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Identification of pheromone-like compounds in male reproductive organs of the oriental locust *Locusta migratoria*

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ABSTRACT

Despite the great economical interest of locusts in agriculture, knowledge on their chemoreception systems is still poor. Phenylacetonitrile is recognised as a pheromone of the desert locust *Schistocerca gregaria*, triggering gregarization, promoting aggregation and inhibiting courtship. However, in the other major locust species, *Locusta migratoria*, pheromones have not been reported. We have identified the two isomers of naphthylpropionitrile from the male reproductive organs of *L. migratoria*. Chemical synthesis has confirmed the identity of the two compounds. Both isomers show significant affinity to CSP91, a protein reported in the testis, but not to three other proteins of the same family (CSP180, CSP540 and CSP884) expressed in female accessory glands. The striking similarity of these compounds with phenylacetonitrile and the unusual nature of such chemicals strongly suggest that naphthylpropionitrile could be pheromones for *L. migratoria*, while their site of expression and binding activity indicate a role in communication between sexes.

1. Introduction

Locusts are responsible for enormous losses in agriculture all over the world. The desert locust *Schistocerca gregaria* is mainly active in Africa, the oriental locust, *Locusta migratoria*, in Asia. Despite their exceptional economical interest, very little is known on their chemoreception system, both regarding semiochemicals and the proteins involved in their recognition. However, in other insect species pheromones and other semiochemicals are efficiently used to control their populations. Locusts are particularly interesting in this respect as they use pheromones to undergo a physiological shift from the "solitary" to the "gregarious" phase, accompanied by some major morphological modification changes, including colour. It is only in the gregarious phase that locust develop the capacity of aggregating into swarms of millions of individuals that cause enormous damage to crops.

Most of the information on locust pheromones is available for the desert locust *S. gregaria* [1–3], where phenylacetonitrile has been identified as the chemical involved in the transition to the gregarious phase and aggregation of "gregarious" locusts [4]. The same compound can also acts as a courtship-inhibiting pheromone and as a self-marking volatile in order to avoid homosexual attacks [5–7]. Cuticular hydrocarbons have been also identified and suggested to play some role in phase transition [8].

In the oriental locust no clear information on pheromones or semiochemicals has been reported so far, apart from some electrophysiologically active phenols and derivatives, identified in the faeces [9]. Although their ligands are still unknown, two binding proteins seem to be involved in the phase transition mechanism [10]: a chemosensory protein, previously reported as CSP-I [11] and another binding protein called "take-out". At the central level, serotonine and dopamine have been reported as the neurotransmitters mediating the phase transition in *S. gregaria* and *L. migratoria*, respectively [12,13].

In a previous paper, we reported on the expression of 17 chemosensory proteins (CSPs) in female reproductive organs of *L. migratoria*, only one of which was also present in male organs, specifically in testis [14]. Such large number of these carrier proteins [15] suggest the presence of semiochemicals in the reproductive organs of locusts. In fact, often pheromones are bound at their production sites to soluble proteins, such as OBPs (odorant-binding

Abbreviations: OBP, odorant-binding protein; CSP, chemosensory protein; 1-NPN, N-phenyl-1-naphthylamine; GC/MS, gas-chromatography/mass spectrometry.

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protein) and CSPs, that help solubilise these hydrophobic compounds and assist their release in the environment. In vertebrates, the best known examples of carrier proteins with such functions are the urinary proteins of mice [16,17] and the salivary proteins of pigs [18,19], proteins that also occur in the olfactory area, where they are involved in detecting the same chemicals. In insects, the pheromone glands of *Lepidoptera* and the mandibular glands of the honeybee contain proteins of the OBP [20] and CSP families [21,22], that are also found in chemosensory organs [11,15,23,24].

Reproductive structures also express proteins of the above families, whose presence in some cases has been put in relationship with semiochemicals [25–27]. The male seminal fluid of some insects, including *Orthoptera*, contains chemicals able to produce physiological or behavioural effects on conspecifics [28,29]. Sex pheromones and other semiochemicals are also produced by males in their seminal fluid and could be transferred to females during copulation [27,30].

Here we report on the identification and synthesis of two pheromone-like compounds in the reproductive organs of *L. migratoria*, 3-(1-naphthyl)propionitrile and 3-(2-naphthyl)propionitrile, and on their affinity to chemosensory proteins expressed in the same organs [14].

2. Materials and methods

2.1. Materials

Locusts (*L. migratoria*) were raised in the Department of Grassland, (China Agricultural University, Beijing, China) at 28–30 °C, relative humidity 60%, and photoperiod of 18 h:6 h (light:dark). Chemicals, unless otherwise stated, were from Sigma–Aldrich, reagent grade.

2.2. Synthesis of naphthylpropionitriles (**1**) and (**2**)

^{1}H NMR (250 MHz) and ^{13}C NMR (62 MHz) were performed on a Brucker Avanche-II in deuterochloroform solutions.

1-Bromomethylnaphthalene (**4**). A mixture of 1-methylnaphthalene (**3**) (Aldrich, 1.4 g, 10 mmol), N-bromosuccinimide (NBS, 1.8 g, 11 mmol) in carbon tetrachloride (20 ml) was heated under reflux in a Pyrex flask while exposed to a sun lamp (Osram UV 300 W) for 30 min. The resulting mixture was partitioned between hexane and diluted thiosulphate; the organic phase was dried over magnesium sulphate, filtered through a short column of silica gel, and evaporated to afford crude (**4**) (1.8 g, 81% yield) as an oil, which was used in the next step without further purification. ^{1}H NMR: delta 4.98 (s, 2H), 7.52 (m, 1H), 7.64 (m, 1H), 7.87 (m, 1H), 8.17 (m, 1H). ^{13}C NMR: 31.99, 123.9, 125.6, 126.4, 127.8, 127.9, 129.0, 130.0, 131.3, 133.4, 134.2.

2-Bromomethylnaphthalene (**6**) was obtained in 85% from 2-methylnaphthalene (**5**) following the same procedure adopted for compound **3**. ^{1}H NMR: 4.68 (s, 2H), 7.45–7.55 (m, 3H), 7.75–7.90 (m, 4H). ^{13}C NMR: 34.2, 126.7, 126.8, 126.9, 127.1, 128.1, 129.4, 135.2, 139.1.

3-(1-Naphthyl)propionitrile (**1**). To a solution of butyllithium (10 mL of a 1.6 M solution in hexane, 16 mmol) in anhydrous tetrahydrofuran (25 mL) stirred under argon in a dry ice/acetone bath acetonitrile (3.2 mL, 60 mmol) was added dropwise. After 20 min, bromide (**4**) (1 g, 5 mmol) dissolved in hexane (5 mL) was added; after 30 min stirring and −78 °C, the reaction was allowed to equilibrate with the room temperature, quenched with saturated ammonium chloride solution and partitioned between water and ethyl acetate. The organic phase was washed with brine, dried over magnesium sulphate, and evaporated to leave a residue from which (**1**) (0.38 g, 42% yield) was obtained by flash-chromatography with hexane–ethyl acetate 4:1. ^{1}H NMR: 2.71 (t, J = 7.5 Hz, 2H), 3.38 (t, J = 7.5 Hz, 2H), 7.40–7, 60 (m, 4H), 7.80 (d, J = 7 Hz, 1H), 7.90 (m, 2H). ^{13}C NMR: 18.5, 28.8, 119.5, 122.8, 125.7, 126.1, 128.1, 129.3, 131.2, 134.1.

3-(2-Naphthyl)propionitrile (**2**) was prepared as described above using bromide (**6**) (60% yield). ^{1}H NMR: 2.71 (t, J = 7.5 Hz, 2H), 3.12 (t, J = 7.5 Hz, 2H), 7.34 (d, J = 7 Hz), 7.47 (m. 2H), 7.51 (s, 1H), 7.75 (m, 3H). ^{13}C NMR: 19.5, 31.9, 119.4, 126.1, 126.5, 126.6, 127.1, 127.8, 127.9, 128.8, 132.7, 133.6, 135.6.

2.3. Analysis of volatiles

Samples of reproductive organs and other parts of the body were dissected and stored at −20 °C until use. Extraction was performed by homogenisation in dichloromethane, followed by centrifugation. The organic phase was concentrated to small volume and 1 μL was used for analysis. The GC–MS system was constituted by a Focus Thermo-Finnigan gas-chromatograph equipped with a Varian CP-WAX-52 capillary column (60 m × 0.32 mm; coating thickness 0.5 μm) and a DSQ Thermo-Finnigan quadrupole mass detector. Analytical conditions were: injector and transfer line at 250 °C; oven temperature was programmed from 50 °C (10 min) to 240 °C at 10 °C/min; carrier gas helium at 1 mL/min; splitless injection. Identification of the constituents was based on comparison of the retention times and mass spectra with those of authentic samples, analysed in the same conditions.

2.4. Protein expression and purification

For the bacterial expression of CSP180, CSP540 and CSP884, constructs were prepared in pET5b containing the genes encoding mature proteins with the addition of an initial methionine. The following oligonucleotides were used to amplify the relative genes:

CSP180-Nde: AACATATGTACACCACCAAGTACGA;
CSP180-Eco: GTGAATTCTTACGCGGAGACCCTCTTGAG;
CSP884-Nde: AACATATGTACACCACCAAGTACGA;
CSP884-Eco: GTGAATTCTTACTCGAAGGGTGATCCAGA;
CSP540-Nde: AACATATGCAGGACAAGTACACCAC;
CSP540-Eco: GTGAATTCTTACGACTTGAGCTTCTTGAT.

Expression and purification of recombinant proteins was accomplished along with standard protocols, previously described for similar proteins [14].

2.5. Fluorescence measurements

Emission fluorescence spectra were recorded on a Jasco FP-750 instrument at 25 °C in a right angle configuration, with a 1 cm light path quartz cuvette and 5 nm slits for both excitation and emission. The protein was dissolved in 50 mM Tris–HCl buffer, pH 7.4, while ligands were added as 1 mM methanol solutions.

2.6. Fluorescence binding assays

The affinity of fluorescent ligand N-phenyl-1-naphthylamine (1-NPN) to recombinant proteins was measured by titration of a 2 μM solution of the protein in 50 mM Tris–HCl, pH 7.4, with aliquots of 1 mM ligand in methanol to final concentrations of 2–16 μM. The probe was excited at 337 nm and emission spectra were recorded between 380 and 450 nm. Binding constant was calculated using Prism software. The affinity of other ligands was measured in competitive binding assays, using 1-NPN as the fluorescent reporter at 2 μM concentration in a 2 μM solution of the protein in Tris buffer, and each competitor at 2–16 μM. Dissociation constants of the competitors were calculated from the corresponding IC_{50} values (the concentration of each ligand halving the initial value of fluorescence), using the equation: $K_D = [IC_{50}]/$