Studies directed to the inhibition of the processionary moth sex pheromone in the laboratory and in the field

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Abstract. In this paper we present a survey of our work, carried out in the period 1980-1997, on the perception process of the processionary moth *Thaumetopoea pityocampa* (Denis and Schiff.). Our aim was to develop analogues of the sex pheromone in the search for mimics and/or inhibitors, capable of mimicking the pheromone action or inhibit the pheromone attractant activity. Several compounds have been found to be good inhibitors of the pheromone perception not only in the laboratory (electroantennogram and wind tunnel) but also in the field, particularly aldehyde **6**, which has also been found to be tuned to a receptor cell different to that of the pheromone. We have discovered a good mimic of the pheromone action, the acetylenic derivative **3**, which is important as a possible replacement of the pheromone in mass trapping experiments. Other analogues, whose activity can be associated to the inhibition of catabolism of the pheromone, have also been found good inhibitors of the pheromone action. Our data show the importance of the acetylenic function in the pheromone structure, support the fact that a strict steric requirement is necessary to achieve an efficient interaction of the analogues with the male antennal receptors, and confirm the predominant role played by the steric size over the polarity of the modified acetate group in the analogue structure.
Key words. *Thaumetopoea pityocampa*, processionary moth, sex pheromone, inhibition, wind tunnel, field tests.

Resumen. *Estudios dirigidos a la inhibición de la feromona de la procesionaria en el laboratorio y el campo*. En este trabajo se presenta un resumen de nuestras investigaciones, realizadas en el período 1980-1997, sobre el proceso de percepción de la procesionaria del pino *Thaumetopoea pityocampa* (Denis and Schiff.) (Lepidoptera, Thaumetopoeidae), en concreto sobre el desarrollo de análogos de la feromona sexual, capaces de mimetizar la acción de la feromona o inhibir la actividad atrayente de la misma tanto en laboratorio como en campo. El conocimiento del efecto inducido por los análogos sobre el comportamiento de los machos es importante no sólo para profundizar sobre su modo de acción sino también para el desarrollo de nuevos compuestos con un uso potencial para el control de plagas. En el laboratorio se han empleado las dos técnicas más útiles en el campo de las feromonas sexuales, esto es el electroantenograma y el túnel de viento. Como inhibidores de la percepción se han preparado una gran variedad de compuestos, previamente diseñados por sustitución isostérica de las tres zonas claves de la feromona (fig. 1), que se suponen interaccionan con el receptor, esto es el grupo enino, la función polar y la cadena alquílica (terminal o central). En forma esquemática las estructuras de los análogos preparados se representan en la figura 2. Se ha procurado que las modificaciones estructurales de la feromona se hagan de una en una, y no varias a la vez, para no modificar severamente la estructura de la feromona. Como buenos miméticos de la acción feromonal en campo se han encontrado el propionato **2** y el acetileno **3** (fig. 3), con una eficiencia relativa del 40 y 65%, respectivamente, en comparación con la de la feromona sintética. En túnel de viento el compuesto **3** es capaz de inducir en los machos de procesionaria la secuencia completa de orientación y cópula con la fuente feromonal. El derivado *trans* de la feromona (**E-1**) es neutro desde un punto de vista comportamental, esto es no inhibe ni incrementa la acción de la feromona tanto en túnel de viento como en el campo, lo que indica que la síntesis de la feromona no requiere procesos extremadamente selectivos. Como buenos inhibidores se han encontrado los fluoroderivados, resultantes de la sustitución de uno o varios átomos de hidrógeno del grupo acetato de la feromona por átomos de flúor, los tioesteres que resultan de la sustitución de un átomo de oxígeno (o los dos) del grupo acetato de la feromona por azufre, así como el alcohol resultante de la hidrólisis de la feromona. Por otro lado, se han estudiado también inhibidores del catabolismo de la feromona, ya que una acumulación de moléculas de feromona en la linfa

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sensilar podría conducir a un incremento del umbral de reconocimiento de nuevas moléculas de feromona por parte de los receptores, esto es una inhibición del proceso de atracción hacia la fuente atrayente. Como inhibidores se han considerado una variedad de trifluorometil cetonas, cuya actividad en laboratorio se ha determinado por presaturación de los receptores antenales con vapores de las mismas o en mezclas con la feromona en diferentes proporciones. Las cetonas más activas han sido las análogas **8** y **9** de la feromona, así como el compuesto **7**, un potente inhibidor de las esterasas antenales de *Spodoptera littoralis*. En el campo los compuestos **8, 9** y **10** han resultado poseer un potente efecto antiferomonal cuando se mezclan con la feromona en proporción 1:1 y 10:1 (fig. 4). Nuestros resultados señalan la importancia de la función acetileno en la estructura de la feromona y confirman el hecho de que, para conseguir una eficiente interacción receptor-inhibidor, se necesita un requerimiento estéreo estricto en la estructura de éste, así como el papel predominante del tamaño estérico sobre la polaridad del grupo acetato modificado.

Introduction

The processionary moth *Thaumetopoea pityocampa* Denis & Schiffermüller (Lepidoptera, Thaumetopoeidae) is one of the most important defoliator pine pests in Southern Europe and North Africa, but some other countries of Central Europe, like Germany, Switzerland, Hungary, Bulgaria, Yugoslavia etc., may also suffer scattered attacks. The larvae leave the nest and march in procession to feed on the young tender branches of the tree. In late February they burrow ca. 10-20 cm deep into the ground around the trees to pupate through May-June. Part of the adults may emerge in the period July-October, depending on the climate, geographic distribution and altitude, and the remaining pupae may enter into diapause for the next year(s) generation. The diapause can last up to 4 consecutive years (Démolin, 1969). The processionary moth is highly resistent to temperature fluctuations, since they can survive in a temperature range of -10ºC to +32º, which explains the appearance of the pest even at high altitutes (1500-2000 m). The moth is particularly active against all species of pines, but can also attack other conifers, like cedars (Montoya & Hernández, 1991). The estimated level of damage caused in Spain in 1977-78 amounted to ca. 10 million dollars, i.e. ca 21% of the total pine grove area. The last instar larvae are also particularly harmful to humans, causing severe irritations on the skin, eyes and the mucous membrane, due to the release of microscopic urticating hairs. Fortunately, there are a number of natural ennemis which help to maintain the population at not very high levels. Birds, mammals and insects, like ants of the genus *Formica*, have been reported to be active predators of the caterpillars, while a variety of hymenopterous, like *Tetrastichus sevardei* Dom. (Hymenoptera, Eulophidae) and *Oencyrtus pityocampae* Mercet. (Hymenoptera, Oencyrtidae), are effective egg parasites (Montoya & Hernández, 1991). However, these natural enemes cannot avoid the increasing population of the pest in many pine stands of our country.

Traditionally, control of the pest in Spain had been carried out by mechanical or insecticide-mediated (DDT, pyrethroids) destruction of the nests. When the use of DDT was banned, other more specific biological control systems were needed (*Bacillus thuringiensis*, cuticle inhibitors like diflubenzuron, semiochemicals...). Among these methods, utilization of sex pheromones emerged as an useful tool due to their features of efficiency, environmentally-friendly and high specificity.

In 1981 we identified the female sex pheromone as (Z)-13-hexadecen-11-ynyl acetate (Z-1) (figure 1) (Guerrero et al., 1981) from extracts of the female pheromone gland. To us this is probably the only component of the pheromone since later experiments directed to find other minor components in the gland resulted unsuccessful (Quero et al., 1997). This is the first structure with an enyne functionality as constituent of the sex pheromone of a moth. The structure of the natural compound was confirmed by synthesis (Camps et al., 1981a; Camps et al., 1981b; Camps et al., 1983; Michelot et al., 1982), and the chemical displayed a remarkable biological activity in the laboratory (electroantennogram and wind tunnel) and in the field (Cuevas et al., 1983; Einhorn et al., 1983).

In order to in-depth study the perception process of insect sex pheromones and to establish structure-activity relationships, we have been engaged in the last years in the design and synthesis of analogues capable of effectively modify the biological activity of the sex pheromone. In this paper we will survey our work in this subject, paying attention to a number of analogues which have been particularly relevant in terms of their activity, either "per se" or in combination with the natural pheromone. As perception inhibitors a large variety of structurally-related analogues have been prepared, all of them designed by isosteric replacement of the three key parts of the pheromone, which are supposed to interact with the receptor: the enyne group, the polar function and the alkyl chain (terminal or central). In schematic form the analogues have the general structure shown in figure 2, wherein Rnp= methyl, ethyl, propyl, butyl; Rp= formate, propionate, haloacetate, alcohol, aldehyde, methyl ketone, methyl ester, ethoxy; and n=9, 10, 11. In some analogues, the double bond of the enyne group has also been replaced by a single bond, an epoxy group, a thiomethylene function, etc., whereas in some others the triple bond of the enyne has been replaced by a double or single bond, or an allene group. All modifications have been carried out in a one-by-one order so as not to severely modify the original structure of the pheromone.

Inhibitors of catabolism have also been considered, since an accumulation of pheromone molecules into the sensillum lymph might lead to an increase of the recognition threshold of new incoming pheromone molecules by the receptors, and therefore to an inhibition of the attraction process to a pheromone source. As catabolism inhibitors a series of trifluoromethyl ketones (TFMKs) have been considered, but in this case their structures may greatly differ from that of the pheromone.

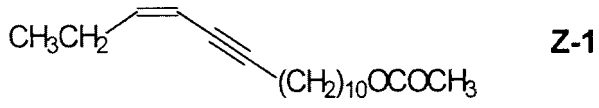

Figure 1. Structure of the sex pheromone of the processionary moth *Thaumetopoea pityocampa*.

Figure 2. General structure of the analogues described in this work. Rnp= non-polar alkyl chain, Rp= polar alkyl chain.

Materials and Methods

Insects. T. pityocampa pupae were collected in infested pine forests in Mora de Rubielos (Teruel), sexed, covered with a 3-6 cm layer of sawdust and sent to our Department. They were kept in wooden boxes in a 16:8 light:dark reversed photoperiod at 23±1°C and 55-65% humidity until emergence. Several toothpicks were placed standing on the sawdust to favor perching of males after emergence. In preliminary assays we noticed that reversal of the photoperiod to carry out the bioassays during working hours did not affect males behavior. Due to the short lifetime of the insects and since young males responded better than the old ones in the wind tunnel experiments, only insects with

Chemicals. A large variety of analogues have been prepared in our laboratory (Camps et al., 1981a; Camps et al., 1987a; Camps et al., 1988; Camps et al., 1990a; Camps et al.; 1990b; Gasol, 1988; Parrilla & Guerrero, 1994; Parrilla et al., 1994). Before tests the chemicals were analyzed for chromatographic and stereochemical purity.

EAG tests. The EAG apparatus used was mainly based on that described by Roelofs (1984) and improved later by us with a new automatic sample injection system (Guerrero et al., 1986). The inhibition assays by presaturation of the antennal receptors were carried out by placing 1-day old individual insects in 12 cm diameter Petri dishes, that contained a piece of filter paper (2 x 2 cm) on which 10, 100, 500 or 1000 mg of one of the test compounds had been absorbed. For the most active compounds lower doses (0.1 and 1 mg) were also assayed. Care was taken to prevent the insects to be in contact with the chemicals. The moths were exposed to vapors of the compounds for 4 h in the dark, taken out to another clean holding container and after 5 minutes their antennae were excised. Between 8 and 12 moths were tested for each treatment. As control the males were exposed only to vapors of hexane. Ten "puffs" on a Pasteur pipette containing 10 mg of the synthetic pheromone were directed over the antennae, and depolarizations recorded at 40 seconds intervals to ensure full recovery of the antennal receptors. The inhibition values were calculated as percent of the relative decrease of the EAG average response to the natural pheromone of treated moths in relation to the mean value displayed by control insects. The results were analysed statistically for significance according to the LSD test ($P<0.05$).

Wind tunnel. The tests were carried out in a wind tunnel of 180x55x50 cm, already setup in our laboratory (Quero et al., 1995). A white board containing irregular-shaped, brown-dark spots was placed on the floor of the tunnel, since the more usual stripped patterns may induce unexpected effects on the orientation of flying insects (David, 1982). The air speed, measured at the center of the flight compartment, was 45 cm/s.

Inhibition tests. Two types of tests were conducted, i.e. study of the behavioral effects induced by the test compounds alone and in combination with the natural pheromone, and study of the inhibition response to the pheromone by presaturation of the antennal receptors. Newly emerged moths perched on the toothpicks were separated from the remaining pupae every day and placed into 31x12x21 cm plastic boxes containing a 2 cm layer of sawdust. The insects were allowed to acclimate to the tunnel conditions for 1 h before they were tested 3-5 h into the scotophase. The temperature of the experiment room was 22±1°C and the relative humidity 60±5%. The toothpicks supporting 1-2 males were carefully taken out, fixed on a polyurethane block and placed into the tunnel on a stainless steel jack at 150 cm downwind from the source. The fan and blower were turned on and the males allowed to respond for 5 min. Moths were utilized only once and then discarded. The compounds were dissolved in nanograde hexane and the required volume to achieve the test doses (0-100 µg) was applied to a 1.5x1 cm cotton wick. The solvent was allowed to evaporate and the lure hanged at 18 cm from the top and 40 cm from the closer screen of the tunnel. Solutions of the compounds were prepared daily, just prior to the experiments, and the wicks discarded after use. Males were scored according to the following behaviors: wing fanning and taking flight (TF), arrival to the middle of the tunnel (65 cm, HW), close approach to the lure (ca. 10 cm, CA), and contact with the source (SC). Only those males arresting at the source for a minimum period of 5 s were recorded as SC. The effect of a specific formulation was calculated as the percentage of males showing a particular behavior relative to the total number of insects released. Generally, 20-30 males were considered for each treatment. The data obtained in different occasions corresponding to the same treatment were not significantly different, so they were combined for statistical analysis using a chi square 2x2 test of independence (Sokal & Rohlf, 1969).

Field tests. The trials were held in Mora de Rubielos (Teruel). The baits were prepared by dissolving the specified amounts of the test compounds and the pheromone in nanograde hexane (1 ml), mixed with paraffin wax (2.5 mg) and transferred to polyethylene vials (3 x 1.1 cm ID). The solvent was evaporated off, the vials capped and used as dispensers. The traps were specially designed for processionary moth catches and contained no glue or insecticide (Montoya, 1984). They were hung on pine branches 1.7-2.0 m high and separated a minimum of 50 m apart. Between parcels the minimum distance was 150 m. The traps were set out in statistically randomized blocks and revised and rotated every day. The inhibition activity of the compounds was measured by the relative trap catch decrease

of the specified blend in comparison with the natural pheromone. Five traps were utilized for each formulation and the data subjected to analysis of variance by LSD test ($P<0.05$).

Results

Most of the compounds studied presented low intrinsic activity in EAG, lower than 40% in comparison to that of the natural pheromone. However, the propionate analogue **2**, the acetylenic derivative **3** and the fluorinated analogue **5** showed potent intrinsic EAG activity (1.5-2.0 mV of depolarization, 60-90% in comparison with the pheromone).

In the field and as expected, the acetylene **3** and the propionate **2** were good pheromone mimics with a 65% and 40% relative trapping efficiency in comparison to the natural pheromone, respectively (Camps et al., 1988).

In contrast, the fluorinated derivative **5** was "per se" only slightly active, catching 12.5 males/trap/week in comparison to 58.4 males/trap/week of the natural material. However, mixtures of **5** and the pheromone in 3:1 ratio exerted a significant 50% inhibitory effect on the activity of the pheromone, while at lower ratios the inhibition was not significant (Camps et al., 1988). In the wind tunnel, the acetylene **3** was also able to induce moths to successfully complete the mate finding sequence, although high doses (50-100 µg) of the analogue were required to elicit similar SC behavior than the pheromone (60-72% of males contacted the source).

However, when mixed with the pheromone in 95:5 and 80:20 ratio the analogue induced a decrease in activity, particularly in CA and SC behaviors (Quero et al., 1995). The *trans* isomer of the pheromone (**E-1**) was a behaviorally-neutral analogue in the field, since mixtures with the pheromone (*cis* isomer, **Z-1**) did not catch a significantly different number of males in comparison with **Z-1**. These results were also confirmed in wind tunnel experiments, wherein 1 µg of a 80:20 mixture of **Z-1** and **E-1** elicited landing at the source on 80% of males, in comparison with 90% when the lure contained **Z-1** alone (Quero et al., 1995).

The alcohol **4**, in contrast, was found to be an inhibitor of the pheromone action since 0.1:1, 1:1 and 10:1 mixtures with the parent material resulted in significantly lower number of catches in comparison with the activity of the pheromone (72, 82 and 64 vs. 540 males caught, respectively) (Camps et al., 1988). This effect was also confirmed in wind tunnel wherein a strong inhibition effect was noted in the number of males taking flight (only 30% in comparison with near 100% with the pheromone) when males were subjected to a 9:91 mixture with the pheromone. At this ratio none of the males contacted the source (Quero et al., 1995). The most potent inhibitor found so far was, however, aldehyde **6**, since addition of 10% to the pheromone-baited lure promoted a dramatic decrease in catches (15-20 males/trap vs. 220 males/trap with the pheromone). Likewise, the presence of only 1% of **6** in the source evoked a complete inhibition of the SC behavior, with the number of males taking flight being only 25% of those attracted to the natural compound alone (Quero et al., 1995).

Haloacetate analogues, which result from the replacement of one or more hydrogen(s) of the acetate group of the pheromone by halogen, are not EAG active but some of them like the mono-, di- and trifluoroacetate derivatives significantly inhibited the attractant activity of the pheromone in the field, particularly the monofluorinated analogue (Camps et al., 1990b). This compound elicited a 68% diminution of male catches when 10% of the chemical was added to the pheromone bait. By contrast, chloroacetate analogues were practically inactive (Camps et al., 1990b).

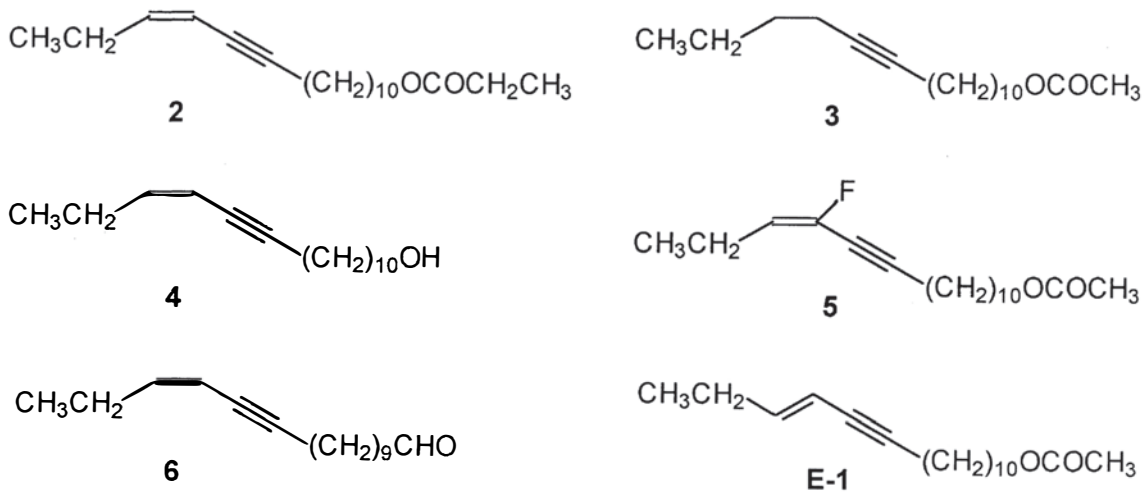

Figure 3. Structures of the analogues **2-6** and **E-1** of the processionary moth sex pheromone.

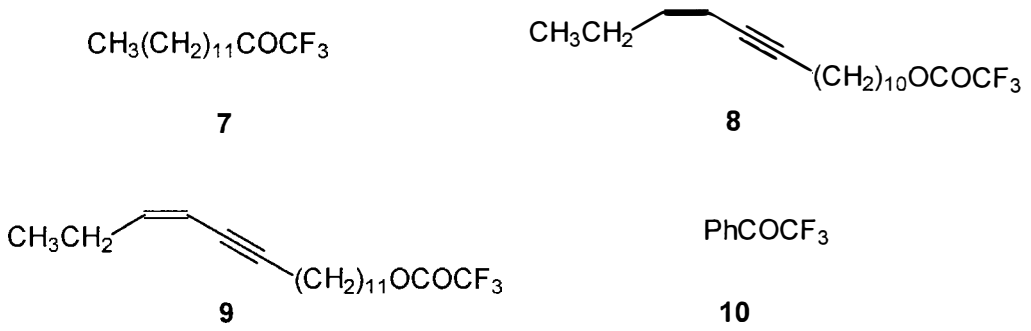

Figure 4. Structures of the trifluoromethyl ketones **7-10** as inhibitors of the pheromone action in the processionary moth.

Among the sulfur analogues tested, only the thioesters, resulting from replacement of the oxygen atom(s) of the acetate group of the pheromone structure by sulfur, have been found to be good antagonists of the pheromone action in the field, the activity (85-97% inhibition) being similar than that of the fluorinated derivatives (Camps et al., 1990a).

As inhibitors of the pheromone catabolism and in presaturation experiments, some of the TFMKs displayed a notable blockage of the pheromone detection on EAG, although they have limited intrinsic EAG activity (Parrilla & Guerrero, 1994). Particularly noteworthy is the activity of compound **7**, which showed a 60% inhibitory action at 100 µg, and compound **8**, which displayed a relatively low (20% inhibition) but statistically differentiated effect at only 1 µg. The effect was dose-dependent (Parrilla & Guerrero, 1994). In the field TFMK **10** and the analogues **8** and **9** showed potent significant disruptant effect when mixed with the pheromone in 1:1 and 10:1 ratios (Parrilla & Guerrero, 1994). In wind tunnel, presaturation of antennal receptors with vapors of 1 µg of **9** induced a clear impairment in the SC behavior with only 36% of males landing at the source. Higher doses of the chemical (10 µg) completely inhibited all types of behavior (Quero et al., 1995). In a different experiment, when 5% of the chemical was added to the pheromone in the source, a significantly lower number of males displayed the CA and SC behaviors in comparison to those subjected to the pheromone alone (Quero et al., 1995).

Discussion

Knowledge of the behavioral effects elicited by analogues as inhibitors, mimics or synergists is important in order to get a better insight of their mode of action and, consequently, to develop new compounds with potential use in pest control (Albans et al., 1984; Camps et al., 1990a; Camps et al., 1990b; Schwarz et al., 1990). The most active compounds were subjected to field tests, not only to study their intrinsic attractant activity, but also to establish their effect on the pheromone activity when mixed with the parent compound in several ratios. Two types of inhibitors have been pursued in our study: inhibitors of pheromone perception by analogues structurally similar to the pheromone, and inhibitors of catabolism which are chemicals able to inhibit the action of the enzymes responsible for the catabolism of the natural attractant.

In the laboratory the electroantennogram (EAG) and wind tunnel are the most useful techniques to evaluate the biological activity of the synthetic analogues. The EAG is one of the first techniques developed to study the action of the pheromone and analogues on the antennal receptors (Schneider, 1957). It has been used successfully for the identification of sex pheromone components in many species of insects (Roelofs & Comeau, 1971) as well as inhibitors and synergists (Kamm & McDonough, 1980). Wind tunnels, on the other hand, have been widely recognized as the most effective technique to study orientation mechanisms of flying insects to their sex pheromone components (Cardé, 1984; Mafra-Neto & Cardé, 1994; Miller & Roelofs, 1978), as well as the inhibitory effect elicited by specific synthetic chemicals (Preiss & Priesner, 1988).

It is clear from our studies that a strict steric requirement is necessary to achieve an efficient competitive interaction with the male antennal receptors. This is particularly evident when we compare the activity of the fluoroanalogues with the corresponding chloroderivatives, wherein the much higher atomic volume of the chlorine atom than that of hydrogen may account for the lack of activity. In contrast, the sterically similar fluorine atom closely mimics the steric requirement of hydrogen at enzyme receptor sites. Moreover, our data also corroborate the predominant role played by the steric size over the polarity of the modified acetate group, as has been shown previously (Prestwich & Streinz, 1988). The antipheromone action of the trifluoroacetate analogue was also found by Albans et al. (1984) in *Heliothis virescens*.

The reduction of EAG responses to pheromone stimuli found in the presaturation experiments can be explained by a long-lasting adaptation of receptor responses through the conditioning stimulus. On the other hand, the reduction of trap captures upon admixture of close structural analogues of the pheromone could be due to improper activation of additional cell types other than the pheromone. It should be noted, in this context, that in single cell recording two types

of receptor cells, found in the long trichoid hairs of the branches of the male antennae, are tuned each one to the pheromone itself and to the aldehyde 6, the most potent inhibitor found by us so far (see above) (Quero et al., 1997).

Our results have led to the discovery of a new potent pheromone mimic, the acetylenic derivative **3**, whose activity in the wind tunnel and in the field may be of the same order than the pheromone at high doses (Camps et al., 1987b). The simplicity of the compound as well as its easy preparation in large scale makes the compound particularly attractive replacing the more costly and less stable pheromone in mass trapping experiments. Other important point emerging from our studies are the following: a) the trans isomer of the pheromone (compound E-1) is not an inhibitor, which is a great advantage from a synthetic point of view; b) the presence of the triple bond in the enyne structure of the pheromone appears to be crucial for biological activity since replacement by a double or single bond induces the complete loss of activity.

Trifluoromethyl ketones have been found to be strong inhibitors of antennal esterases of insects (Prestwich, 1987; Durán, et al., 1993), the enzymes responsible for catabolism of pheromones, due to formation of hemiketals with a serine residue present at the active site of the enzyme (Linderman et al., 1988, Rosell et al., 1996). Inhibition of enzymatic catabolism of odorant molecules has been considered as a potential approach for the disruption of pheromone reception in the search for new strategies for pest control (Prestwich, 1987). In the processionary moth these chemicals have shown very low intrinsic electro-antennographic activity, as described in other species (Prestwich & Streinz, 1988, Riba et al., 1994). After presaturation of the receptors, an EAG inhibition ranging from 20 to 90% was observed. It should be noted the activity of compound **7**, which had shown a prominent antennal esterase inhibition on *S. littoralis* (Lepidoptera, Noctuidae) (I5● 1.6 (M) (Durán et al., 1993). Presaturation of receptors, however, on *Sesamia nonagrioides* (Lef.) with a TFMK analogue of the pheromone resulted in a weak inhibition of the EAG response (Riba et al., 1994). In the field some TFMKs have displayed potent inhibitory activity on processionary male catches when mixed with the pheromone, but this type of effect was not confirmed when the corresponding analogue was added to the pheromone of *S. nonagrioides*, wherein a clear synergistic effect was observed (Riba et al., 1994). In our wind tunnel experiments, the inhibitory action of the analogue **9** was corroborated but, again, in a different insect, like *Ostrinia nubilalis*, a similar TFMK analogue of the sex pheromone did not inhibit male attraction when was co-evaporated with the pheromone (Klun et al., 1991). In general, we have found a good agreement between the activity of the TFMKs on the EAG and on male behavior in wind tunnel, but more recent studies from our laboratory have shown that their activity is not fully correlated with the antiesterase effect (Renou et al., 1997).

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