Naturwissenschaften 79, 184 – 185 (1992)

An Amino Acid Derivative as the Sex Pheromone of a Scarab Beetle

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Since the identification of bombykol, a myriad of compounds have been reported as sex pheromones. Nevertheless, as highlighted by the examples of female Lepidoptera, which by and large utilize alcohols, acetates, aldehydes, and hydrocarbons, including epoxyhydrocarbons, pheromones are restricted to some groups of chemicals with marked structural similarities. Even in the Coleoptera, with more diversity in molecular structures, structurally related compounds are used by insects of the same family as evidenced by the scarab beetles; the sex pheromones of *Popillia japonica*, *Anomala rufocuprea*, and *A. cuprea* are (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one [1], methyl (*Z*)-tetradec-5-enoate [2] and (*R*,*Z*)-5-(−)-(oct-1-enyl) oxacyclopentan-2-one [3], respectively. On the other hand, *Costelytra zealandica* and *Kheper lamarcki* of the same family have been reported to utilize quite different chemicals, viz., phenol [4] and a mixture of hexadecanoic acid, 2,6-dimethyl-5-heptenoic acid, and (*E*)-nerolidol along with a polypeptide pheromone carrier [5], respectively.

We report here that the large black chafer *Holotrichia parallela* (Mots.) (Coleoptera: Scarabaeidae), *ookurokogane* in Japanese, utilizes a unique sex pheromone, L-isoleucine methyl ester.

H. parallela is an important agricultural pest in Japan, which was initially called *Lachnosterna morosa* Waterhouse [6] and later renamed [7]. Adults of the beetle remain in the soil during daytime, coming to the surface every other day soon after sunset [8]. Females, after reaching the leaves of the host plants, assume a calling position by extruding an abdominal gland. That behavior, resembling the calling of moths, lasts no longer than 15 min.

In a preliminary screening for the sex pheromone(s), the abdominal tips of calling females were washed in various solvents and bioassayed. The solutions, containing 1 – 5 female equivalent (FE), were transferred to pieces of cotton, which were set 2 m away from each other on the topmost leaves of mulberry trees. Males were attracted only by the samples generated by polar solvents, namely, ether and dichloromethane.

An ether extract of 50 FE was separated on an SiO_2 column by successively eluting with hexane/ether mixtures, 100:0, 95:5, 90:10, 80:20, 50:50, and 0:100. Activity in the field bioassay was recovered in the 100% ether fraction, which gave one-peak GC profiles on two capillary columns. The single peak appeared at t_R 9.50 min on a DB-23 (30 m × 0.254 mm; 0.25 μm) operated at 50 °C for 1 min and programmed at 8 °C/min to 210 °C and held at this temperature for 10 min [50(1)-210(10)/8] and t_R 12.56 min on HP-1 (25 m × 0.2 mm; 0.33 μm) operated at 50(1)-210(10)/8-230(10)/20. The difference in the retention indices ΔI 440 [*I*(DB-23) 1468; *I*(HP-1) 1028] indicated a polar compound.

That the pheromonal activity was due to a single compound was corroborated by GC-EAD [9] recorded with a modified station for scarab beetles [10] using male antennae. Crude extract and the active ether fraction generated only one EAD signal at t_R 9.50 min (DB-23). Vapor phase IR of the active peak displayed characteristic bands of a methyl ester at 1755 ($\nu C=O$) and 1173 cm^{-1} ($\nu C-O$). A library search (Hewlett Packard) indicated methyl 2- and 3-methylpentanoate as the best fittings. However, these structures were ruled out, based on their polarities. Furthermore, they would not explain the weak band at 1622 cm^{-1}, which was considered to be due to NH_2 bending mode. El-MS exhibited a base peak at m/z 86 along with the following fragments: 41(13), 44(8) 69(13), 74(11), 88(40), 113(0.2), 116(0.4), 130(0.2), 145(0.3), and 146(0.3). The molecular peak was confirmed to be m/z 145 by chemical ionization with ammonia [146 (M + 1), base peak; 163 (M + 18)] and isobutane [146 (M + 1), base peak; 186 (M + 41)]. Therefore, the pheromone was considered an α-amino ester; fragmentations of the C1-C2 and C2-C3 bonds would give rise to the El-MS peaks at m/z 86 and 88, respectively [11]. The reaction of the natural product with acetyl chloride in ether and which was catalyzed by pyridine gave a new peak on DB-23 at t_R 19.24 min (M^+ 186; base peak, m/z 128). These data suggested as possible structures methyl esters of norleucine, leucine, and isoleucine.

Methyl esterification of these amino acids was obtained according to [12]. Leucine and norleucine methyl esters had retention times shorter and longer, respectively, than the natural product. Their MS also differed from that of the pheromone chiefly in the fragment at m/z 88, which was less intense due to the lack of a substituent on C3. On the other hand, both the MS and the retention time of DL-isoleucine methyl ester were identical to those of the natural product.

Chromatographic resolution of the four isomers of isoleucine methyl ester was achieved on a CP-cyclodextrin β-236-M-19 (50 m × 0.25 mm; 0.25 μm) operated at 95 °C (Fig. 1a). By co-injection of each authentic isomer and a DL-mixture, the peaks were assigned as L-*allo*- [methyl (2*S*,3*R*)-2-amino-3-

methylpentanoate], t_R 24.109, D-*allo*-[methyl (2*R*,3*S*)-2-amino-3-methylpentanoate], t_R 24.465, D- [methyl (2*R*,3*R*)-2-amino-3-methylpentanoate], t_R 24.865, and L-isoleucine methyl ester [methyl (2*S*,3*S*)-2-amino-3-methylpentanoate], t_R 25.479 min. Resolution was also accomplished after derivatization with acetyl (*S*)-lactyl chloride, as described for alcohols [13]. On a DB-wax column (30 m × 0.25 mm; 0.25 μm) operated at 100(1)-210(1)/4-220(10)/10, the derived peaks appeared at 25.66 (D-*allo*), 25.97 (D-), 26.10 (L-*allo*), and 26.42 (L-). The natural product gave a major peak corresponding to methyl (2*S*,3*S*)-2-amino-3-methylpentanoate and its derivative on the chiral (Fig. 1 b) and the achiral columns, respectively. The composition of the pheromone was estimated to be 83.6 % of L-, 9.9 % of D-, 4.5 % of D-*allo*-, and 2 % of L-*allo*-isoleucine methyl ester. Our synthetic methyl ester, obtained from L-isoleucine (Nakalai Tesque), also contained the same three minor isomers as the natural product (Fig. 1 c), although in slightly different proportions.

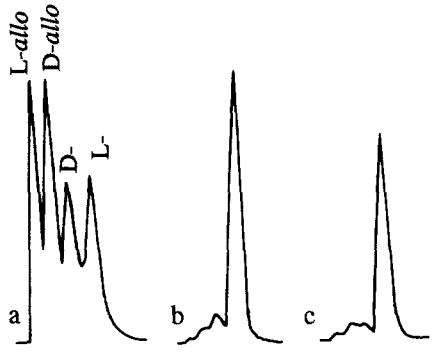

Fig 1. Chiral resolution of isoleucine methyl esters, a) synthetic DL-mixture, b) the sex pheromone of *H. parallela*, c) synthetic mixture derived from L-isoleucine

In the field test, five males were attracted to a natural composition of the pheromone at the 1 000-ng level (1 FE). They approached the pheromone source by ca. 2 cm, kept searching for 2 or 3 min, followed the cotton when it was deliberately moved, and flew away without landing. However, further experiments in the next season(s) are required to clarify whether the minor components are *sine qua non* for pheromonal activity.

Received December 4, 1991 and January 10, 1992

1. Tumlinson, J. H., et al.: Science *197*, 789 (1977)
2. Tamaki, Y., et al.: Appl. Entomol. Zool. *20*, 359 (1985)
3. Leal, W. S.: Naturwissenschaften *78*, 521 (1991)
4. Henzell, R. F., Lowe, M. D.: Science *168*, 1005 (1970)
5. Burger, B. V., et al.: Z. Naturforsch. *38c*, 848 (1983); *45c*, 863 (1990)
6. Anonymous: Major Insects and Other Pests of Economic Plants in Japan. Tokyo: Japan Plant Protection Assoc. 1980
7. Japan Soc. Appl. Entomol. Zool. (ed.): Major Insects and Other Pests of Economic Plants in Japan. Tokyo: Japan Plant Protection Assoc. 1987
8. Yoshioka, K., Yamasaki, Y.: Jap. J. Appl. Entomol. Zool. *27*, 52 (1983)
9. Struble, D. L., Arn, H., in: Techniques in Pheromone Research. New York: Springer 1984
10. Leal, W. S., et al.: Appl. Entomol. Zool. (in press)
11. Anderson, C. O., et al.: Arkiv Kemi *19*, 405 (1962) [cf. CA *58*, 119e]
12. Brenner, M., Huber, W.: Helv. Chim. Acta *36*, 1109 (1953)
13. Slessor, K. N., et al.: J. Chem. Ecol. *11*, 1659 (1985)

Naturwissenschaften 79, 185 – 187 (1992)

Hormonal Regulation of Dopadecarboxylase Activity and Chitin Synthesis in an Epithelial Cell Line from *Chironomus tentans*

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Cuticle formation is one oft the most important physiological processes regulated by molting hormones, the ecdysteroids. This physiological pathway is restricted to epidermal tissue and imaginal disks. Several insect cell lines are able to synthesize at least parts of the cuticle in vitro [1]. The epithelial cell line from *Chironomus tentans* [2] synthesizes chitin [3] and responds to 20-OH-ecdysone with a reduction of this synthesis [4]. In this paper we will demonstrate that dopadecarboxylase [5], a key enzyme in the sclerotization process [6], is also present in these cells and is regulated by molting hormones. Hormone specificity and the site of hormonal regulation of both dopadecarboxylase and chitin synthesis in the cell line from *C. tentans* will be demonstrated.

Since the *C. tentans* cell line does not metabolize added ecdysteroid significantly [7], long-term hormone incubations with definite hormone concentrations are possible. Dopadecarboxylase activity was measured according to [8]. Cells from 20 – 30 ml cell culture were harvested by centrifugation, washed once with saline, and the cell pellet frozen in liquid nitrogen. For enzyme determination the cells were homogenized with an all-glass homogenizer in 500 μl 100 m*M* phosphate buffer + 0.25 *M* sucrose, pH 7. The homogenate was centrifuged at 4 000 *g* at 4 °C for 15 min and the supernatant was taken for enzyme activity measurements. ^{14}C-labeled dihydroxyphenylalanine (30 nCi/assay; 10^{-4} *M*) and 10 m*M* pyridoxalphosphate were incubated with 60 μl cell extract for 30 min at 25 °C; 100 μl water was added and the synthesized dopamine separated by adsorption to a cation exchanger DEHP (= Bis(2-ethylhexyl)-hydrogenphosphate) dissolved in 200 μl $CHCl_3$. The $CHCl_3$ phase was reextracted with 200 μl 50 m*M* phosphate buffer, pH 7. The $CHCl_3$ phase was evaporated at 60 °C and the radioactivity of the residue de-