Oviposition Responses of *Culex pipiens* to a Synthetic Racemic *Culex quinquefasciatus* Oviposition Aggregation Pheromone

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The oviposition pheromone of *Culex quinquefasciatus* was synthesized in a racemic form in a simple (five steps), efficient, high yielding (45% total yield), and low cost way (use of relatively low cost reagents). Our synthetic racemic pheromone (SRP) was tested in the laboratory for its bioactivity on *Culex pipiens* biotype *molestus*, which is a member of the species complex that *Culex quinquefasciatus* belongs. In the testing conditions, bioactivity at the doses of 0.01, 0.1, 1, and 10 μg per cage was found with the best bioactivity achieved at 1 μg per cage. The effectiveness of our SRP offers a capable tool for improving mosquito oviposition traps for surveillance or even control programs.

**KEYWORDS:** Mosquito; oviposition; pheromone; *Culex pipiens* biotype *molestus*; racemic synthesis; 6-acetoxy-5-hexadecanolid

**INTRODUCTION**

*Culex pipiens* represents a mosquito species complex whose taxonomical status is still a matter of argument. According to the recent generic and subgeneric changes, the formerly known two separated species or subspecies *Cx. pipiens pipiens* and *Cx. pipiens molestus* are now considered as a single species with two different biotypes (1). The most common and widespread members of the complex are as follows: *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus*, and *Culex quinquefasciatus*. The aforementioned two biotypes possess significant ecological and physiological differences. The *molestus* biotype prefers hypogeous habitats (underground urban environments, such as cellars, sanitary spaces under buildings, and septic tanks), it is autogenous (does not require a blood meal to produce its first batch of eggs), it is homodynamous (does not hibernate), and it is mammophilous (feeding from mammals). In contrast, the *pipiens* biotype prefers epigeous habitats (breeds in rural, open air collection of water), it is anautogenous (requires blood meal to produce its first batch of eggs), it is eurygamous (capable of mating in cages in the laboratory and other confined spaces), it is heterodynamous (able to hibernate during the winter), and it is primarily ornithophilous (tendency to feed on birds).

*Culex quinquefasciatus* has many similarities with the *molestus* biotype (homodynamous, stenogamous, and anautogenous), but it is usually found in tropical areas whereas *Cx. pipiens* is mainly found in temperate or even colder climates (2).

The *Cx. pipiens* complex has a worldwide distribution, and members of the complex play a significant role in the transmission of arboviruses and other vector-borne diseases (3). Thus, any minimal change in local climates could force the population as well as the pathogens that they transmit to move to new areas (4, 5). One very important flavivirus disease is the West Nile (WN) virus, which is transmitted in natural cycles between birds and mosquitoes but also can infect humans (6). WN occurs in Middle East, Africa, India, the United States, and also in Europe (1). *Cx. quinquefasciatus* as well as *Cx. pipiens* biotype *pipiens* are the most likely WN vectors, but Lundström (3) suggested that biotype *molestus* should also be collected and processed for isolation of WN virus in order to evaluate the occurrence of the virus in an area.

Female *Culex* mosquitoes deposit their eggs in the form of egg rafts on the water surface. Some of these species form a droplet at the apex of each egg in the egg raft, which affects the oviposition behavior of intraspecific gravid females (7–9). The main volatile compound present in the apical droplets of the *Cx. quinquefasciatus* egg rafts is (-)-(5R,6S)-6-acetoxy-5-hexadecanolid, which acts as an attractant to other gravid females in order to oviposit the pheromone release nearby (10, 11). The interspecific activity of the previously mentioned pheromone was demonstrated by Bruno and Laurence in 1979.
(7) when they reported that a Cx. quinquefasciatus egg raft acted as an attractant to gravid Cx. pipiens biotype pipiens females.

The isolation and characterization of the \((-\)-(5R,6S)-6-\)acetoxy-5-hexadecanole from Cx. quinquefasciatus egg rafts provided the impetus for several attempts toward its syntheses. Sugars or amino acids (12), chiral auxiliaries (13), as well as Sharpless asymmetric dihydroxylation (14) and epoxidation reactions (15) have been used for the introduction of the asymmetry (16) while Wittig coupling, Knoevenagel condensation, and Julia olefination have been applied for the construction of the carbon skeleton. However, most of these syntheses suffer from various drawbacks as they require either uncommon reagents and/or usually expensive intermediates or entail many steps giving low overall yields.

Hwang et al. in 1987 (17) reported that the only enantiomer bioactive to Cx. quinquefasciatus was the \((-\)-(5R,6S) isomer whereas the other three were inactive but not repellent. Sakakibara et al. in 1984 (18) stated that \(\alpha\)-ene steroids \(\beta\)-methoxy-3,4-tetrahydro-4-aza-methylphenyl ether was the \((5\alpha,6\alpha)\) (whereas the other three were inactive but not repellant. The synthetic racemic oviposition stimulant C. pipiens provided the impetus for several attempts toward its syntheses. Biotype and reported that they all showed a significant bioactivity, but Clements in 1999 (19) stated that these results could not be taken as proven, since there was not a clear specification of their synthetic procedure.

The unclear evidence for the bioactivity possessed by all four isomers in combination with the need to simplify the synthetic procedure and reduce the cost led us to develop the racemic pheromone in a simple, efficient, high yielding, and less expensive way. Toward that, a synthetic racemic oviposition pheromone (SRP) of Cx. quinquefasciatus was synthesized in a five step procedure and its bioactivity was evaluated against Cx. pipiens biotype molestus in laboratory bioassays.

**MATERIALS AND METHODS**

**General.** All chemical reactions were carried out under anhydrous conditions and argon atmosphere using dry, freshly distilled solvents, unless otherwise noted. Tetrahydrofuran (THF) was distilled from sodium/benzophenone, dichlormethane (CH2Cl2) was distilled from CaH2, and pyridine was first dried over solid sodium hydroxide and distilled through an efficient fractionating column. Yields refer to chromatographically and spectroscopically (\(1\)H NMR) homogeneous products, unless otherwise stated. All reagents were purchased at the highest commercial quality and used without further purification, unless otherwise mentioned. All reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm Merck silica gel plates (60 F254) by using UV light as the visualizing agent and ethanolic \(\cdot\)lactol as a colorless oil (3.6 g, 88%). The volatile lactol was used in the following reactions without phase separation, the aqueous phase was washed twice with EtOAc, filtered off through Celite, and the product was purified by flash column chromatography. NMR spectra were recorded on a Varian Unity Inova 600 MHz spectrometer. Solution state (\(\mathrm{CDCl}_3\)) spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer (Kifissia, Greece) for more than 2 decades was used. Adults were kept in wooden framed cages (33 cm \(\times\) 33 cm \(\times\) 33 cm) with 32 \(\times\) 32 cm. Mosquito Rearing.

A Cx. pipiens biotype molestus colony was maintained in the laboratory of Benaki Phytopathological Institute (Kifissia, Greece) for more than 2 decades was used. Adults were kept in wooden framed cages (33 cm \(\times\) 33 cm \(\times\) 33 cm) with 32 \(\times\) 32 cm.
mesh at 20 ± 2 °C, 80 ± 2% relative humidity, and a photoperiod of 14:10 (L:D) h. Cotton wicks saturated with 10% sucrose solution were provided to the mosquitoes as a food source. Females laid eggs in round, plastic containers (10 cm diameter × 5 cm depth) filled with 150 mL of tap water. Egg rafts were removed daily and placed in cylindrical (mesh at 20 °C, 80%. (d) K3Fe(CN)6, K2OsO2(OH)4, K2CO3, t-BuOH/H2O/acetone 2.8:4:1, 0 °C, 12 h, 80%. (e) Ac2O, pyr, 0–5 °C, 3 h, 80%.

RESULTS AND DISCUSSION

The synthetic procedure of SRP is demonstrated in Scheme 1. Compound 1 was synthesized by using the following reaction sequence: reduction, Wittig coupling, oxidation, dihydroxylation, and finally lactonization. According to Couladourovs et al. in 1999 (14b) following the methodology for the synthesis of γ- and δ-lactones, the key intermediates 3 and 4 were easily produced from δ-valerolactone (2) in two steps involving reduction of the molecule with 1.1 equiv of disobutyaluminum hydride (DIBAL-H) leading to the corresponding lactol and subsequent enlargement of the carbon skeleton by Wittig reaction. Oxidation, cis-dihydroxylation on the 9:1 mixture of cis and trans double bonds, and consequent lactonization led to the formation of the erythro and threo isomers, respectively (see Scheme 1). Thus, a mixture of four stereoisomers of 6-acetoxy-5-hexadecanolide (1) containing 45% of the natural oviposition aggregation pheromone [(-)-(5S,6R)]], 45% of its enantiomer [(+)-(5S,6R)], and 10% of the respective threo enantiomeric pair [(-)-(5S,6S) and (+)-(5S,6S)] was synthesized.

By this approach, a bioactive mixture (see following bioassays) was prepared using a short and high yielding procedure (five steps, with 45% total yield) employing a less expensive, commercially available starting material. Olagbemiro et al. (20) also reported a semibiotechnological method to prepare racemic pheromone (as a mixture of erythro isomers) employing glycerides of (Z)-5-hexadecenoic acid as the starting material, which has to be derived after cultivation of Kochia scoparia (Chenopodiaceae) and extraction of the seeds.

According to behavioral and electrophysiological studies, it is suggested that the oviposition pheromone acts as an attractant and a stimulant in Cx. quinquefasciatus (21). In Culex mosquitoes, the oviposition attractant pheromone is stereospecific and the receptor accepts only the (−)-(5R,6S) enantiomer and not the other three isomers, which are inactive (17).

Our SRP of Cx. quinquefasciatus was tested as an oviposition attractant for gravid Cx. pipiens biotype molestus at the doses of 0.01, 0.1, 1, 10, and 100 μg per testing cage. Oviposition was elicited with the presence of the SRP at the doses of 0.01, 0.1, 1, and 10 μg per cage (see Table 1). The highest response was recorded at the dose of 1 μg per cage resulting in an oviposition attraction level of 72.3%. From these data, it is obvious that SRP is effective on Cx. pipiens biotype molestus even though it is the oviposition pheromone of the Cx. quinquefasciatus and it is also demonstrated that the pheromone of one species could be active to other species from the same complex. In contrast, the 100 μg per cage dose did not elicit any oviposition response. In Figure 1, the oviposition response in relation to doses is shown. The fitting curve was

\[
\text{response} = 0.73 (±0.0079) - 0.024 (±0.0036) \times \log(\text{dose}) - 0.039 (±0.003) \times \log^2(\text{dose})
\]

where \(R_{(\text{adjustment})}^2\) is 0.9.
The high pheromone concentration (100 μg per cage) could result in either confusion of the gravid females for the oviposition sites or even repulsion of the gravid females of laying their eggs. Although Millar et al. in 1994 (22) reported that attraction was stable to very high pheromone concentrations (1000 μg per cage), similar results to ours were stated from Hwang et al. in 1987 (17) and Blackwell et al. in 1993 (21) when they recorded the same pattern of behavior in the oviposition pheromone of *Cx. quinquefasciatus* gravid females. Specifically, Blackwell et al. in 1993 (21) found that the synthetic oviposition pheromone, with the two isomers (erythro-6-acetoxy-5-hexadecanolide), increased egg laying by *Cx. quinquefasciatus* females in a dose-dependent manner over a dose range of 0.01–80 μg. With 100 μg of pheromone added to the laying pot, there was a statistically significant reduction in the oviposition behavior. The attraction (% eggs laid in test pot) in this experiment was almost 85%.

Our attraction was almost 72%, although another member of the same species complex is used and these results indicated that there is a very good response of *Cx. pipiens* biotype molestus to the SRP of *Cx. quinquefasciatus*. In addition, the presence of the three other isomers into our SRP did not cause any reverse effect on its bioactivity, which is in agreement with previously cited studies (8, 23).

These results are very promising toward the use of our SRP in field conditions. To achieve that, further studies could be contacted as to determine the best possible way of using it. Fields of studies could be the search of possible synergy with other reported attractants such as chemical substances, grass infusions, etc. (21, 22, 24, 25). Effective doses in open air, stability of the SRP in natural conditions, ways of applying it, etc. are also research areas. The development of a pheromone oviposition mosquito trap, which is not species specific, could be of great value for surveillance or even control programs if it could be combined with larvicide.

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