Makisterone A and 24-methylenecholesterol from the ovaries of the honey bee, *Apis mellifera* L.

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Summary. Makisterone A, a 28-carbon ecdysteroid (molting hormone) has been isolated from the ovaries of queen bees. Analysis by reversed-phase and silica high performance liquid chromatography (HPLC) in conjunction with a radioimmune assay (RIA) revealed about 11 ng of makisterone A present per gram of ovaries on a fresh weight basis. No C_{27} ecdysteroids were detected. The predominant neutral sterol present was 24-methylenecholesterol.

Key words. Ecdysteroids; molting hormones; makisterone A; HPLC; RIA; honey bee; ovaries; *Apis mellifera;* neutral sterols; 24-methylenecholesterol.

The honey bee, *Apis mellifera,* is an insect incapable of converting dietary C_{28} and C_{29} phytosterols to cholesterol[1], a pathway common to most phytophagous insects[2]. Recently, makisterone A (2β, 3β, 14α, 20R, 22R, 25-hexahydroxy-24-methyl-5β-cholest-7-en-6-one), a 28-carbon ecdysteroid (molting hormone), has been identified as the major free ecdysteroid in honey bee pupae at peak titer[3]. Since neutral sterols serve as the precursors for ecdysteroids[4] and ecdysteroids may play an important role in caste determination in Hymenoptera[5–9], we have examined the sterol composition and ecdysteroid content of the ovaries of queen bees.

Materials and methods. Ovaries from gravid, laying queen bees (n = 33) maintained at Beltsville were dissected out in saline and extracted twice by homogenization in 100% methanol followed by extraction with 75% methanol/water (v/v). The homogenates were centrifuged at 1000 g, the supernatants were combined, dried in vacuo and then partitioned between 70% methanol/water (v/v) and hexane as previously described[3,10]. The hexane phase was analyzed by gas-liquid chromatography (GLC) for sterol composition[11], while the methanolic phase was further purified by butanol/water partitioning and open column chromatography on silicic acid[3]. The resulting purified extract was then analyzed by high performance liquid chromatography (HPLC) and radioimmune assay (RIA). Ovarian ecdysteroids were fractionated isocratically on both a reversed-phase C_8 column (150 mm × 4.6 mm I.D.; 5 µm particle size; IBM, Danbury, CT) with 35% methanol/water (v/v) and on a silica column (150 mm × 4.6 mm I.D.; 5 µm particle size; Rainin, Woburn, MA) with methylene chloride/2-propanol/water (125/25/2; v/v/v) at flow rates of 1 ml/min. The eluant was monitored at 254 nm and the retention times of unknown ecdysteroids were compared to retention times of ecdysteroid standards. One-ml fractions were collected for RIA analysis[12]. The cross-reactivity factors for makisterone A and 20-hydroxyecdysone were determined to be 8.65 and 4.85, respectively, by comparing the mass of each ecdysteroid (Simes; Milano, Italy) required to displace 50% of the radiolabeled ligand ([23,24-^{3}H]ecdysone; sp. act 55–60 Ci/mmole) relative to the mass of ecdysone required.

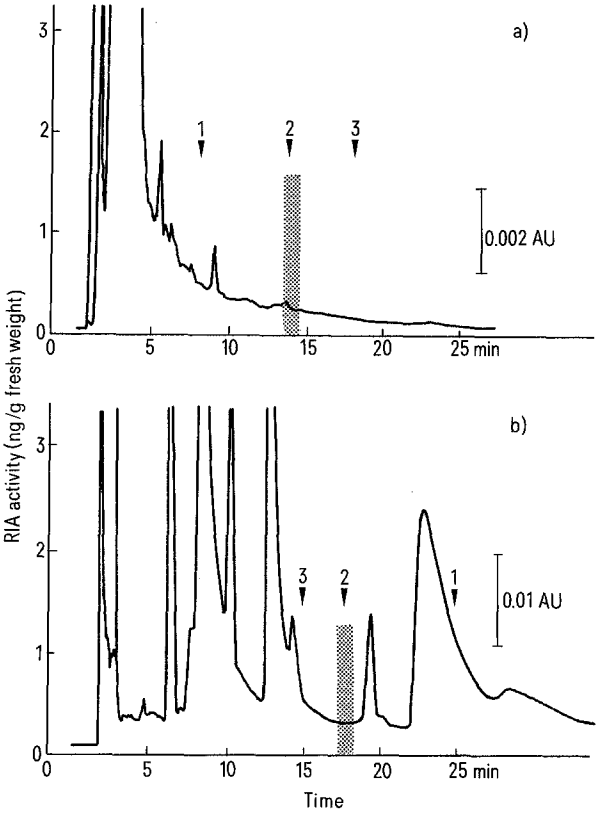
Results and discussion. The predominant sterol in queen bee ovaries is 24-methylenecholesterol (table), which accounted for over 53% of the total sterols present. Sitosterol (19.0%) and isofucosterol (14.3%) were the next most abundant sterols. C_{27} sterols accounted for only about 2% of the total sterols, consistent with previous reports of the inability of this species to dealkylate C_{28} or C_{29} phytosterols at the C-24 position[1]. 24-methylenecholesterol has previously been identified as the major sterol from honey bee workers[11,13], queens[13], and prepupae[11]. Interestingly, honey bee workers must possess a mechanism for selectively transferring certain sterols to the developing brood, since 24-methylenecholesterol was identified as the major brood sterol regardless of the sterol composition of the diet fed to the workers[11,14]. The exact mechanism of this selective transfer, however, has yet to be elucidated.

The normal and reversed-phase HPLC separations of the ovarian extract, along with their respective RIA analyses, is shown in the figure. In both solvent systems, a single, immuno-

Neutral sterols from queen bee ovaries*

Sterol	Relative %**
Cholesterol	0.7
Desmosterol	1.3
24-Methylenecholesterol	53.1
Campesterol	10.0
Stigmasterol	1.0
Sitosterol	19.0
Iso-fucosterol	14.3

* Sterols were identified by comparison of relative retention times (cholestane = 1.0) with those of authentic sterol standards in GLC on a fused silica capillary column (J&W, Durabond DB-1; 15 m × 0.25 mm i.d.; film thickness = 0.25 µm) at 236°. ** Total does not equal 100%, as several unidentified minor sterols were not included.



HPLC traces (254 nm) and RIA analyses of ecdysteroids from queen bee ovaries. *a* Column: C_8 (150 mm × 4.6 mm I.D.); mobile phase: 35% methanol/water; flowrate: 1 ml/min; column temp: 33°. *b* Column: Silica (150 mm × 4.6 mm I.D.); mobile phase: methylene chloride/2-propanol/water, 125/25/2; flow rate: 1 ml/min; column temp: 33°. The shaded areas represent RIA activity, expressed as ng of ecdysone equivalents per gram fresh weight of ovaries, and are not corrected for cross-reactivity. The approximate elution times of ecdysteroid standards are indicated: 1 = 20-hydroxyecdysone; 2 = makisterone A; 3 = ecdysone.

reactive peak that had a retention time identical to that of authentic makisterone A was evident. When the immunoreactive area from the reversed-phase fractionation (fig. a; fraction 14) was injected on silica, a single immunoreactive peak was also revealed that likewise matched the retention time of authentic makisterone A, although no UV-absorbance was observed. After correcting for cross-reactivity, the concentration was calculated to be 10.6 (silica) to 12.6 (reversed-phase) ng of makisterone A per gram of ovaries on a fresh weight basis. No immunoreactivity was observed in fractions co-eluting with the C_{27} ecdysteroids, 20-hydroxyecdysone or ecdysone, which were well-separated from the makisterone A fraction under both reversed-phase and silica chromatographic conditions (fig. a and b).

Since ovarian ecdysteroids are frequently present as conjugates[15–17], we enzymatically hydrolyzed the contents of the aqueous phase resulting from the butanol/water partition[10,18]. Subsequent HPLC/RIA analysis revealed that only 2.25 ng of makisterone A per gram fresh weight of ovaries were liberated by hydrolysis.

These data represent the first report of a 28-carbon molting hormone from an adult holometabolous insect and along with previous work involving honey bee pupae[3], strongly suggest that the C_{28} ecdysteroid, makisterone A, is the major molting hormone in *A. mellifera*. We have been unable to verify the presence of C_{27} ecdysteroids as previously suggested[6]. The nature of the molting hormone in other Hymenoptera, however, remains unclear. Ecdysone and 20-hydroxyecdysone have been reported from two species of ants, *Pheidole pallidula* queens[7] and *Plagiolepsis pygmaea* larvae[8]. The separation and identification of these ecdysteroids, however, was based upon thin-layer chromatography (TLC), a procedure that would not necessarily detect makisterone A if this compound were present, due to the relatively poor resolution by TLC. The nature of the neutral sterols in these species would be of interest as it might give an indication as to whether C_{27} or C_{28} ecdysteroids are synthesized. For example, analysis of brain and whole body extracts of another ant species, the leaf-cutting ant, *Atta cephalotes isthmicola*, revealed the presence of only 28-carbon sterols[19] (C-24 alkyl), an indication that this particular species cannot convert plant sterols to cholesterol and therefore may produce a 28-carbon molting hormone.

The sterol composition and correlation to ecdysteroid content in the Hemiptera (true bugs)[20,21] may have an interesting analogy in the Hymenoptera. Previous studies have shown that blood-sucking hemipterans, which have a preponderance of cholesterol in their diets, produce the C_{27} ecdysteroids, ecdysone and 20-hydroxyecdysone, while other phytophagous hemipterans, whose diets are rich in C_{28} and C_{29} phytosterols and cannot convert these sterols to cholesterol[20], utilize makisterone A as their molting hormone[21,22]. Analysis of sterols in six species of Hymenoptera reveal a similar pattern, at least with regard to sterol composition[23]. Phytophagous species contain relatively high levels of 24-methylenecholesterol, whereas omnivorous or predatory species contain mostly cholesterol. Future investigations aimed at elucidating the ecdysteroid content of insects containing predominantly 28-carbon sterols may reveal that C_{28} molting hormones, like makisterone A, are more prevalent than originally thought.

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Hydroxylation of menthols and cineoles with *m*-chloroperbenzoic acid

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Summary. Reaction of menthols and cineoles with *m*-chloroperbenzoic acid afforded tertiary, secondary, and primary alcohols, some of which were natural products having potent plant growth regulatory activity or were mammalian metabolites.
Key words. 1-Menthol; *iso*-menthol; *neo*-menthol; 1,4-cineole; 1,8-cineole; *m*-chloroperbenzoic acid; hydroxylation.

We have been studying the hydroxylation of terpenoids in mammals[1]. Such reactions have some synthetic utility in the preparation of insect pheromones and perfume protecting agents. Although several methods for the introduction of a hy-