Biorational Approaches for Insect Control by Enzymatic Inhibition

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INTRODUCTION

Enzymatic inhibition is a well-known tool to block many important biochemical and physiological processes, resulting in the elucidation of new metabolic pathways as well as in a in-depth knowledge of many kinetic mechanisms of enzyme-driven reactions. For this reason, the development of potent inhibitors is an important area of research in the pharmaceutical and agrochemical fields [1,2], among others. Illustrative of the significance of these compounds is, for instance, the development of inhibitors of human neutrophil elastase for treatment of pulmonary emphysema [3], inhibitors of neuropathy target esterase, the target site of certain neurotoxic organophosphorous compounds [4], or renin inhibitors as therapeutic agents for the control of hypertension [5].

At present, the main tools for controlling insect pests are broad-spectrum neurotoxic insecticides. These chemicals have a number of serious drawbacks, including their toxicity to mammals, including humans,ugend,ing and non-target organisms as well as their persistence in the environment. Another major problem associated with their utilization is the possible development by the insects of genetically-based resistance to insecticides, which may lead to unexpected control failures [6]. Therefore, the development of novel biorational, specific and non-toxic approaches to pest control is highly desirable. In this work we present an overview of the enzymatic inhibitors that have been found in the recent literature to affect the physiology, growth and development of insect larvae and/or modify the intraspecific communication of adults, so that they can be envisioned as potential biorational control agents. The enzymes covered include pheromone degrading enzymes (esterases, aldehyde dehydrogenases, oxidases), pheromone biosynthetic enzymes, oxidoreductases, juvenile hormones, juvenile hormone epoxide hydrolases, proteases, molting hormones and phenoloxidases. Although these approaches refer to control of insect pests, many of them can be in principle also considered suitable for medicinal chemistry studies, since the mechanism of action of these inhibitors on related enzymes is quite similar, if not equal, in both fields.

Keywords: Enzyme inhibitors, pest control, pheromone degrading enzymes, pheromone biosynthetic enzymes, oxidoreductases, juvenile hormones, proteases, molting hormones, phenoloxidases

1. PHEROMONE-DEGRADING ENZYMES

Insects have a highly specialized and sensitive pheromone olfactory system, which is crucial to their survival. For example, many insects use sex attractants as an integral part of their mate-finding strategy [7,8]. The olfactory system consists of hollow hairs, usually located on the mouthparts of the antennae. The lumen of these hairs is filled with a protein-rich solution, the sensillar lymph, which contains abundant pheromone-binding proteins (PBPs). These proteins are thought to transport the pheromone to the dendrite of the sensory neuron, which protrudes into the lumen of the hair. Once detected, the pheromone is degraded by pheromone-degrading enzymes (PDEs), which include monooxygenases, aldehyde oxidases, aldehyde dehydrogenases, esterases and glutathione-S-transferases. The subject of insect pheromone olfaction as a new target has been reviewed recently [9] and we shall present further details below.

1.1 Pheromone Esterases

General and pheromone-specific esterases are widely distributed on the body surfaces in order to degrade conspecific and heterospecific pheromone components which can adsorb on the cuticle [10]. Localization of sensory hair specific esterase isozymes has been demonstrated in the silk moth Antheraea polyphemus [11,12]. Despite the suggested role in the olfaction process, odorant-degrading enzymes (ODEs) have not been isolated or characterized, possibly because of the very tiny amount present in the male antennae (four orders of magnitude lower than that of pheromone-binding proteins). Recently, a bioinformatic approach has led to the cloning of cDNAs encoding a putative odorant.
degrading enzyme (ApolODE) and a putative integumental esterase from *A. polyphemus* [13]. The ApolODE has a predicted molecular mass of 59.9 kDa, pI of 6.63, three potential N-glycosylation sites and a putative catalytic serine site with the sequence Gly195-Glu-Ser-Ala-Gly-Ala [13].

Incorporation of polyfluoroketone moieties into inhibitors exhibiting close structural analogy to the substrates has proven to be a useful strategy for generating strong inhibitors of diverse serine hydrolases, many members of this type of enzymes being pharmaceutically attractive targets. Among others, enzymes inhibited by fluoroketones include acetylcholinesterase [14], chymotrypsin [15], trypsin [16], juvenile hormone esterase [17], human liver microsomal carboxylesterases [18], cytosolic human phospholipase A2 [19], or human phospholipase A2 [20]. Inhibition studies carried out with these fluorinated derivatives have led, for instance, to the development of renin or angiotensin converting enzyme inhibitors as medicinally useful antihypertensive agents [20]. The fluorinated ketones function as transition-state analogues of the enzyme, with the inhibition activity arising by formation of an adduct of tetrahedral geometry between the serine residue, present at the active site of the enzyme, with the highly electrophilic carbonyl moiety [21,22]. A crystal structure has been obtained for the covalent complex of porcine pancreatic elastase and peptidyl α,α-difluoro-β-keto amide with the inhibitor bound at the active site as a hemiketal with a Ser-195 [23].

Use of inhibitors of the catabolism of the odorant molecules, as disruptants of pheromone reception, has been proposed as a potential approach for insect control [24]. Trifluoromethyl ketones (TFMKs) reversibly inhibit the antennal esterases responsible for the pheromone catabolism in male olfactory tissues. A variety of TFMKs have been prepared and their inhibitory activity established in different moth species [11,22,25,26]. In the processionary moth *Thaumetopoea pityocampa*, the most closely related analogues to the pheromone displayed a potent inhibitory effect on male catches when mixed with the pheromone in different ratios [27].

In *Spodoptera littoralis* a good agreement has been found between the activity of several TFMKs (Fig. 1) on the electroantennogram (EAG) and on male behaviour in a wind tunnel. Particularly effective are 3-ocylthiotrifluoroprop- onone (OTFP, 1a) and (Z,E)-9,11-tetradecadienyl trifluoromethyl ketone (3), the most closely related analogue of the major component of the pheromone 2, which decreased the EAG response to the natural attractant [28], and significantly reduced the percentage of insects landing at the source [29]. Moreover, males treated with these inhibitors exhibited erratic progress towards the plume, flying across the wind with high number of intersections with the plume. Interestingly, the non-fluorinated analogue did not affect the regular flight to the pheromone source, confirming the key role played by fluorine in the inhibitory action of these molecules [29]. Both compounds 1a and 3 had displayed good antiesterase activity (IC50<10 µM) in "in vitro" assays [22,26]. In the Mediterranean corn borer *S. nonagrioides*, similar type of effect was also noticed in wind tunnel after topical application of the analogue 5 to the male antenna [29]. In crossed specificity studies the inhibition effect of several TFMKs on antennal extracts of the two latter insects had low specificity for the enzyme [30].

OTFP (1a) behaved also as an oviposition deterrent on larvae reducing diet consumption and growth, pupation and adult emergence when administered to the 2nd instar larvae of *S. littoralis* and *S. nonagrioides* [31]. In addition, adult males, which had been treated with the chemical at the larval stage, were less attracted to the pheromone or to virgin females than untreated males [31].

In other insects, such as *Ostrinia nubilalis* and *Plutella xylostella*, however, TFMK analogues of the pheromone have resulted weak inhibitors of crude antennal homogenates [25,32]. Some aliphatic TFMKs have been reported to be bound to a 15-KDa PBP, present in the sensory hairs of the processionary moth males, and transported through the haemolymph, thus facilitating a productive inhibition of the esterases responsible for pheromone catabolism [33]. Also, 3-decylthio-1,1,1-trifluoropropanone (1b, DTFP) appeared to bind the PBP of *A. polyphemus* [34].

With regard to toxicology, TFMKs 1 and 5 showed little toxicity to mice, with an LD50 of 1 g/kg after the 6th day of administration, whereas the major component of the pheromone of *S. nonagrioides* 4 elicited a LD50 of 5 g/kg.

![Fig. (1). Structures of trifluoromethyl ketones 1a, 1b, 3 and 5 and phosphorofluoridate 6 as inhibitors of pheromone esterases.](image-url)

Compounds 3 and 5 are analogues of the major components of the pheromone of *Spodoptera littoralis* 2 and *Sesamia nonagrioides* 4.
after the same period of treatment [35]. These data agree with previous studies on the low acute toxicity to mice displayed by several substituted TFMKs [36,37]. So far, all the gathered data point out to the possible application of this type of chemicals in future biorational strategies for pest control [38].

With the aim of preparing pheromone carboxylesterase inhibitors, several dialkyl phosphorofluoridates and alkyl methyl phosphorofluoridates, such as compound 6 (Fig. 1), analogue of the main pheromone component of the Oriental fruit moth Grapholita molesta, have been developed [39]. The compounds disrupted the pheromone-mediated behavior in a wind tunnel, possibly by inactivation of the pheromone carboxylesterase.

1.2 Aldehyde Dehydrogenases

Aldehyde dehydrogenases are the primary degrading enzymes found in both leg and antennal tissues of male and female Heliothis virescens, as determined in studies using tritium-labelled Z9-14:Ald or Z11-16:Ald (11) as substrates [40]. Several analogues of the major component of the pheromone 11 have been shown to display antipheromone activity, among them N-methyl carbamate 7, trifluoroacetate 8, trichloroacetate 9 and methyl ketone 10 [41] (Fig. 2).

These chemicals acted as competitive antipheromones displaying reversible inhibition of electrophysiological and behavioral responses of males, and methyl carbamate 7 was also found to reduce oviposition on cotton [42]. The proposed mechanism of action involved tight binding of the chemicals to a nucleophilic receptor site forming a stable tetrahedral hemiketal adduct, similar to the mechanism proposed for the esterase inhibition by TFMKs. In the same context, Prestwich [24,43] developed acyl fluoride 12 by replacement of the aldehyde hydrogen by fluorine in the structure of pheromone 11. Compound 12 resulted to be a potent hyperagonist in H. virescens males at high doses and a disorientation agent at lower doses in wind tunnel [24]. The author proposed a mechanism involving irreversible N-acylation of the receptor protein by reaction of the acyl fluoride function with a free amino group of the protein.

1.3 Oxidases

Aldehyde oxidizing enzymes (AOEs), which convert pheromone aldehydes to inactive carboxylic acids, are present at high levels in antennal tissues of adult moths. The AOE may include oxygen-requiring aldehyde oxidases and nucleotide-cofactor-requiring aldehyde dehydrogenases. Several types of analogues for AOE inhibition of H. virescens pheromone, such as cyclopropanols, α-fluorinated aldehydes and α,β-unsaturated carbonyl compounds have been prepared. Cyclopropanols, such as 13 (Fig. 3), appeared to act as AOE inhibitors possibly via alcohol oxidase-mediated oxidation to the unstable cyclopropanones. Cyclopropanones would be acting as transition state analogues by formation of stable tetrahedral adducts with an active cysteine residue of the antennal AOE [44]. α-Fluoro (compound 14, Fig. 3) and α,α-difluoro substituted (compound 15, Fig. 3) analogues of (Z)-11-hexadecenal, one of the major components of the pheromone of H. virescens, were shown to be modest inhibitors of both the alcohol oxidase and AOE. The most potent inhibitor found (IC50<0.1 μM) was the vinyl ketone 16 (Fig. 3), and the inhibition appeared to be irreversible. The proposed mode of action involved a Michael type addition of the thiol group of a cysteine residue at the AOE active site, giving an adduct unable to undergo further processing by the enzyme. Further studies on the activity of these chemicals on male behaviour have not been pursued.

![Fig. (2). Structures of the N-methyl carbamate 7, trifluoroacetate 8, trichloroacetate 9, methyl ketone 10 and acyl fluoride 12, analogues of the main pheromone component of Heliothis virescens 11, as compounds with antipheromone activity.](image)

![Fig. (3). Structures of cyclopropanol 13, fluoro derivatives 14, 15 and α,β-unsaturated aldehyde 16, analogues of the major pheromone component of Heliothis virescens 11, as inhibitors of aldehyde oxidizing enzymes.](image)

2. PHEROMONE BIOSYNTHETIC ENZYMES

It is known that biosynthesis of insect sex pheromones in Lepidoptera takes place mainly through the action of the following enzymes: fatty acid synthase, desaturases, specific
chain shortening enzymes and one or more of the following: a reductase, an acetyl transferase, an alcohol oxidase or an acetate esterase [45]. Particularly appealing is the study of desaturases, which allow the stereoselective introduction of specific unsaturations in certain position(s) of the fatty acyl chain, resulting in unsaturated fatty acid products with Z9, E9, Z10, Z11, E11, Z12, E12, Z14 and E14 double bonds, and multiple unsaturated fatty acids by the action on unsaturated substrates [46].

The first acyl-CoA desaturase described was a stearoyl-CoA desaturase purified from rat liver in 1974. Since then, many desaturases have been cloned and sequenced, and the investigations have shown that the pheromone gland $\Delta_{11}$ desaturases of several species of moths share a number of biochemical properties with the ubiquitous metabolic integral membrane acyl-CoA $\Delta_9$ desaturase of animal cells [46]. However, it should be emphasized that $\Delta_{11}$ desaturases are particularly interesting targets in prospective approaches for pest control because mammals and plants seem to lack that particular regioselectivity. Other insect orders such as Diptera, Hymenoptera and Coleoptera also have pheromone biosynthetic pathways involving similar types of enzymes, but Coleoptera can also produce pheromones in one or more of three major ways: by sequestration of host compounds, by structural modification of dietary host material and by “de novo” biosynthesis through the classical isoprenoids pathway regulated by JHIII [45]. Elucidation of the biosynthetic pathways of most pheromonal structures has been mainly carried out by feeding experiments with stable isotopically-labelled precursors [47].

Based on previously shown inhibition of desaturation of stearic to oleic acid by a cyclopropenic C-18 fatty acid, several inhibitors of Z-11 and Z-9 desaturation of palmitic acid in the biosynthetic pathway of S. littoralis were reported [48]. Cyclopropenic fatty acids with the cyclopropene ring at positions 10-11, 11-12 and 12-13 (compound 17, Fig. 4) inhibited the biosynthesis of two key components of the pheromone, (Z)-9-tetradecenyl acetate and (Z,E)-9,11-tetracadienyl acetate. In the same vein, 2-halofatty acids have also been found to be inhibitors of the sex pheromone production in S. littoralis, T. pityocampa and Bombyx mori [49]. Among them, 2-bromohexadecanoic acid (18) was particularly active, being the Z-11 desaturase and acetyl transferase the target enzymes.

In spite of the promising features that these types of inhibitors display for "in vivo" application, only one report was found in the literature on the "in vivo" activity of these fatty acids. Several cyclopropenic analogues of the pheromones of three different insects, Musca domestica. P. xylostella and Ephestia eleutella, in which the Z double bond was replaced by a cyclopropene moiety, were synthesized as desaturase inhibitors [50]. The analogues interfered with the mating behaviour of the insect but no further details of the biological activity of these chemicals were reported.

The $\beta$-oxidation or chain-shortening step is also one of the primary steps in the biosynthetic pathways of insect sex pheromones. We have prepared a variety of monofluorinated, such as compound 19 (Fig. 4)) [51], acetylenic (compound 20), and cyclopropene fatty acids (compounds 21-23) [52], that have resulted good inhibitors "in vitro" and "in vivo" of the first step of the biosynthesis of the major component of the pheromone of S. littoralis. This $\beta$-oxidation step involves transformation of palmitic into myristic acid through an acyl-CoA dehydrogenase and the compounds were designed to block the enzymatic oxidation at carbons 2 and 3 of the parent palmitic acid. The most potent inhibitors found were the 2,3-dichlorocyclopropane analogue of palmitic acid 23 in the experiments "in vitro" and 2-bromopalmitic acid (18) in the “in vivo” assays [53].

3. OTHER ESTERASES

3.1 General Esterases

TFMKs also inhibit esterase-mediated resistance to insecticides and therefore they can also be considered potential insecticide synergists. In this regard, several TFMKs have resulted good insecticide synergists for azinphosmethyl-resistant tufted apple bud moths Platynota idaeosalis [54]. From whole body homogenates of azinphosmethyl-resistant adult females, two 1-naphthyl acetate resistance-associated esterases were isolated and these enzymes were inhibited by OTFP (1a) with an IC$_50$ of $10^{-8.5}$ M. Similarly, in the Colorado potato beetle Leptinotarsa decemlineata, OTFP has also been found an effective inhibitor of a resistance-associated esterase and 3-nonylthio-1,1,1-trifluoropropan-2-one (NTFP), the C$_9$ homologue of OTFP, enhanced 2.3x the toxicity of malathion to mice [18]. The synergistic effect of these compounds is somewhat surprising since TFMKs are reversible inhibitors and, as such, they commonly display weaker effects in vivo than the irreversible inhibitors, such as malathion. As noted above, toxicological studies showed that the TFMKs elicited little toxicity to mice. Thus, administration of NTFP to Swiss-Webster mice at doses 125-250 mg/kg induced no mortality over a period of 3 months [18], whereas OTFP (1a) and compound 5 induced no apparent symptoms of toxicity at 500 mg/kg over a 7-day period [35]. These data suggest the possible application of these chemicals as new insecticide synergists [55].

3.2 Acetylcholinesterase

Plant terpenes are usually a good source of compounds for pest management, since they are environmentally friendly
and offer strong resistance to insect attack. This is the case, for instance, of limonoids from Meliaceae plants, flavonoids from Asteraceae, sesquiterpenes from Celastraceae, etc.

The activity of some of these compounds has been associated with a mechanism involving inhibition of AChE. *Ent*-clerodanes from aerial parts of *Gutierrezia microcephala* (Asteraceae) and β-dihydroagarofurans, isolated from *Maytenus sp.*, have exhibited insecticidal activity inhibiting growth, pupation and emergence on larvae of *Spodoptera frugiperda* [56]. The latter compounds were potent inhibitors of AChE (78-100% inhibition at 15 ppm) with the most potent toxic agent exhibiting a LD$_{50}$ of 1.9 ppm on adults [57]. Similar results were reported with insecticidal triterpenes isolated from *Parthenium argentatum* [58].

**3.3 Juvenile Hormone Esterase**

Insect juvenile hormones (JH) regulate many aspects of insect growth, metamorphosis and reproduction. A decrease in JH levels is correlated with an increase in the rate of JH degradation, and it is believed that degradation by specific haemolymph JH esterases along with changes in the rate of JH biosynthesis is responsible for the regulation of JH titer and insect development [59]. As noted above, TFMKs are potent inhibitors of JHE [36], particularly those containing a sulfur atom in β position to the carbonyl, like OTFP (1a) [60]. It was hypothesized that the increased potency of the OTFP series was the result of mimicking the α,β-unsaturation system of JH by sulfur [61]. However, the presence of an intramolecular hydrogen bond between the hydroxyl group of the hydrate form and the sulfur in the most stable conformation of 3, the TFMK analogue of *S. littoralis* pheromone [62], proves that the sulfur does more than simply mimicking the site of unsaturation in the natural substrate. The extent of hydration of these compounds may also be an important factor for esterase inhibition [63,64] although our own studies revealed that no clear correlation between hydration rate and inhibition potency could be established [30]. In this context, complete oxidation of OTFP afforded the corresponding sulfone which was entirely hydrated. This diol was a potent "in vitro" selective inhibitor of JHE (I$_{50}$ 1.2 nM) whereas "in vivo" elicited juvenile activity when applied to larval and adult cabbage looper *Trichoplusia ni* [65]. The alcohol precursor of OTFP as well as its α-methyl analogue are also effective inhibitors of JHE at the nM range [66]. Other β-thioderivatives display "in vitro" inhibition activity on JHE of *T. ni* at the nM range, two-three orders of magnitude higher than the sulfur-lacking TFMKs [67]. In "in vivo" experiments, application of OTFP and other β-thio-TFMKs on fifth-instar larvae of *T. ni* delayed pupation and suppressed JHE activity in a dose-dependent manner [67].

In *H. virescens* larvae JHE synthesis can be inhibited by a recombinant baculovirus expressing antisense JHE mRNA [68]. A considerable proportion of larvae showed intermediate developmental forms, such as larval segmentation, pupal cuticle, size, and behavior, as result of attempted but not accomplished larval-pupal molt. These features are similar to those induced by application of JH or JHE inhibitors. Expression of genes coding for insect JHE has resulted in recombinant baculovirus with a prospective utilization as biological insecticides [69]. These viruses are efficacious in the laboratory, greenhouse and field and dramatically reduce damage caused by insect feeding. Moreover, the recombinant viruses synergize and can be synergized by classical pesticides, such as pyrethroids, and since they are highly selective for insect pests, they can be used without disrupting biological control. As a consequence, a number of baculoviruses are currently used to control several species of Lepidoptera [69].

### 4. JUVENILE HORMONES

In holometabolous insects and in the absence of JH, the larval-pupal molt is regulated by 20-hydroxyecdysone. Therefore, the presence of JH or a JH analogue (JHA) during that period results in a supernumerary larva that is unable to give rise to normal adults, and, as consequence, many JHAs have been prepared and tested for insecticidal activity [70]. Some of them, like methoprene (24, Fig. 5), resemble JH in their basic structure, but several highly active compounds with less apparent similarity to JHs have also been synthesized and tested, for instance fenoxycarb (25), pyriproxyfen and diofenolan. Application of these compounds may produce morphological deformity and sterility in adults, suppress egg production by females, and inhibit oviposition. Thus, fenoxycarb has been used for the control of a number of stored wheat and rice pests, coding moth, leafrollers, pear psyllids, diaspidid scales, fleas and mosquitoes [71]. Pyriproxyfen is active against mosquitoes, houseflies, scales whiteflies, aphids and pear psylla, and diofenolan can be used against lepidopteran pests in citrus, grapes and olives as well as against scale insects [71].

![Fig. 5. Structures of methoprene (24) and fenoxycarb (25) as juvenile hormone analogues and tebufenozide (26) as ecdysteroid mimic.](image-url)

Inhibition of JH biosynthesis is also an attractive target for the development of new biorational insect control agents [72-74]. Among the most active inhibitors of JH biosynthesis it should be noted compactin [75], piperonyl butoxide [76], fluoromevalonate [77] (see below) and fluvastatin [78]. A number of nitrogen heterocycles, as ligands of cytochrome P450 monoxygenase, have resulted also as remarkable inhibitors of JH synthesis. Thus, imidazoles with a JH-like terpene chain induced precocious metamorphosis in the silkworm *B. mori* after topical application to the larvae or when administered to the diet
[79], and 1,5-disubstituted imidazoles have been described to inhibit JH synthesis "in vitro" and "in vivo" in the cockroach 
Diplopoda punctata (IC₅₀ 64-820 nM) [80]. Brevioxin, a heterocyclic oxime from extracts of 
Penicillium brevicompactum, displayed precocious metamorphosis on Oncopeltus fasciatus by inhibition of the 
final steps of JHIII biosynthesis [81]. Several fluoromevalonates have been prepared as anti-JH (AJH) 
compounds [77], and among them 6-fluoromevalonate was the most active on Manduca sexta (ED₅₀ 0.7 mg/g body 
weight) and prevented normal ecysis to pupa when it was administered to the diet of T. ni larvae. The AJH activity 
was postulated to be due to inhibition of JH biosynthesis at the level of enzymatic phosphorylation of mevalonate 
and homomevalonate. Allatostatins are neuropeptides which also reversibly inhibit JH biosynthesis by the corpora allata 
of moths, cockroaches and crickets [82]. However, so far no attempts have been made to extend these studies for the 
development of new approaches for pest control.

5. JUVENILE HORMONE EPOXIDE HYDROLASES

The two primary metabolic degradation pathways of JH in insects are the ester hydrolysis by JH esterase and the 
epoxide hydration by an epoxy hydrolase (EH). While in general JH esterase is more important than JH-EH in 
Lepidoptera, in the southern house mosquito Culex quinquemaculatus activity of the latter enzyme exceeded that 
of JH esterase throughout most of the 4th stadium by ca. 6-fold. This suggests that JH-EH and not JH esterase has a 
dynamic role in the initiation of metamorphosis [83]. Therefore, development of JH-EH inhibitors may be also 
important not only to demonstrate the biological role of this enzyme but also for their potential application in pest 
control strategies. Several glycidyl ethers and epoxy alcohol JH analogues have been examined as inhibitors of JH-EH on 
M. sexta but only the epoxy analogues provided significant levels (µM) of inhibition [84]. Linderman and coworkers 
have found that those inhibitors designed to mimic a polarized or ionic transition state resulted moderately active 
against T. ni JH-EH [85], and that the potency of the inhibitors was dependent on the absolute configuration of 
the epoxy group [86].

6. PROTEASES

The possible role of protease inhibitors (PIs) in plant 
protection was investigated as early as 1954 when trypsin 
inhibitors present in soybean were shown to be toxic to the larvae of the flour beetle Tribolium confusum. Since then, 
there have been many reports of PIs in "in vitro" assays against gut proteases and in "in vivo" when applied on 
artificial diets [87]. According to the active amino acid in the "reaction centre", protease inhibitors can be classified as 
serine, cysteine, aspartic and metallo-proteases inhibitors.

6.1 Serine Plant Inhibitors

Serine proteinases, such as trypsin, chymotrypsin and 
elastase, are responsible for the initial digestion of proteins 
in the gut of most higher animals [88]. These types of 
proteinases differ on their mode of action: trypsin specifically cleaves the C-terminal to residues carrying a 
basic side chain (Lys, Arg), chymotrypsin acts on residues carrying a large hydrophobic side chain (Phe, Tyr, Leu), and 
elastase on residues carrying a small neutral side chain (Ala, Gly). Inhibitors of serine proteinases have been described in many plant species, being trypsin inhibitors the most 
common type. This fact, along with that mammalian trypsin is readily available, have made this class of proteinase 
inhibitors the subject of intense research. Such studies have provided a basic understanding of the mechanism of action 
that applies to most serine proteinase inhibitor families, except to α₂-macroglobulin. This mechanism involves 
reaction of the reactive site of the inhibitor (a peptide bond) with the active site of the enzyme in a substrate-like manner 
[89]. In each case contact occurs over a small portion of the enzyme and the inhibitor, but over this portion the fit is 
excellent and numerous van der Waal interactions, hydrogen bonds and salt bridges are formed. The complex formation 
occurs with relatively small conformational changes although a small conformational adaptation of the inhibitor 
readily takes place.

Serine plant inhibitors induce antinutritional effects on 
several lepidopteran insect species, reducing larval growth and producing mortality at certain doses. α₂- 
Macroglobulins, high molecular weight proteins that “inhibit” other proteinases by digesting them, reduced larval 
growth inducing occasional death in the Australian sheep blowfly Lucilia cuprina, when incorporated into the artificial 
diet [90]. Also, saponins, a group of compounds which protect plants against insects attack, have been shown to 
reduce larval growth in the flower beetle Tenebrio molitor [91] and in the European corn borer O. nubilalis [92], 
among others. These results suggest that these proteases are key in protein digestion in these insects and that their 
inhibition leads to an almost complete blockade of 
digestion. Upon administration to S. littoralis larvae, alfalfa 
saponins elicited prolongation of the larval and pupal stages, retarded growth, increased mortality and reduced fecundity 
and fertility [93]. The authors suggested that inhibition of the digestive enzymes and interference with the sterol 
metabolism could be involved in the activity of these compounds. Other insects, which have been sensitive to 
serine inhibitors, are H. zea, Spodoptera exigua, 
Callosobruchus maculatus, M. sexta [88] or the black field 
cricket Teleogryllus commodus [94].

In S. nonagrioides, serine proteinases and exopeptidases 
(carboxypeptidase A, B and leucine aminopeptidase) are the 
major proteolytic enzymes [95]. "In vitro", the esterase 
inhibitor DIMBOA (2,4-dihydroxy-7-methoxy-1,4-
benzoazin-3-one), present in maize and other cereals, 
inhibited the activity of esterase, carboxypeptidases and 
aminopeptidase, but exhibited no effect on trypsin, 
chymotrypsin and elastase. "In vivo", DIMBOA reduced 
growth of S. nonagrioides larvae causing larval and pupal 
mortality when larvae were fed on maize inbred plants [96]. 
The authors suggested that the activity of the compound 
could be attributed to the reaction of the chemical with 
nucleophilic residues in the active centre of the enzyme [97], 
a similar mechanism of action proposed for the action of 
TFMKs (see above).
6.2 Cysteine Proteinase Inhibitors

The discovery that many Coleoptera and Hemiptera utilize cysteine proteinases (papain, calpains, asparaginases and cystatins) for digestive proteolysis has implicated cysteine proteinase inhibitors (CPIs) as plant defensive proteins [98,99]. Plant defensive CPIs are, predominantly, inhibitors of the cystatin superfamily, which occurs ubiquitously among plants, animals and microorganisms. In animals, cystatins are involved in the regulation of protein turnover and in the attenuation of pathological processes, such as viral replication, bacterial infection, chronic inflammation and tumor metastasis [87]. As an example of the prospects of utilization of CPIs, a single major proteinase was effectively inhibited by the potato CPI-10' (IC\textsubscript{50} 31 nM) and chicken egg white cystatin (IC\textsubscript{50} 121 nM) in the southern corn rootworm Diabrotica virgifera virgifera [100]. Incorporation of the potato CPI into the diet resulted in significant increase in larval mortality and weight loss, suggesting that expression of these inhibitors by transgenic corn plants in the field is a potentially attractive method to increase resistance to the pest.

7. MOLTING HORMONE MIMICS

Molting hormones regulate molting in immature stages of insects. At certain application times the molting hormones elicit abnormal molting with lethal developmental and reproductive disorders [101]. However, their high cost, rapid degradation and low species specificity has precluded attempts for practical application. Nevertheless, compounds mimicking the action of natural ecdyso steroid hormones have been used to control insect pests, such as azadirachtin [102]. Azadirachtin elicits a delay or a permanent block of molting by reducing ec dys teroid titer or by inhibiting the conversion of ecdysone to the more active 20-hydroxyecdysone. At sublethal concentrations, azadirachtin prolonged larval instars and reduced food intake on S. littoralis when incorporated into artificial diet [103]. Other compounds with ecdysteroid activity mimic the action of natural ecdysteroid hormones by binding to the same hormone receptors, and therefore elicit accelerated molting and disruption of reproduction [104]. Among them, several bisacylhydrazines have been found to display potent ecdysteroid activity resulting, after ingestion with the diet, in premature lethal molts in several lepidopteran, dipteran and coleopteran larvae, stop feeding within 4-16 h, reduction of egg production, ovicidal activity and disruption of normal spermatogenesis [71]. In addition, bisacylhydrazines exert low acute toxicity to mammals, birds and fish. Among the most potent bisacylhydrazines there should be noted tebufenozide (RH-5992) (26, Fig. 5), halofenozide (RH-0345) and methoxyfenozide (RH-2485). In the field tebufenozide is highly active against Cydia pomonella and leafrollers in apples, S. exigua in cotton, L. dispar in forestry, Diatraea saccharalis in sugarcane and numerous other lepidopteran pests in vegetables and ornamentals. Halofenozide, in turn, is active against the scarab beetles, such as Popillia japonica, Phyllophaga spp., etc., caterpillar pests of apple, corn, cotton, grape, rice and vegetables. The practical use of ecdysteroid agonists and azadirachtin alone or combined, is spreading rapidly for control some insect populations [104].

8. PHENOLOXIDASE INHIBITORS

Phenoloxidase (PO), the enzyme responsible for the biosynthesis of melanin, is considered as an important component of insects' immune system. The enzyme is not only involved in defense reactions but also in other physiologically important processes, such as sclerotization of the cuticle, an essential step for the survival of all insects [105]. PO, in association with other cuticular enzymes, generate a number of reactive intermediates that crosslink structural proteins and chitin, causing hardening of the cuticle. Moreover, in wound healing massive amounts of haemolymph loss is partly prevented by the action of PO by rapidly depositing melanin pigment at the wounding site [106]. In addition, PO-generated cytotoxic quinones may play an important role in killing opportunistic intruder microorganisms at the wounding site. POs are stored as inactive proenzymes and, as such, many of them have been purified and their cDNAs sequenced [107].

The first inhibitors of PO were isolated from pupal extracts of the house fly M. domestica and characterized as three low molecular weight proteins (range 2350-3350 D). These inhibitors are heat and pH stable and elicited competitive inhibition of endogenous PO activity [108,109]. The primary sequence of one of the inhibitors contains dopa as an unusual amino acid at position 32 and has a cystine motif commonly found in snail and spider toxins [109]. Another high molecular weight PO inhibitor from M. sexta larval haemolymph has been characterized as a pH and heat labile glycoprotein [110]. It forms a tight complex with PO, that cannot dissociate by the action of SDS and Triton X-100. Another inhibitor of PO is oxalate, a non-protein inhibitor, which appears to inhibit the PO responsible for the sclerotization of cockroach ootheca, thereby protecting the eggs [111]. However, in spite of their important role in regulation of PO oxidase, it is not clear at present whether these inhibitors are associated with the insect immune system or not. In addition, no studies on the possible application of these inhibitors on pest control have been undertaken so far.

CONCLUSIONS

Pest management continues to be an important challenge for the agricultural community. The continuously increasing concern over environmental health and public safety has led to the prohibition of some highly effective broad-spectrum chemicals from the market, and therefore new alternative approaches have been developed to improve crop protection. As a result, new more specific and less toxic biorational agents have been discovered and their mode of action elucidated. However, many of the new biorational products, which only constitute 2-3% of the insecticide market, have two important drawbacks: high production cost and limited applicability. Therefore, new studies at the target enzyme level will serve as the basis for the development of efficient and environmentally-friendly new insect control agents. In this context, application of the latest advances in biotechnology and genetic engineering, utilization of emergent recombinant DNA technology and expression of pesticide proteins to induce plant natural defensive responses should play a central role in the discovery of such agents.
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ABBREVIATIONS

PBP = Pheromone binding protein
PDE = Pheromone degrading enzyme
ODE = Odorant degrading enzyme
TFMK = Trifluoromethyl ketone
OTFP = 3-oclythiotrifluoropropanone
EAG = Electroantennogram
AOE = Aldehyde oxidizing enzyme
NTFP = 3-nonylthiotrifluoropropanone
ACH = Acetylcholinesterase
JH = Juvenile hormone
JHA = Juvenile hormone analogue
EH = Epoxide hydrolase
AJH = Antijuvenile hormone
PI = Protease inhibitor
CP = Cysteine protease
CPI = Cysteine protease inhibitor
PO = Phenol oxidase

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