Original Article

Differential Saturation Electroantennogram of Germacrene-D, a Sex Pheromone Mimic of the American Cockroach

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By applying differential saturation EAG (DS-EAG) technique, the olfactory receptor for germacrene-D (**1**), a sex pheromone mimic of the American cockroach, was investigated using natural sex pheromones [periplanone-A (**2**) and periplanone-B (**3**)], a general odor (camphor) and this mimic. The mimic was consequently revealed to be involved in the sex pheromone receptor responsible for periplanone-A in preference to that for periplanone-B.

INTRODUCTION

Since a plant component, germacrene-D (**1**), was reported as a sex pheromone mimic of the American cockroach (*Periplaneta americana* L.),¹⁾ several investigators^{2,3)} have made effort to deduce the structural factors important for sex pheromonal activity included in **1** by preparing analogs of **1**. Major interest in these works was based on the structural similarity between **1** and periplanone-B (**3**)⁴⁻⁶⁾ which is a natural sex pheromone isolated from females of the insect together with another sex pheromone, periplanone-A (**2**).^{7,8)} Clear structural relationship between **1** and **3** has not been deduced.

Quite recently, we⁹⁾ employed the differential saturation electroantennogram (DS-EAG) method in order to elucidate the olfactory receptor system for the monoterpene sex pheromone mimics^{10,11)} of the cockroach. The mimics were consequently revealed to be involved in the sex pheromone receptors responsible for **3**.

In this work, an investigation on the olfactory receptor system for **1** was conducted by employing DS-EAG technique in order to establish a basic conception for elucidating

structural relation of **1** with the natural pheromone(s).

MATERIALS AND METHODS

1. Odorous Compounds for DS-EAG

Germacrene-D (**1**) was isolated from a Compositae plant, *Solidago altissima* L. Pure natural sex pheromones, **2** and **3**,¹²⁾ isolated from the female cockroaches were used. Camphor as a general odor was obtained commercially. Five hundreds micrograms of **1** and camphor, and, 1×10^{-2} μ g of **2** and **3** were suitable quantities as the primary odorous stimulation to saturate the antennae, while 100 μ g of **1** and camphor, and 2×10^{-3} of the pheromones were used for the secondary stimulation to the saturated antennae.

2. Isolation of **1**²⁾

The aerial parts except for flowers (12.5 kg) (collected near Tokyo in October) were extracted with methanol (48 l) for 1 month at

room temperature. After removal of the solvent to 4 l aqueous concentrate, the ethyl acetate extract from the aqueous solution was equally divided into 3 portions. For each portion, Florisil (495 g) column chromatography (column: 4.5×60 cm) was performed by eluting with *n*-hexane (1 fraction=1.3 l). Fractions with same numbers were combined and analyzed by GC (4% OV-1 in a 2 m×3 mm column, 145°C, N₂ 50 ml/min). The presence of **1** was shown in fractions 1–3 (9.0 g). For 1 g of the fractions, 10% AgNO₃-Florisil column chromatography (120 g) was performed eluting with *n*-hexane containing increasing proportions (0, 5, 10,..., 90, 95 and 100%) of chloroform (column: 3×60 cm, 1 fraction=200 ml). Subsequently, the column was eluted with a mixture of chloroform and ether, in which the concentration of ether was increased in successive 5% steps. Germacrene-D (**1**) was included in 50–100% ether in chloroform fractions in each of the chromatography with 1 g sample. Such chromatography was repeated 9 times. The fractions (5.8 g) were chromatographed over neutral aluminum oxide (50 g) (column: 3.5×40 cm) eluting with *n*-hexane. Fractions having **1** (2.7 g) were subjected to HPLC [sample: 100 mg, Develosil 30-3 (3 μm) in a 4 mm×15 cm, column, solvent: *n*-hexane (1.1 ml/min)]. Finally 2.7 g of the fractions gave 1.9 g of pure **1**; [α]_D²⁵ –59.8° (*c*=1.00, *n*-hexane), –19.2° (*c*=1.00, MeOH) (based on the value by Niwa,¹³) our **1** was composed of a mixture of (–)- and (+)-**1** with 53 : 47 ratio); UV (*n*-hexane): $\lambda_{\max}$ 259 nm (ϵ =4500); IR (film): $\nu_{\max}$ 3070, 1630, 1605, 1390, 1375, 880 cm^{–1}; PMR (CCl₄, 60 MHz): $\delta_{\text{ppm}}^{\text{TMS}}$ 0.80 [3 H, doublet (d), *J*=6 Hz], 0.86 [3 H, d, *J*=6 Hz], 1.46 [3 H, broad singlet (bs)], 4.69 (2 H, bs), 5.10 (1 H, multiplet), 5.18 (1 H, double doublet, *J*=16 and 9 Hz), 5.73 (1 H, d, *J*=16 Hz); MS: *m/z* 204 (M⁺, C₁₅H₂₄), 161 (M⁺–43, base peak), 120, 105, 81, 79, 41; GC retention time (*t*_R, min) 7.1; HPLC: *t*_R 4.8.

3. Odor Delivery System,⁹⁾ Antennal Preparation and Response Recording in DS-EAG (Fig. 1)

By turning a 3-way stop-cock (b), we introduced a continuous air-stream (a, 30 ml/sec)

escaping from the path d into a 10 ml syringe (e₁) through the path c. A filter paper (f₁, 1.3 cm diameter) impregnated with a high concentration of a primary odor for saturation was placed on a vinyl holding tube (g) in the syringe, so that the air-stream (h) after having passed through the filter paper (f₁) contained sufficient quantities of the primary odor. While the antenna was saturated by the primary odor, secondary odorous stimulation was carried out by pressing the plunger (i) of another 10 ml syringe (e₂) in which a filter paper (f₂) impregnated with the secondary odor was equipped. Consequently, an air-stream (j) with both primary and secondary odors was applied to an antenna (k).

Fig. 1 Schematic drawing of odor delivery system and antennal preparation for the differential saturation EAG (DS-EAG) recording.

a: air-stream, b: 3-way stopcock, c: path for odor saturation, d: path for escaping, e₁ and e₂: 10 ml syringe, f₁: filter paper with primary odor, f₂: filter paper with secondary odor, g: vinyl tubing holder, h: air-stream with primary odor, i: syringe plunger, j: air-stream with both primary and secondary odors, k: male antenna, l: glass slide, m: rubber stand, n: cockroach sarine, o: Petri dish, p: indifferent electrode, q: recording electrode, r: probe, s: micro-electrode amplifier, t: biophysical amplifier, u: wave memory scope.

After 1 mm of the tip of an excised antenna (k) of an adult male of the cockroach was cut off the antenna was fixed with small pieces of adhesive tape on a glass slide (l) on an angled rubber stand (m) in a 50 mm diameter Petri dish (o) filled with cockroach saline solution (n). The scape end of the antenna was immersed into a medium permitting electrical connection of the antenna with an indifferent electrode (p, spiral chlorided silver wire). A distal end of the antenna was inserted into a recording electrode (g) (glass capillary composed of chloridized silver wire and the saline solution). The recording electrode was connected to a microelectrode amplifier [s, Model DPZ-11, Dia-medical System Co. (Tokyo)] via a probe (r, Model DPZ-101). The amplified signal was further amplified with a biophysical amplifier (t, Model DPA-100F). The EAG response was visible on an oscilloscope of a wave memory apparatus (u, Model DPM-202). One antenna was used for the saturation with one primary odor. While the antenna was saturated, secondary odorous stimulations were performed using all the secondary odors. Such test was repeated 3 times. The average EAG amplitudes were obtained from the 3 tests with standard deviations (see Table 1).

RESULTS

Typical EAG response patterns in DS-EAG are shown in Fig. 2, and the expression with figures (average amplitude $\pm$ standard deviation of 3 tests) is shown in Table 1.

The quantities of every compound used elicited large responses more than 0.8 mV [Fig. 2-I, *a* (camphor, 100 μ g), *b* (**1**, 100 μ g), *c* (**2**, 2×10^{-3} μ g) and *d* (**3**, 2×10^{-3} μ g)] in the usual EAG recording. These responses are much higher than the control response (I-*e*, without compound). Saturated bases [II-A (camphor, 500 μ g), III-B (**1**, 500 μ g), IV-C (**2**, 1×10^{-2} μ g) and V-D (**3**, 1×10^{-2} μ g)] elicited 0.7–1.5 mV. Against the saturated bases, secondary stimulation with a compound, when the same compound was used for saturation, gave small or negligible responses [II-*a/A* (0.25 mV), III-*b/B* (0.15 mV), IV-*c/C* (0.01 mV) and V-*d/D* (0.10 mV)]. These responses were comparable to the control response (II-*e/A*) in which air without

Fig. 2 Typical EAG responses recorded in DS-EAG experiments by stimulation with camphor (general odor), germacrene-D (**1**, sex pheromone mimic), periplanone-A and periplanone-B (**2** and **3** respectively, sex pheromones).

I: responses induced by 100 μ g of camphor (*a*), 100 μ g of **1** (*b*), 2×10^{-3} μ g of **2**, (*c*), 2×10^{-3} μ g of **3** (*d*) and air (*e*) to the base line given by continuous air-stream only. II: responses induced by secondary stimulations with the above quantities of the compounds to the base line saturated with 500 μ g of camphor (A), in which, for example, *a/A* indicates a response obtained from secondary stimulation with 100 μ g of camphor to the base saturated with 500 μ g of camphor. Similar stimulations were performed to the base lines produced by saturation with 500 μ g of **1** (III-B), 1×10^{-2} μ g of **2** (IV-C) and 1×10^{-2} μ g of **3** (V-D).

odor was used as secondary stimulation. This indicates that receptor saturation by the primary odorous compounds was perfect.

To the camphor-saturated base line (II-A), the compounds active as sex pheromone evoked large secondary responses [II-*b/A* (0.51 mV), II-*c/A* (0.50 mV) and II-*d/A* (0.75 mV)]. On the other hand, camphor evoked large secondary responses against the base

Table 1 EAG amplitudes (mV) of the adult male American cockroach in differential saturation experiments.

		Primary odor for saturation			
		Camphor (500 μ g)	1 (500 μ g)	2 (1×10^{-2} μ g)	3 (1×10^{-2} μ g)
Saturated base line		1.18 $\pm$ 0.28	0.73 $\pm$ 0.17	1.50 $\pm$ 0.18	0.86 $\pm$ 0.13
Odor for secondary stimulation (amplitude from saturated base line)	Camphor (100 μ g)	0.25 $\pm$ 0.13	0.51 $\pm$ 0.13	0.50 $\pm$ 0.09	0.75 $\pm$ 0.17
	1 (100 μ g)	0.48 $\pm$ 0.10	0.15 $\pm$ 0.08	0.19 $\pm$ 0.03	0.35 $\pm$ 0.08
	2 (2×10^{-3} μ g)	0.88 $\pm$ 0.25	0.44 $\pm$ 0.18	0.01 $\pm$ 0.02	0.32 $\pm$ 0.07
	3 (2×10^{-3} μ g)	0.95 $\pm$ 0.25	0.64 $\pm$ 0.26	0.31 $\pm$ 0.04	0.10 $\pm$ 0.06

Each amplitude is given by average value $\pm$ standard deviation of 3 tests. Values in the line "saturated base line" means the amplitudes after having been saturated by compounds and thus became base lines for secondary odorous stimulations (see II-A, III-B, IV-C and V-D in Fig. 2). The other amplitudes were measured for the responses from the saturated base lines whose shapes are typically shown in *a/A*, *b/A*,, *c/D* and *d/D* in Fig. 2.

lines saturated with compounds active as sex pheromone [III-*a/B* (0.48 mV); IV-*a/C* (0.88 mV) and V-*a/D* (0.95 mV)]. These facts imply that receptors responsible for camphor are different from those for the sex pheromonal compounds. "General odor receptor" has been proposed by the authors¹⁴⁾ for the receptors responsible for general odors such as food odors including camphor. The receptors are scattered on the antennae of both male and female. On the other hand, "special odor receptors"¹⁴⁾ are sensitive to special odors such as sex pheromones, and exist on male antennae only.

In the saturation experiments with **1** (Fig. 2-III), although both natural pheromones gave significant secondary EAG responses, the response by **2** [III-*c/B* (0.44 mV)] was weaker than that by **3** [III-*d/B* (0.64 mV)]. In this case, camphor evoked somewhat weaker secondary response as compared with the other saturation experiments (see III-*a/B* comparing with IV-*a/C* and V-*a/D*). This suggests that **1** is weakly associated with general odor receptors. It is known that **1** stimulates significantly general odor receptors on the female antennae of the cockroach.¹⁴⁾ When the antennae were saturated with **2** (see IV), the secondary stimulation with **1** elicited weaker response [IV-*b/C* (0.19 mV)] as compared with **3** [IV-*d/C* (0.31 mV)]. In the case of saturation with **3** (see V), secondary responses by **1** and **2** (V-*b/D* and *c/D*)

yielded similar amplitudes (0.35 and 0.32 mV, respectively). From these evidences, the following conclusions may be induced: (1) Either sex pheromone (**2** or **3**) has a specific receptor (periplanone-A receptor or periplanone-B receptor), which supports the conclusions reported in the single cell recording work¹⁵⁾ and our previous EAG work⁹⁾; (2) Germacrene-D (**1**) is associated with both of the sex pheromone receptors, but seems to be involved preferentially in the periplanone-A receptor to in the periplanone-B receptor.

DISCUSSION

DS-EAG technique developed for beetles^{16,17)} and moths¹⁸⁾ involves an aspect that insect olfactory receptors successfully saturated with a high concentration of a compound in a continuous air-stream (primary odorous stimulation) will not respond to further stimulation (secondary odorous stimulation) with the saturating compound, but will still respond from other antennal receptors to secondary stimulation with other types of compounds.

Using this technique, we discovered important facts for the olfactory receptor systems on the male antennae of the American cockroach in a previous work⁹⁾ which was undertaken for solving a problem of the structural relationship between the mono-terpenoid sex pheromone mimics with the verbenyl skeleton and the natural sex pheromones. The following aspects have been

revealed from the study, (i) the presence of general odor receptors which are different from sex pheromone receptors; (ii) the presence of two types of sex pheromone receptors specifically responsible for **2** and **3** and (iii) strong interaction of the verbenyl monoterpene sex pheromone mimics with the periplanone-B receptor. According to the above DS-EAG results, we are inspecting the structural overlap between a monoterpene mimic [(+)-verbanyl propionate] and **3** employing Dreiding models and computer graphics. Since the structural overlap between (+)-bornyl acetate and (+)-*trans*-verbenyl acetate was elucidated,¹⁹⁾ all the monoterpene sex pheromone mimics seem to be involved in periplanone-B receptor.

Burrows *et al.*¹⁵⁾ have reported the first two [(i) and (ii)] of the above findings in their single cell recording studies in which they recognized two types of sex pheromone receptor cells, one being selectively responsible to **2** and the other to **3**, and the presence of cells for food (general) odors.

In the present EAG work, surprisingly **1** seems to interact strongly with the periplanone-A receptor rather than the periplanone-B receptor. However, in order to give a structural correlation between **1** and **2**, it may be further required to synthesize oxygen-containing germacrene-D derivatives and to evaluate their sex pheromonal activity. For syntheses of these compounds, an information is proposed by Niwa *et al.*²⁰⁾ who derived two compounds with the same carbon skeleton as that of **2** from epoxygermacrene-D, a derivative of **1**, upon treatment with basic alumina.

Concerning general odor receptors, two types of receptors which are responsible for carbonyl and alcoholic compounds are known from our EAG works.^{21,22)} In a single cell recording study,²³⁾ the presence of general odor receptors for amines, acids, esters and unsaturated (cf. **1**) compounds together with the above two types of cells are recognized.

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要 約

ワモンゴキブリの性フェロモンミミックである
germacrene-D の応差飽和触角電図

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ワモンゴキブリの性フェロモンミミックである germacrene-D (1) と, 天然の性フェロモンである periplanone-B (3) との構造が類似しているが, この2物質間の構造的相関は明確には説明されていない。本研究では, 1 に対する受容器を知るために, 1 のほかに性フェロモンで

ある periplanone-A (2) と 3 および camphor を刺激物質とした応差飽和触角電図法 (DS-EAG) を応用した。1 で飽和したときに生じる 2 および 3 による二次的な EAG 反応, 同様に 2 で飽和した場合の 1 と 3 の反応, そして 3 で飽和したときの 1, 2 による二次反応から, 1 は 2 および 3 の受容器に受容されるが, 2 の受容器に受容される可能性のほうがより大きいことがわかった。この結果は, 1 と 2 の構造的な関連性を新たに示唆するものであった。