Characterization of the nymph recognition pheromone of a cockroach, *Nauphoeta cinerea* (Olivier) (Dictyoptera: Blaberidae), that depresses wing-raising activity in adult males

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(Received 2 March 1998; Accepted 21 October 1998)

Abstract

The cuticular chemicals of the cockroach, *Nauphoeta cinerea*, that depress wing-raising activity in adult males were characterized from the cuticular wax of post-teneral-stage last-instar nymphs. Using spectral methods, the chemical structures of the depressants were determined to be a mixture of *cis*-25,26-epoxy-29-methylpentacontadiene and five *cis*-25,26-epoxyhenpentacontadienes. The mixture was considered to function as a nymph recognition pheromone by conspecific mature adult males through antennal contact.

Key words: Cockroach, *Nauphoeta cinerea*, nymph recognition pheromone, *cis*-25,26-epoxy-29-methylpentacontadiene, *cis*-25,26-epoxyhenpentacontadienes

INTRODUCTION

The cuticular wax of the body surface of many insects is generally and primarily considered to play an essential role in waterproofing and in creating a barrier against infection from microorganisms (Jackson and Blomquist, 1976; Blomquist et al., 1987). In addition to these functions, the chemicals, particularly hydrocarbons and their derivatives, are also employed for communication, transmitting information for the purpose of species, sex and nest mate recognition, and for cues serving as aphrodisiacs, anti-aphrodisiacs, aggregation pheromones and other functions (Blomquist et al., 1993). An increasing number of papers has been published in the past two decades on these semiochemicals in cockroaches (Nishida and Fukami, 1983; Fukui and Takahashi, 1983b; Schal et al., 1990), fruit flies (Antony and Jallon, 1982; Jallon, 1984; Antony et al., 1985), a rover beetle (Peschke, 1985; Peschke and Metzler, 1987), paper wasps (Singer et al., 1992) and many other insects (see Blomquist et al., 1993). In an interesting case, adult males of the cockroach, *Nauphoeta cinerea*, uses cuticular wax, octadecyl (*Z*)-9-tetracosenoate, as an intermale recognition pheromone (IMRP) to discriminate

between other adult males (Fukui and Takahashi, 1983a, b).

Adult males of *N. cinerea* discriminate adult females on a chemical basis through antennal contact. Both males and females possess a mixture of hydrocarbons on the body surface that induces wing-raising (Wr) behavior in mature males (Takahashi and Fukui, 1980). As they mature, the males secrete an intermale recognition pheromone, octadecyl (*Z*)-9-tetracosenoate, which suppresses Wr behavior and facilitates the recognition of other mature males (Fukui and Takahashi, 1983a, b). A male which recognizes another mature male through antennal contact often displays aggressive behavior (Fukui and Takahashi, 1991). However, mature males do not show aggressive behavior toward teneral males, but display Wr behavior, since they perceive the presence of hydrocarbons as a wing-raising stimulant (Takahashi and Fukui, 1980), but not IMRP, which is not secreted on the body surface of younger adult males (Fukui and Takahashi, 1983b).

Nymph recognition by mature adult males occurs in a manner similar to intermale recognition. The behavioral observations of Fukui and Takahashi (1991) confirmed that the mature males are able to discriminate post-teneral

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nymphs from teneral ones through antennal contact. The mature males demonstrated Wr behavior toward teneral nymphs but showed neither conspicuous Wr nor agonistic behavior toward post-teneral nymphs. This absence of Wr behavior of adult males upon contact with post-teneral nymphs was considered to be due to the perception of nymph-specific contact chemical(s) that are secreted on the body surface of post-teneral nymphs and that depress the Wr behavior of the males (Fukui and Takahashi, 1991). In this paper we describe the characterization of the nymph recognition pheromone.

MATERIALS AND METHODS

Insects. Insects were reared according to the methods of Fukui and Takahashi (1991). Newly moulted males and females of last instar nymphs and newly emerged adults were removed daily from a laboratory culture, separated by sex, and housed in plastic containers (21 × 27 × 10 cm) along with mouse food and water, until the extraction and behavioral tests. During housing, the insects were maintained at $26 \pm 1^\circ\text{C}$ and $60 \pm 10\%$ R.H. under a 14L–10D photoperiod.

Extraction. Groups of 10-d-old male or female nymphs (40–50 nymphs per group) were separately anesthetized by 5 to 7 min exposure to ether vapor, then immersed three times in hexane (total ca. 5 ml per insect) for 3 to 5 min. Each of the hexane extracts from a total 3,000 females and 2,000 males was dried over anhydrous Na_2SO_4 and concentrated, and then subjected to characterization of Wr depressants.

In order to confirm the specificity of the Wr depressants to post-teneral nymphs, every other individual of 3-h- and 14-d-old male or female adults, or 3-h- and 10-d-old males or females of nymphs were immersed in 10 ml of hexane for 5 min, after which the solvent was evaporated. The residue was dissolved in 0.2 ml of hexane. Five samples were prepared for each age and sex, and subjected to quantitative analysis.

Behavioral test. The antennae of 3-h- and 10-d-old nymphs were cut off at the base; and the Wr response by adult males toward the detached antennae was examined. The detached antennae of 10-d-old nymphs were then washed by mild shaking in 2 ml of solvent (hexane,

ether or MeOH) for 5 s, and examined to determine whether or not each was covered with Wr depressants.

The hexane-washed antennae were also used to evaluate the activity of the depressant as follows. Each of the antennae was passed three times through a 5 μl droplet of test solution on the tip of a micropipette, and then the solvent was evaporated. All the test solutions were prepared by decadal dilution from 1 to 100 nymph equivalents per 1 ml CCl_4 . The treated antennae were then subjected to a behavioral test. The hexane-washed antennae were used for a control.

Each of thirty 20-d-old adult males was confined individually in a glass pot (11 cm dia., 7 cm ht.) 15 min prior to its behavioral test. The base of test antenna prepared as above was attached to the tip of a glass rod and then intermittently brought into contact with the antennae of the adult male. This contact was continued for about 30 s and repeated three times at intervals of about 3 min for every test male. The number of males that raised their wings at least once during the three repetitions was recorded. This trial was replicated four or six times and the mean percentage of the Wr response was calculated.

Behavioral tests were performed in a room illuminated with a red light (20–30 lx) from 8 to 11 h after 7 a.m., when the lights were turned on during the 14L–10D photoperiod. The room was controlled at $26 \pm 1^\circ\text{C}$ and $60 \pm 10\%$ R.H.

Chromatography. Each of the hexane extracts of male and female nymphs was subjected to column chromatography on a silica gel (100 g, Wakogel C-200, Wako Pure Chemical) using hexane, 5%, 10%, 20%, 50% ether/hexane, ether and MeOH (1,000 ml each). Subsequent column chromatography on 16.7% AgNO_3 -impregnated silica gel ($\text{AgNO}_3 : \text{SiO}_2 = 1 : 5$ (w/w); 80 g Wakogel C-200) was performed using hexane, 1%, 2%, 5%, 10%, 20% and 50% ether/hexane (800 ml each), and the eluate was collected into 16 fractions (400 ml each). Final purification was carried out by high performance liquid chromatography (HPLC) with a gel permeation chromatograph (GPC) (TSKgel G1000H8 + G2000H8 + G2000H8, 7.5 mm i.d. × 60 cm each; TOSOH) and a Hitachi

L-6000 pump system equipped with a Waters R401 differential refractometer. Chloroform as the eluting solvent was flowed at a rate of 1 ml/min. The purity of all fractions was checked using thin layer silica gel plates (5 × 20 cm, 0.25 mm thick; Kieselgel 60 GF, Merck) that were impregnated with or without 16.7% of AgNO₃.

Gas chromatography (GC) was performed on a Shimadzu GC-14A instrument equipped with a flame ionization detector and a Shimadzu Chromatopack CR6-A integrator. The GC with a crosslinked methyl siloxane HP-1 fused capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) was programmed from 30°C to 320°C at 6°C/min with a 5 min initial isothermal hold. The column head pressure was set at 98 kPa. Helium was used as the carrier gas.

For quantitative analyses of wing-raising depressants from the last instar nymphs and adults, HPLC was conducted using a Nucleosil 5NO₂ column (4 mm i.d. × 25 cm, Chemco Scientific Co., Ltd.). An eluting solvent of 0.2% ether/hexane was flowed at a rate of 1 ml/min. The amounts of both wing-raising depressants were estimated by comparison of peak area to that of the purified wing-raising depressants.

Reductive ozonolysis. Ozonolysis was carried out by bubbling oxygen containing ozone into 2 mg of the purified material in 1 ml of hexane solution at 1 below -50°C for 1 min. The reaction mixture was immediately injected into the GC.

Spectrometry analysis. The mass spectrum (MS), including high resolution mode (HR-MS), was measured on a Hitachi M-80A mass spectrometer using an in-beam direct insertion technique. MS measurement of the ozonolysis product was done on a Hitachi M-80B mass spectrometer with an OV-1 glass column (4 mm i.d. × 2 m) programmed from 100°C (2 min holding) to 320°C at a rate of 10°C/min. Both spectrometers were operated in the EI mode at 70 eV. The infrared (IR) spectrum was obtained on a Nicolet 7000 FT-IR spectrometer using a film method. Proton nuclear magnetic resonance (¹H-NMR) and ¹³C-NMR spectra were measured on a JEOL JNM-FX 90Q spectrometer at 90 MHz and 22.50 MHz, respectively. INEPT/R analysis was performed to confirm the multiplicity of ¹³C signals determined from the ¹³C-NMR spectra (off-resonance decoupling). All spectra were recorded in CDCl₃.

RESULTS

Wing-raising response of adult males toward nymph antennae and solvent-washed antennae of different age-groups

Figure 1A shows the percentages of Wr response by mature adult males toward the detached intact antennae of 3-h- and 10-d-old nymphs. Intact antennae of 3-h-old female and male nymphs elicited Wr behavior from 76.7% and 83.3% of males, respectively. On the other hand, 1.11% and 1.65% of males showed Wr

Fig. 1. Wing-raising activity of 20-d-old adult males induced by the intact antennae of 3-h- and 10-d-old nymphs (A), and by the intact and solvent-washed (hexane, ether, or MeOH) antennae of 10-d-old nymphs (B). Each test was replicated six times. Means ($\pm$ SE) indicated with the same letters are not significantly different ($p < 0.05$; Kruskal-Wallis test followed by Dunn's test) (see text for details).

behavior toward the antennae of 10-d-old female and male nymphs, respectively.

Adult males elicited Wr behavior of 58%, 52% and 20% toward 10-d-old female nymph antennae washed with hexane, ether and MeOH; and 50%, 54% and 22% toward male nymph antennae (Fig. 1B).

Purification of wing-raising depressant

Hexane extract of 10-d-old male nymphs (2.2 g) was fractionated on a silica gel column. Elution of 5% ether/hexane depressed Wr activity in test males (Table 1). The elution was applied on an AgNO₃-impregnated silica gel column. The sixth fraction, that is, the second eluate of the 5% ether/hexane, was active and formed a single spot on an AgNO₃-impregnated silica gel TLC (*R_f*=0.33, developing solvent: 50% benzene/hexane). Further purification was performed using an HPLC equipped with GPC columns. Forty-three-point-eight milligrams of active colorless material was eluted at 31.2 min.

Hexane extract of 10-d-old female nymphs (1.4 g) was also separated in the manner described above. Depressant activity from the hexane extract of female nymphs appeared in the same fraction as that from male nymphs; a final yield of 30.1 mg of colorless material was purified.

On the other hand, the hexane eluates of the extracts of both male and female nymphs elicited Wr behavior from adult males (Tables 1, 2).

Characterization

Purified materials from 10-d-old nymphs of both sexes were analyzed spectrometrically. Since the spectrometric data for the two sexes were coincident, all the data described below are from male nymphs: MS *m/z* (% relative intensity): 726 (M⁺, 24), 708 (M⁺−18, 41), 391 (32), 377 (35), 83 (100) (Fig. 2); ¹H-NMR: δ 5.35 (4H, br t), 2.90 (2H, m), 1.98 (8H, m), 1.50 (4H, m), 1.38–1.22 (74H, m), 0.88 (6H, t); ¹³C-NMR (INEPT/R): δ 129.96 (d), 57.26 (d), 32.0 (t),

Table 1. Wing-raising (Wr) response (%) of 20-d-old adult males toward the hexane extract of 10-d-old nymphs and the silica-gel-column eluates of this extract

Test sample ^a	1 NE ^b	10 NE	100 NE	Judgement
Hexane extract	30.0*/38.3* ^c	22.5*/8.0*	10.8*/22.5*	Wr suppressing
Hexane eluate	98.3*/88.3*	100*/100	100*/100*	Wr stimulating
5% ether/hexane eluate	33.3*/28.3*	1.7*/0*	0*/0.8*	Wr suppressing
≥10% ether/hexane eluate ^d	55.8/54.0	55.0/50.8	48.3/32*	inactive
Control ^e	55.8/53.3	56.7/52.5	52.5/54.2	

^aEach test sample was prepared in the purification process of wing-raising suppressant. ^bNymph equivalent. ^cPercent mean of Wr response toward female extract or eluate/percent mean of Wr response toward male extract or eluate. ^dA mixture of 10% ether/hexane to methanol eluate was used as the test sample. ^eHexane-washed antennae of 10-d-old female or male nymphs. For each test, 30 males were used and four replications were performed. * denotes significant difference against control (*p*<0.05; Mann-Whitney *U*-test).

Table 2. Wing-raising response (%) of 20-d-old adult males toward the hexane extract of 3-h-old nymphs and the silica-gel-column eluates of this extract

Test sample ^a	1 NE	10 NE	100 NE	Judgement
Hexane crude extract	89.2*/80.8*	90.8*/99.2*	99.2*/100*	Wr stimulating
Hexane eluate	90.8*/85.8*	97.5*/100*	98.3*/100*	Wr stimulating
5% ether/hexane eluate	51.7/57.5	52.0/60.8	56.0/53.3	inactive
≥10% ether/hexane eluate	55.8/50.8	51.7/59.2	52.5/52.5	inactive
Control	52.5/54.2	53.3/55.8	55.0/53.3	

^aEach test sample was prepared on a silica gel column using the hexane extract and corresponding eluates of 200, 3-h-old nymphs (See the legend of Table 1 for details).

29.7 (t), 29.6 (t), 29.4 (t), 27.9 (t), 27.3 (t), 26.7 (t), 22.7 (t), 14.1 (q); IR: 3009, 2956, 2918, 2850, 1464, 845, 720 cm^{-1} .

HR-MS gave a molecular formula of $\text{C}_{51}\text{H}_{98}\text{O}$ [obs. 726.7614, calc. 726.7622], indicating three unsaturations. Water elimination from the molecular ion [m/z 708 ($\text{M}^+ - 18$); HR-MS $\text{C}_{51}\text{H}_{96}$: obs. 708.7485, calc. 708.7517] indicated that the material had an oxygen-containing moiety, such as alcohol, aldehyde or cyclic ether (Clerc et al., 1981) (Fig. 2). The IR spectrum revealed no absorption corresponding to an alcohol or aldehyde group, and thus the oxygen-containing moiety was determined to be restricted to a cyclic ether. A common 42 mass unit resulting from the summation of fragments at MS m/z 391 [HR-MS $\text{C}_{27}\text{H}_{51}\text{O}$: obs. 391.3959, calc. 391.3942] and 377 [$\text{C}_{26}\text{H}_{49}\text{O}$: obs. 377.3792, calc. 377.3786], generated by alpha-cleavage at the cyclic ether, indicated that the purified material had an epoxy group. Fragmentation further positioned the epoxy at C-25, 26.

The epoxy group was also confirmed by NMR spectra. A chemical shift at δ 57.96 in the ^{13}C -NMR spectrum was assigned to epoxide carbon atoms, since chemical shifts for epoxide carbon atoms have been reported in the range of δ 40–74 by Stephen and Whitham (1975). As for the geometric isomerism of epoxide, Iwaki et al. (1974) observed signals at δ 2.92 and δ 2.65 for the methine proton of *cis*- and *trans*-7,8-epoxy-2-methyloctadecanes, respectively. Thus, the chemical shift of the methylene protons at δ 2.90 in the ^1H -NMR spectrum for the purified

material, indicating the geometry of the epoxide group, is *cis*. The remaining two unsaturations were assigned to two olefinic double bonds, since integration of the proton spectrum at δ 5.35 (br t) (^1H -NMR) on the olefinic double bond carbons was shown to consist of four protons. The IR spectrum of the purified material showed no absorption at around 970 cm^{-1} , indicating that the geometry of both consisted of a *cis*-configuration. Taken together, the above data conclusively indicate that the purified material is *cis*-25,26-epoxyhenpentacontadiene.

The purified material was subjected to a reductive ozonolysis reaction to determine the position of olefin. By the reaction and subsequent GC and GC-MS analyses, six aldehydes and five monoepoxydials were detected, indicating that the purified material consisted of several compounds.

The aldehydes identified by GC analysis were hexanal (t_R : 9.0 min), heptanal (12.9 min), octanal (16.4 min), nonanal (19.5 min), decanal (22.5 min), and undecanal (25.4 min) based on a comparison with the authentic compounds. The relative ratio of the concentrations of a series of aldehydes as calculated from gas chromatogram peaks was determined to be 12 : 15 : 55 : 15 : 33 : 70. On the other hand, the monoepoxydials identified by GC-MS analysis consisted of a monomethyl branched monoepoxydial and four non-branched monoepoxydials (Table 3). The former was 14,15-epoxy-18-methyl-1,31-hentriacontanediol [1]. The latter monoepoxydials were 14,15-epoxy-1,30-triacontanediol [2],

Fig. 2. Mass spectrum of the purified wing-raising suppressant in the last instar male nymph.

14,15-epoxy-1,32-dotriacontanedial [3], 14,15-epoxy-1,34-tetratriacontanedial [4] and 16,17-epoxy-1,34-tetratriacontanedial [5]. The relative ratio of the concentrations of a series of monoepoxydials calculated from the total and selected ion chromatograms was determined to be 19 : 18 : 21 : 12 : 30.

Every epoxydial was made up of an even number of carbons, indicating that each was produced with two aldehydes in ozonolysis which have an odd and an even number of carbons, based on the odd number of carbons in the purified material. Thus four non-branched monoepoxydials ([1], [2], [3] and [4]) were produced together with undecanal pairing with octanal, decanal, octanal and hexanal, respectively (ratio: 19 : 18 : 21 : 12); a monomethyl branched monoepoxydial ([5]) with heptanal and nonanal (ratio: 15 : 15) pairing with decanal and octanal (ratio: 15 : 15) respectively (Tables 3 and 4).

These results indicated that the purified material consisted of the following six compounds (Table 4): (Z,Z)-11,42-*cis*-25,26-epoxy-

29-methylpentacontadiene [6], and (Z,Z)-7,41-, (Z,Z)-9,43-, (Z,Z)-11,41-, (Z,Z)-11,43- and (Z,Z)-11,45-*cis*-25,26-epoxyhenpentacontadienes [7], [8], [9], [10] and [11]. The relative ratio of the concentrations of the six compounds, each of which consisted of purified material, was determined to be 19 : 15 : 15 : 18 : 21 : 12 (Table 4).

Activity of purified wing-raising depressant and its synthesized candidates

Forty percent, 2.7%, and 0% of adult males (mean of 6 replications) showed Wr response toward 1, 10, and 100 nymph equivalent (NE) materials that were purified from female nymphs, respectively, and 38.3%, 2.2%, and 0% of adult males showed Wr response toward the corresponding purified materials from the male nymphs, respectively. On the other hand, for the same NE, 52%, 54% and 38% of the adult males, respectively (mean of 4 replications) showed Wr response toward (9Z,25S,26R,43Z)-25,26-epoxy-9,43-henpentacontadiene, which was synthesized by Mori and Argade (1994), over the range from 1 to 100 NE; and

Table 3. Monoepoxydials identified with GC-MS from the reductive ozonolysis product of purified wing-raising suppressant, prominent ions of their mass spectra, and relative ratios of their amounts

Monoepoxydial	Prominent ions	Relative ratio
14,15-epoxy-18-methyl-1,31-hentriacontanedial [1] ^a	492 (M ⁺), 474 (M ⁺ - 18), <u>295</u> , <u>239</u> , <u>225</u>	19
14,15-epoxy-1,30-triacontanedial [2]	464 (M ⁺), 446 (M ⁺ - 18), <u>267</u> , <u>239</u>	18
14,15-epoxy-1,32-dotriacontanedial [3]	492 (M ⁺), 474 (M ⁺ - 18), <u>295</u> , <u>239</u>	21
14,15-epoxy-1,34-tetratriacontanedial [4]	520 (M ⁺), 502 (M ⁺ - 18), <u>323</u> , <u>239</u>	12
16,17-epoxy-1,34-tetratriacontanedial [5]	520 (M ⁺), 502 (M ⁺ - 18), <u>295</u> , <u>267</u>	30

^a Reference number of each monoepoxydial. Single underlined figures show cleavages at the position of epoxide. Double underlined figure shows cleavage at the position of branched monomethyl.

Table 4. Characterized compounds from wing-raising suppressant, relative ratios of their amounts, and combinations of ozonolysis products

Characterized compound	Relative ratio	Ozonolysis products
(Z,Z)-11,42- <i>cis</i> -25,26-epoxy-29-methylpentacontadiene [6] ^a	19	1^b + octanal ^c + undecanal
(Z,Z)-7,41- <i>cis</i> -25,26-epoxyhenpentacontadiene [7]	15	5 + heptanal + decanal
(Z,Z)-9,43- <i>cis</i> -25,26-epoxyhenpentacontadiene [8]	15	5 + octanal + nonanal
(Z,Z)-11,41- <i>cis</i> -25,26-epoxyhenpentacontadiene [9]	18	2 + decanal + undecanal
(Z,Z)-11,43- <i>cis</i> -25,26-epoxyhenpentacontadiene [10]	21	3 + octanal + undecanal
(Z,Z)-11,45- <i>cis</i> -25,26-epoxyhenpentacontadiene [11]	12	4 + hexanal + undecanal

^a Reference number of characterized compound. ^b Bold figures indicate monoepoxydial in Table 3. ^c The ratio of six aldehydes is as follows: hexanal : heptanal : octanal : nonanal : decanal : undecanal = 12 : 15 : (19 + 15 + 21) : 15 : (15 + 18) : (19 + 18 + 21 + 12) = 12 : 15 : 55 : 15 : 33 : 70.

50%, 54% and 38% of the adult males, respectively (mean of 4 replications) showed Wr response toward the antipode.

HPLC profiles of cuticular chemicals

HPLC analyses of cuticular waxes on a small scale revealed that Wr depressant on the body surface of 10-d-old male and female nymphs showed the same retention time (19.6 min) on a 5NO₂ column. Each of these 10-d-old males and females had approximately 15 μ g of depressant on its body surface. On the other hand, less than one twenty-fifth of this amount was detected on the body surfaces. A small amount of Wr depressant was found in the cuticular waxes of 3-h- and 14-d-old adult females. Intermale recognition pheromone (IMRP) on the body surface of 14-d-old adult males was detected at a different retention time (16.5 min) from that of Wr depressant, and the amount of IMRP per insect was estimated as ca. 5 μ g.

The amount of Wr depressants was estimated as follows: for last instar male nymphs, $15.3 \pm 0.182 \mu$ g (mean $\pm$ SE, $n=5$) (10-d-old) and $0.57 \pm 0.016 \mu$ g (3-h-old); for adult males, 0 μ g (14-d-old) and $0.143 \pm 0.015 \mu$ g (3-h-old); for last instar female nymphs, $15.02 \pm 0.132 \mu$ g (10-d-old) and $0.475 \pm 0.019 \mu$ g (3-h-old); for adult females, $0.295 \pm 0.005 \mu$ g (14-d-old) and $0.333 \pm 0.009 \mu$ g (3-h-old). On the other hand, the amount of IMRP per 14-d-old adult male was estimated as $5.2 \pm 0.122 \mu$ g.

DISCUSSION

The detached intact antennae of post-teneral 10-d-old nymphs of both sexes in *N. cinerea* scarcely elicited Wr behavior from adult males, though most of test males showed Wr behavior to the antennae of 3-h-old male and female nymphs (Fig. 1A). However, half of the test males showed Wr behavior toward the 10-d-old male and female antennae washed with hexane and ether, whereas the antennae washed with a polar solvent, i.e. MeOH, elicited Wr behavior toward only a fifth of the test males (Fig. 1B). These results suggested that the antenna of 10-d-old nymphs induces Wr behavior by itself, but is covered with a Wr depressant which is soluble in hexane and ether and partially soluble in MeOH (Fig. 1). Since the presence of the

depressant has been indicated on the surface of other appendages, such as legs and wings in *N. cinerea* (Fukui and Takahashi, 1991, personal observation), the whole body of the 10-d-old nymphs was subjected to the extraction. The Wr depressant activity was found in the 5% ether/hexane fraction of the whole body extract of 10-d-old nymphs (Table 1). On the other hand, Wr stimulant was found in the hexane fraction of the whole body extracts of 3-h- and 10-d-old nymphs (Tables 1, 2).

The Wr depressants purified from the cuticular extracts of 10-d-old males and females were estimated at ca. 15 μ g per insect, and the composition of the depressants characterized from both sexes was the same. The composition of the depressants consisted of (Z,Z)-11,42-*cis*-25,26-epoxy-29-methylpentacontadiene, and (Z,Z)-7,41-, (Z,Z)-9,43-, (Z,Z)-11,41-, (Z,Z)-11,43- and (Z,Z)-11,45-*cis*-25,26-epoxyhenpentacontadienes (Table 4). However, the Wr depressant activity of two synthetic stereoisomers of (Z,Z)-9,43-*cis*-25,26-epoxyhenpentacontadiene (Mori and Argade, 1994) was apparently lower than the purified materials from the extracts of both sexes of 10-d-old nymphs. This indicates that multiple compounds might depress the Wr activity. Further synthetic study of Wr depressant candidates excluding the two synthetic stereoisomers will clarify which compounds contained in the candidates are responsible for the depressant activity.

Adult males can recognize other adult males through the perception of Wr depressant, which functions as an intermale recognition pheromone, on their body surface. Upon recognition, males often behave aggressively toward other males (Fukui and Takahashi, 1983a, 1991). However, adult males exhibit no response upon perceiving Wr depressant or nymph recognition pheromone secreted on the body surface of post-teneral nymphs (Table 1). This difference between adult male behavior toward other males and adult male behavior toward post-teneral nymphs is believed to result from mutual depressant discrimination between adult males and nymphs. The Wr depressant that was purified and characterized in post-teneral 10-d-old nymphs might be used as a nymph recognition pheromone by mature adult males. Thus we

have assigned the name, "nymph recognition pheromone," to the nymph-specific Wr depressant. In the case of encountering teneral nymphs, however, mature males display Wr behavior through the perception of hydrocarbon(s), since Wr depressant is scarcely secreted on the body surface of teneral 3-h-old nymphs (Table 2). However, since 1-d-old male and female nymphs did not elicit Wr behavior from the mature males (Fukui and Takahashi, 1991), Wr depressant is apparently secreted onto the body surface of both nymphs and where it suppresses the Wr display of mature males.

Why did the adult mature males of *N. cinerea* recognize nymphs? In cockroaches, the generations generally overlap and nymphs and adults contact one another through the formation of aggregations. The nymphs of *N. cinerea* live together in an aggregation, whereas the adult males court adult females and behave aggressively toward other males (Farine et al., 1981). Under such circumstances *N. cinerea* males may fight each other in order to establish territories and social dominance, and agonistic encounters between the males are very intense (Ewing, 1972, 1973). To establish territories and social dominance, the males must first recognize other adult males; in fact, they recognize other males through an intermale recognition pheromone, octadecyl (Z)-9-tetracosenoate (Fukui and Takahashi, 1983a). Also the males must recognize nymphs and females because nymphs and females are not focal insects in establishing territory and social dominance. Thus, the ability of adult males to recognize nymphs and females through some labels (cues) should facilitate establishment of territory and social dominance without mistaking nymphs or females for adult males in an aggregation that is formed by overlapping generations. Consequently, such social interaction between adult males may lead them to recognize nymphs through a nymph-specific label, i.e. nymph recognition pheromone, whereas they recognize adult females through a female recognition pheromone (Fukui and Takahashi, 1983a, b). In order to confirm the above speculation, further observations of social interactions among adult males and females, and nymphs are needed. Although sex

discrimination by the male of an Australian field cricket, *Teleogryllus commodus*, is also known to be accomplished through contact pheromones that have not yet been isolated (Rence and Loher, 1977), whether males are able to recognize nymphs is not known. However, the present study has indicated that the adult males of *N. cinerea* recognize nymphs through a nymph recognition pheromone characterized from their cuticular wax. To the best of our knowledge, this is the first such record.

ACKNOWLEDGEMENTS

We would like to express our grateful thanks to Dr. H. Naoki of the Suntory Institute of Bioorganic Research for the measurements of GC-MS (M-80B) and IR spectra, and to Prof. K. Mori, the University of Tokyo, for his gift of the synthetic compounds.

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