrescentiae* was reared in dry yeast containing 15% water at 25 °C for 2 months. Water saturated with sodium chloride was added to a mixture of mites and dry yeast. The mite bodies which floated on the surface of the water were collected and repeatedly poured into water saturated with sodium chloride until the bodies were free of dry yeast, after which they were washed with distilled water and stored in a deep freeze (−80 °C). A 185 g sample of lyophilized mite bodies was obtained from a 5 kg culture of dry yeast.

The lyophilized mite bodies were dipped in 800 ml of 70% aqueous methanol at 4 °C for one night, then removed by centrifugation. The supernatant was mixed with 800 ml of *n*-hexane and the lower layer was concentrated in vacuo to a residual water solution. This water layer was extracted with 60 ml of *n*-butanol three times. RIA was performed on all layers which showed that the *n*-butanol layer contained the most ecdysteroid. The *n*-butanol layer was applied to a silica gel column with a benzene-methanol solvent system. RIA showed that the 10% methanol eluate was the most active fraction. This fraction was concentrated, then purified by HPLC on an ODS column (Develosil ODS-5, 8.0 × 250 mm, Nomura Kagaku, Seto, Japan) eluted with a CH_3CN-H_2O linear gradient system at a flow rate of 3 ml/min. The fraction eluted from 9–18 min (24–33% CH_3CN) showed a little activity in the RIA and was assumed to contain 20-OH-Ecd (Rt 11.5 min) and Ecd (15.5 min). The main fraction was the eluate with a retention time of 20–22 min (35–37% CH_3CN) which corresponded to 2-dEcd (Rt 21.0 min). The minor and major fractions were further purified by HPLC on an ODS column (Develosil ODS-5, 4.6 × 250 mm) eluted with a CH_3CN-H_2O linear gradient system at a flow rate of 1 ml/min.

Results and discussion

The most active fraction of the minor fraction had the same retention time as authentic Ecd and the amount of Ecd present was calculated from the RIA as 0.066 µg/100 g dry mite bodies. The most active fraction of the major fraction had the same retention time as 2-dEcd and its content of 2-dEcd was calculated from the RIA as 5.3 µg/100 g mite bodies (fig. 1).

To confirm the chemical structure of the ecdysteroids contained in these two fractions, each ecdysteroid sample was derivatized with trimethylsilylimidazole at 130 °C for 14 h and then analyzed with GC-MS[2]. Fully trimethylsilylated ecdysteroids derived from the mite bodies were separated by GC on a capillary column (DB-1, 0.261 mm × 15 m, J & W Scientific, Folson, CA) programmed for a 50–300 °C temperature gradient, then analyzed with a mass spectrometer (JMS DX-705L, JEOL). The mass chromatogram of Ecd was monitored at m/z 636, 567 and 2-dEcd at m/z 548, 479, 171. The minor fraction that was estimated to contain Ecd was analyzed by GC-MS but we did not succeed in identify-

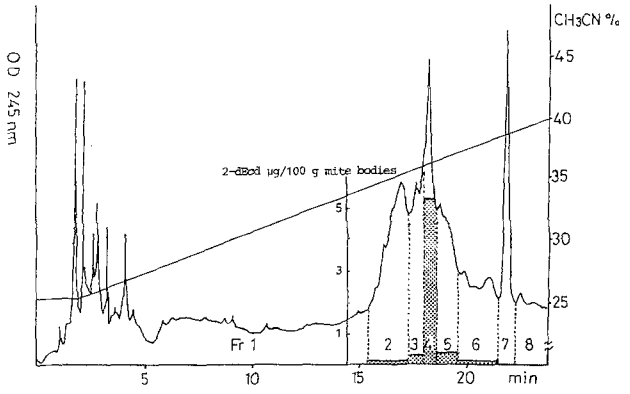

Figure 1. Final HPLC purification of the ecdysone from the mite, *T. putrescentiae*. Samples of the major fraction derived from the first HPLC were purified and 8 fractions collected manually. From results of the RIA we calculated the contents of 2-dEcd (µg/100 g mite bodies): No. 1 (not detectable), No. 2 (0.11), No. 3 (0.32), No. 4 (5.3), No. 5 (0.38) No. 6 (0.08), No. 7 (0.06), No. 8 (0.03).

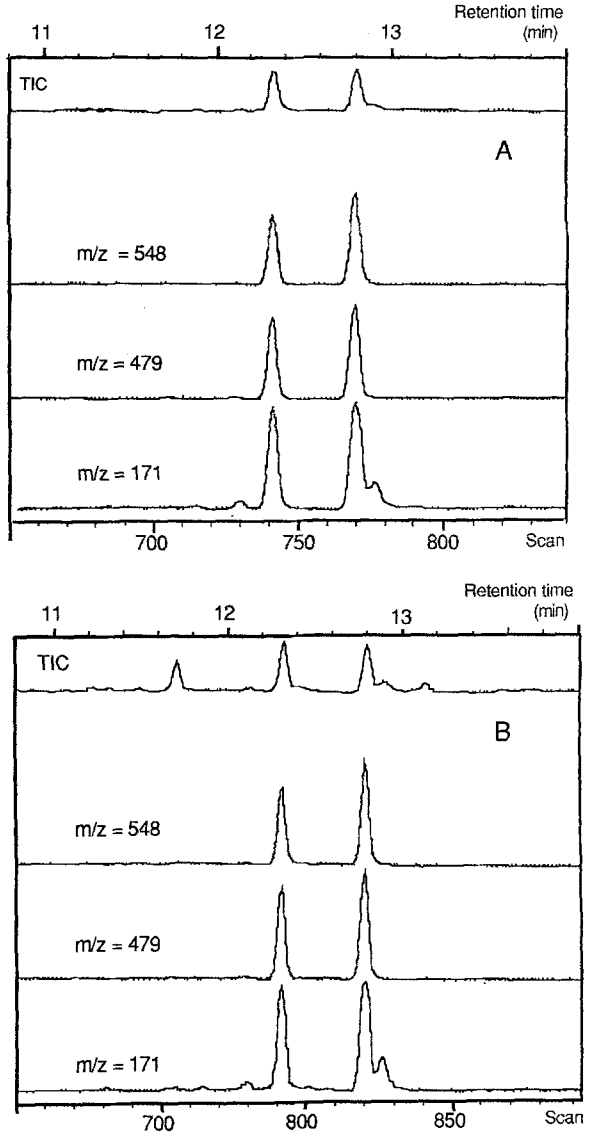

Figure 2. GC-MS analysis of tetratrimethylsilylated 2-deoxyecdysone. *A* Mass chromatogram of tetratrimethylsilylated authentic 2-dEcd monitored by TIC (total ion current), m/z 548, 479 and 171. *B* Mass chromatogram of trimethylsilylated fraction No. 4 from the last HPLC monitored by TIC, m/z 548, 479 and 171.

ing Ecd in this fraction. In the major HPLC fraction 2-dEcd was identified by GC-MS analysis (fig. 2) and the amount present calculated as 1.9 µg/100 g mite bodies after calibration with fully trimethylsilylated authentic 2-dEcd. Both authentic and natural 2-dEcd, however, appeared as two peaks on the mass chromatogram, both peaks having parent ions of m/z 736, indicating full trimethylsilylation. To resolve this question, we treated authentic 2-dEcd (500 µg) with DMF or DMF and imidazole at 130 °C for 14 h. After this treatment, 2-dEcd (together with a small amount of another compound) was purified by preparative HPLC. The other compound was eluted earlier than 2-dEcd and had a yield of 7% from treatment with DMF and 15% with DMF containing imidazole. The NMR spectrum (in CD_3OD, FX-200 JEOL) of the unknown compound was very similar to that of 2-dEcd. Its methyl signals were assignable though its methylene and methyne signals were not clearly assignable because of the small amount of sample. Since the chemical shift of the 19-methyl of the unknown compound was 0.1 ppm lower than that of 2-dEcd, it should be the 5 α isomer; i.e., the A-B ring junction changed from *cis* to *trans*[3]. We concluded that 2-dEcd was isomerized during the derivatization reaction and possibly also during the GC analysis.

GC-MS analysis has been used to identify and quantify the ecdysteroid, but does have some problems. For example, to obtain quantitative amounts of fully trimethylsilylated samples requires very drastic derivatization conditions and a high temperature for GC analysis. Therefore we attempted to use a recently developed LC-MS system, suitable for analyzing ecdysteroids at room temperature without derivatization. An ODS column (Develosil ODS-5, 2.0 × 250 mm) was used for HPLC separation and a flit FAB system was adopted for mass spectrometry and ionization[4, 5]. We succeeded in establishing an LC-MS system for the analysis of ecdysteroids, 20-OH-Ecd, Ecd and 2-dEcd. The details of these results will be reported elsewhere. 2-Deoxyecdysone was identified from the No. 4 fraction (fig. 1) and the amount present calculated to be 3.9 µg/100 g mite bodies by the LC-MS system using the calibration curve derived from authentic 2-dEcd (fig. 3). Although the amounts of 2-dEcd were variable with three different systems, the major ecdysteroid in the mite, *T. putrescentiae*, was identified as 2-dEcd.

Ecdysone and 20-OH-ecdysone have been identified in ticks and the biological role of ecdysteroids has been studied[6]. Little is known about mite ecdysteroids, our study being the first to demonstrate ecdysteroids in the mite. Because 2-dEcd is a precursor of Ecd and 20-OH-Ecd in insects, these two ecdysteroids may exist in small

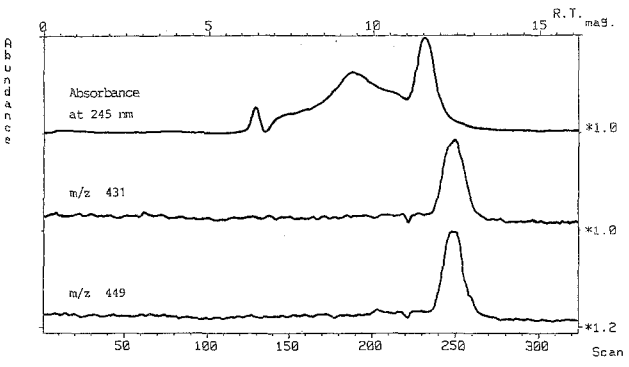

Figure 3. LC-MS analysis of the 2-deoxyecdysone from the mite, *T. putrescentiae*. The sample was from fraction 4 of the final HPLC (fig. 1). A 2 µl sample of this solution (equivalent to 4.1 g mite bodies) was analyzed, monitoring at 245 nm absorbance and m/z 449(M+1) and 431 (M−18+1). The delay between the UV peak and the peak of the mass chromatogram represents the connecting tube volume between the UV detector and the mass spectrometer.

amounts in the mite. We could not observe biological activity of Ecd, 20-OH-Ecd or 2-dEcd with our bioassay systems; however, after further chemical studies, we hope to show the biological role of ecdysteroids on different sexes and at different growth stages of mites.

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