Interactions between piperonyl butoxide analogues and metabolic enzymes conferring insecticide resistance

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A thesis submitted for the Degree of Doctor of Philosophy
from Imperial College London
To my Savvas
Declaration

I hereby declare that I have composed this thesis. The work within this thesis is a record of my own work; any collaborative work has been specifically acknowledged.

Despina Philippou
June 2010
Abstract

The insecticide synergist, piperonyl butoxide (PBO), has been used to reduce resistance factors and to characterise metabolic resistance resulting from mixed function oxidases (MFOs). Studies have also shown that PBO can interact with resistance-associated esterases.

It is well-documented that the mechanism by which PBO inhibits P450s is the interaction of the methylenedioxyphenyl (MDP) moiety of PBO molecule with the haem moiety of the enzyme. To investigate the interactions between PBO and esterases, a structure activity relationship (SAR) study was carried out using analogues of PBO and E4, a resistance-associated esterase from the peach-potato aphid *Myzus persicae*. Results indicate that the polyether and the alkyl chain were fundamentally important in this interaction. Removing one oxygen atom from the MDP moiety of PBO did not affect the binding affinity, but increased affinities resulted from the replacement of the polyether with an alkynyl ether chain or by increasing the length of the alkyl chain.

An analogue, EN 16/5-1, that retains the ability to interact with esterases but loses the ability to act on P450s, due to a modification to the MDP moiety, was used in conjunction with PBO to facilitate characterisation of metabolic resistance in *M. persicae* and pollen beetle *Meligethes aeneus*.

Selected analogues of PBO containing the alkynyl ether side chain exhibited high synergistic effects *in vivo* and were found to be potent inhibitors of O-deethylation of 7-ethoxycoumarin when tested against pyrethroid resistant *M. aeneus*.

The capability of some of the analogues to act as insecticide synergists was investigated *in vivo* against two *M. persicae* clones possessing different metabolic resistance profiles.

Finally, the potential of PBO analogues to provide potent and/or specific synergism to overcome insecticide resistance and reduce insecticide titre applied to crops is considered.
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Abbreviations

AChE  Acetylcholinesterase
Arg   Arginine
Asp   Aspartate
ATChI Acetylthiocholine iodide
CL    Confidence limits
DTNB  5, 5’ Dithio-bis (2-nitrobenzoic acid)
DEAE  Diethylaminoethyl
DTT   Dithiothreitol
E4    Esterase isozyme
ECOD  O-deethylation of 7- Ethoxycoumarin
EDTA  Ethylenediaminetetraacetic acid
E_{pb} Semi-purified pollen beetle esterases
FE4   Variant of E4
FUₘₙ Fluorometric Units
GLM   Generalized Linear Model
Gly   Glycine amino acid
GST   Glutathione-S-transferases
I     Index value: Percentage of binding affinity of analogues to E4
IC₅₀  Inhibitor concentration required to give 50% inhibition of enzyme activity
IPM   Integrated pest management
IRM   Integrated resistance management
kdr   Knockdown resistance
LD₅₀  Lethal concentration to kill 50% of the population
Leu   Leucine
LSD   Least Significant Difference
MDP   Methylenedioxyphenyl
Met   Methionine
MFOs  Mixed function oxidases
mOD   Milli Optical Density
n     Number of replicates per treatment
N     Total number of experimental replicates
NADPH Nicotinamide adenine dinucleotide phosphate tetrasodium salt
OP    Organophosphorus insecticides
P450  Cytochrome P450 mono-oxygenases
PAGE  Polyacrylamide Gel Electrophoresis
PBO   Piperonyl butoxide
Phe   Phenylalanine
PMSF  Phenylmethanesulfonyl
ppm   Parts per million
PTU   Phenylthiourea
RF    Resistance factor
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<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VGSC</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>1-NA</td>
<td>1-naphthyl acetate</td>
</tr>
<tr>
<td>7-EC</td>
<td>7-Ethoxycoumarin</td>
</tr>
<tr>
<td>7-OH</td>
<td>7-Hydroxycoumarin</td>
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Chapter 1

Introduction

1.1 Insecticide resistance

Insects are the largest animal group in the world in terms of number of species (75% of all animal species are insects) and are of great ecological and economical importance. In agricultural systems insects can either be of beneficial importance, for example honeybees, *Apis mellifera* Linnaeus (Hymenoptera: Apidae) a major pollinator of food plants and producer of honey, or be pests, destroying up to 30% of the potential annual harvest (Grimmelikhuijzen *et al.*, 2007). Additionally, many insect species are vectors of human and animal diseases (Lounibos, 2002).

The use of insecticides plays a major role in controlling populations of insect pests, but widespread use of such chemicals has resulted in selection of genotypes which display a resistant phenotype and many species have developed mechanisms to resist insecticide treatments (Feyereisen, 1995). Indeed, resistance to many classes of insecticides is now widespread and is viewed as an extremely serious threat to crop protection and control of insect vectors worldwide (Nauen, 2007).

Insecticide resistance is a “pre-adapted” phenomenon and its development depends on the genetic variability present in an insect population. In any insect population individual insects may have mutations that allow them to survive insecticide treatment (Denholm and Devine, 2001). This is best demonstrated when considering that the response to a stimulus, such as exposure to insecticide, in an insect population will be normally distributed. This means that some individual insects will be controlled at a low dose, others will require a somewhat higher dose and a few will survive an even higher dose. In the next generation, insects derived from the surviving individuals will not significantly shift the response dose of the resulting population. These individuals represent the extreme of a normal distributed population and are unable to contribute to the resistance development. However, if the population contains individuals that carry pre-existing genetic alterations, i.e. random mutation which confers the
ability to survive exposure to an insecticide, then this genetic trait will be passed on to the next generation. Individuals that do not possess the genetic trait will not survive and such a process will “select” those individuals which have the “resistance gene”. Therefore, the gene pool is enriched with such genes and “selection for resistance” occurs as the level of resistance in the population rises (Price, 1991).

Many definitions of insecticide resistance have been proposed and probably that proposed by Sawicki (1987) and subsequently modified by Feyereisen (1995) is the most inclusive: “Resistance marks a genetic change in response to selection. Individuals carrying genetic traits for coping with the chemically hostile environment survive and reproduce, thereby passing on these traits to their progeny. Continued selection pressure exerted by the insecticide rapidly increases the frequency of the genetic trait (resistance) in the population.”

The intensity, dose rate and timing of applications of an insecticide to control insect populations result in the selection of resistant insect forms carrying resistance genes, which survive and pass the resistance trait to their offsprings (Nauen, 2007). The rate at which resistance develops also depends on the reproduction, the migration and the host range of the pest. Insects with short life cycles and high reproductive rates can rapidly build up resistant populations (Hemingway and Ranson, 2000). Development of resistance can occur more rapidly in greenhouses, where insects have a short generation time, the gene pool remains restricted as there is little or no immigration of susceptible individuals and the rate and frequency of insecticide applications are high (Comins, 1977).

There are four main mechanisms that enable insects to resist insecticides and these are: increased metabolism, changes to the target protein, reduced penetration of insecticides through the insect cuticle (not discussed in the present work) and behavioural resistance (not discussed in the present work) (Oppenoorth, 1985; Sparks et al., 1989).

### 1.2 Metabolic Resistance
A major factor in the development of insecticide resistance is the ability of an insect to detoxify the insecticide at a rate to limit the accumulation of the active material at the target
site. Insecticide detoxification by insects results from a variety of metabolic processes in which the active material is converted into a non-toxic substrate or into a form suitable for rapid elimination from the body (Fukuto, 1990; Nebbia, 2001). Three main groups of enzymes, esterases, mixed function oxidases (MFOs) and glutathione-S-transferases (GSTs) have been reported to confer resistance in several insect species (Oppenoorth, 1985).

### 1.2.1 Esterases

Esterases are enzymes that catalyse the hydrolysis of ester-containing compounds to their corresponding alcohol and acid (Figure 1.1). A common and widely recognised classification proposed by Aldridge groups esterases into three types (A, B and C) based on their interaction with organophosphorus insecticides (OPs). A-esterases have an active site cysteine residue and hydrolyse OPs; B-esterases hydrolyse OPs and have an active site serine; C-esterases do not degrade OPs (Aldridge, 1953).

![Figure 1.1 Esterase hydrolysis. Esterases hydrolyse an ester via the addition of water to form the corresponding alcohol and acid (adapted from Wheelock et al., 2005)](image)

Esterases play an important role in the metabolism and detoxification of many exogenous ester-containing compounds such as OP, carbamate and pyrethroid insecticides (Wheelock et al., 2005). Metabolic resistance associated with esterases involves hydrolysis of ester bonds and/or sequestration of the insecticides (Devonshire and Moores, 1982; Gupta and Dettbarn, 1993; Casida and Quistad, 2004; Oakeshott et al., 2005; Wheelock et al., 2005). Overproduction of esterases (quantitative) and/or qualitative changes in enzyme structure are both mechanisms by which esterases are associated with resistance to insecticides (Yan et al., 2009).
Overexpression of esterases associated with metabolic insecticide resistance can occur either by amplification of esterase genes or by upregulation, or a combination of both and has been documented in numerous insect species (reviewed by Li et al., 2007). The overproduction of a specific carboxylesterase, E4 or its closely related variant FE4, by the resistant peach-potato aphid Myzus persicae Sulzer (Hemiptera: Aphididae) is perhaps the most extensively studied example of insecticide detoxification by gene amplification (Field et al., 1988, 1993; Field and Devonshire, 1998). These esterases with broad overlapping substrate specificities can account for as much as 1% of the aphid’s total protein and can hydrolyse and/or sequester insecticides resulting in high levels of insecticide resistance (Devonshire and Moores, 1982). In highly resistant aphids the elevated levels of E4/FE4 enzymes result from the amplified genes, with gene copy number rising up to approximately 80 copies (Field et al., 1999). Although amplified E4/FE4 are not normally found together in a single aphid or even population, the two genes were present together in the progeny of laboratory crosses between E4 and FE4 clones (Blackman et al., 1996) and the combination has been found in a small number of aphids collected from Greece (Blackman et al., 1999). Amplified esterases associated with insecticide resistance have also been found in mosquitoes of the Culex genus such as Culex pipiens pipiens, C. p. quinquefasciatus and C. tritaeniorhynchus Linnaeus (Diptera: Culicidae) (reviewed by Hemingway et al., 2004). For example, it was found that amplified esterase-based mechanisms in C. p. quinquefasciatus involved the co-amplification of two different esterases, both present in 80 copies in the Pel RR strain (Paton et al., 2000). Gene amplification of an identical gene to that in insecticide-susceptible insects was also found to be the mechanism underlying elevated esterase (NI-EST1) in OP resistant brown planthoppers Nilaparvata lugens Stal (Hemiptera: Delphacidae) (Small and Hemingway, 2000).

Overexpression of carboxylesterases associated with insecticide resistance may also occur via upregulation of the corresponding genes. In OP resistant Aphis gossypii Glover (Hemiptera: Aphididae) it was found that the relative transcription level and gene copy number of a carboxylesterase were 4.54- and 0.97-fold higher respectively, compared to the susceptible strain, indicating that the increased expression of the carboxylesterase resulted from increased transcription levels rather than gene amplification (Cao et al., 2008). In OP resistant B-biotype Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae) it was found that two carboxylesterase
genes (coe1 and coe2) were associated with resistance with coe1 being overexpressed approximately ~ 4-fold in the OP resistant strain and that the elevated expression was not related to gene amplification. It was further suggested that OP resistance in this strain was associated with modified transcriptional control (Alon et al., 2008). Overproduction of resistance-associated esterases has been also demonstrated in many other important agricultural insect pests including the two-spotted spider mite Tetanychus urticae Koch (Acari: Tetranychidae) (Van Leeuwen and Tirry, 2007) and the western flower thrip Frankliniella occidentalis Pergande (Thysanoptera: Thripidae) (López-Soler et al., 2008).

Metabolic resistance may also result from qualitative changes in esterase activity as demonstrated in early studies on OP resistant houseflies, Musca domestica Linnaeus (Diptera: Muscidae). In this case, resistant houseflies were shown to be associated with decreased esterase activity compared to susceptibles, due to structural changes in the enzyme that facilitated the hydrolysis of OP insecticides but prevented or reduced hydrolysis of model substrates conventionally used for biochemical characterisation of esterase activity. This became known as the “mutant ali-esterase theory” (Oppenoorth and van Asperen, 1960). A mutant esterase has also been reported in malathion and diazinon resistant sheep blowfly, Lucilia cuprina Wiedemann (Diptera: Calliphoridae) where structural changes in a specific esterase, isozyme E3, have a null phenotype on gels stained using standard esterase substrates (Campbell et al., 1997). It was shown that the gene encoding E3 in the diazinon resistant sheep blowfly differed at five amino acid sites compared to the OP susceptible. One of these substitutions, replacement of glycine to aspartate (Gly137Asp) was found to be within the active site of the enzyme and alone was responsible for both the loss of carboxylesterase activity and the elevated OP hydrolase activity. Modelling of Asp137 in the homologous position in acetylcholinesterase suggests that Asp137 may act as a base to orientate a water molecule in the appropriate position for hydrolysis of the phosphorylated enzyme intermediate (Newcomb et al., 1997). Another mutation, Trp251Leu, was also identified in OP resistant L. cuprina conferring malathion resistance (Campbell et al., 1998). Both above mutations were found in OP resistant M. domestica conferring diazinon and malathion resistance, respectively (Claudianos et al., 1999). Additionally, the same tryptophan residue was found to be mutated to glycine in the parasitoid wasp Anisopteromalus calandrae Howard (Hymenoptera: 23
Pteromalidae) (Zhu et al., 1999). Qualitative changes in esterase activity have also been documented in other OP resistant insect species including the mosquito C. tarsalis (Ziegler et al., 1987), the diazinon resistant screwworm Cochliomyia hominivorax Coquerel (Diptera: Calliphoridae) (Carvalho et al., 2006, 2009) and the malathion resistant parasitoid Habrobracon hebetor Say (Hymenoptera: Braconidae) (Mendoza et al., 2000).

**1.2.2 Mixed function oxidases**

Mixed function oxidases (MFOs) are an important family of enzymes involved in the detoxification of xenobiotics such as insecticides and plant toxins and for metabolising endogenous compounds such as hormones (Agosin, 1985). Cytochrome P450 mono-oxygenases (P450s) are the most important and extensively studied component of the MFOs (reviewed by Werck-Reichhart and Feyereisen, 2000).

The P450s belong to the group of heme-thiolate proteins, which feature a proximal thiolate ligand from the amino acid cysteine (Figure 1.2). The P450 enzymes derived their name from the formed prominent peak at about 450 nm in the carbon monoxide Soret optical difference spectrum (Omura and Sato, 1964). This enzyme catalyses the reaction when one atom of molecular oxygen is incorporated into a substrate (e.g. insecticide) and the second atom of oxygen is reduced to produce a molecule of water according to the reaction:

\[
\text{Substrate (S) + NADPH + H}^+ + O_2 \rightarrow \text{SO + NADP}^+ + H_2O
\]

Activation of this reaction requires two electrons provided by the flavoprotein NADPH cytochrome P450 reductase although sometimes the second electron is provided by cytochrome b5 (Feyereisen, 1999; Murataliev et al., 2008).
P450s are found in virtually all aerobic organisms including mammals, birds, insects, plants and bacteria. For insects, P450s can be detected in a wide range of tissues such as midgut, fat bodies and malpighian tubules (reviewed by Scott, 1999) and the levels and activities can vary with different stages of development (Chung et al., 2009). Existence of multiple P450 isoforms within individual organisms, different expression patterns and wide substrate spectra confer high diversity (Scott and Wen, 2001).

Insect P450s are known to play an important role in the metabolism of many insecticides including the activation/detoxification of OPs (Hodgson et al., 1995a). Due to the broad substrate specificity of P450s, detoxification affects several classes of insecticides and thereby can confer cross resistance to unrelated compounds (Hodgson, 1985; Oppenoorth, 1985). Insecticide resistance has been associated with enhanced detoxification of insecticides by increased levels of P450 activity usually from gene overexpression rather than qualitative changes. Genes belonging to the families CYP4, CYP6, CYP9, and CYP12 have all been reported to be associated with insecticide detoxification (reviewed by Li et al., 2007).

In many cases the role of P450 in resistance has been implicated by correlation using model substrates such as, 7-ethoxycoumarin and 7-methoxycoumarin or molecular techniques or by a combination of both methods. Overexpression of P450 activity results, usually, from increased transcription rather than gene amplification (reviewed by Li et al., 2007). Enhanced level of P450 activity was reported to be correlated with permethrin resistance in the head louse, *Pediculus humanus capitis* De Geer (Anoplura: Pediculidae) (Audino et al., 2005) and in the

![Figure 1.2 P450 porphorin moiety in CYP3A4 visualised using Pymol, (DeLano, 2002). Oxygen atoms are shown in red, nitrogen in blue, sulphur in yellow and the iron in orange. Carbon atoms are shown in green as bond only and the hydrogen atoms have been omitted for clarity.](image)
cotton bollworm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Yang *et al.*, 2005). In the latter, *H. armigera* exhibited resistance to various pyrethroid insecticides after selection with fenvalerate and phoxim. It was found that the monooxygenase activities in the midguts of sixth-instar larvae of the selected strain to various substrates such as p-nitroanisole, 7-ethoxycoumarin and 7-methoxycoumarin were 3.7-, 4.7- and 10-fold higher respectively, compared with that of the unselected strain (Yang *et al.*, 2005). Elevated P450 activity has also been reported in combination with high esterase levels in a field resistant strain of *T. urticae* exhibiting resistance to bifenthrin, dicofol and fenbutatin (Van Leeuwen *et al.*, 2005). With imidacloprid, correlation between elevated P450 activity and resistance has been reported in B-biotype *B. tabaci* (Rauch and Nauen, 2003; Wang *et al.*, 2009) and recently it has been found that overexpression (up to 17-fold) of a single P450 gene, CYP6CM1, was correlated with imidacloprid resistance in both B- and Q- biotypes (Karunker *et al.*, 2008, 2009). Overexpression of P450 activity by co-upregulation of three P450 genes has been reported in a permethrin resistant *M. domestica* strain after continual exposure to this insecticide (Zhu *et al.*, 2008).

Elevated P450 activities have also been reported in human disease vectors such as mosquitoes (reviewed by Hemingway *et al.*, 2004). Overexpression of P450 activities by upregulation was reported in OP and pyrethroid resistant *Aedes aegypti* Linnaeus (Diptera: Culisidae) in combination with a target site mutation (Marcombe *et al.*, 2009). Additionally, it has been found that five P450 genes were upregulated (>2.5-fold) in a pyrethroid resistant strain of *C. quinquefasciatus* and the expression ratio for the three highest (CYP9M10, CYP4H34 and CYP6Z10) was found to be 264-, 8.3- and 3.9- fold respectively, when compared to the susceptible (Komagata *et al.*, 2010). Although overproduction of P450 activity by amplification is not common, an amplification has been reported in the malaria vector *Anopheles funestus* Giles (Diptera: Culicidae) where two genes (CYP6P9 and CYP6P4) were amplified 25- and 51- times in pyrethroid resistant females (Wondji *et al.*, 2009).

Although metabolic resistance due to a qualitative change in P450 activity is also a rare event pyrethroid resistance in a strain of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) has been identified. This mutant P450, resulting from three amino acid substitutions
(Arg335Ser, Leu336Val and Val476Leu) in CYP6A2, was associated with DDT resistance in the RDDTR strain, with the recombinant protein showing an enhanced ability to metabolise DDT (Amichot et al., 2004).

1.2.3 Glutathione-S-Transferases

Glutathione-S-transferases (GSTs) are a large family of enzymes found in vertebrates, plants, insects, yeast and aerobic bacteria involved in the detoxification of a wide range of endogenous and exogenous compounds (Salinas and Wong, 1999).

Generally, GSTs metabolise a wide range of hydrophobic toxic compounds such as insecticides and other endogenous substrates, by catalysing the conjugate of endogenous GSH to the hydrophilic centre of the toxic substrates. This conjugation process converts the products to more water soluble forms, allowing them to be more easily excreted from the cells (Dauterman, 1985).

The implication that GSTs could be involved in the metabolism of insecticides was made initially by the correlation of enhanced GST activity in insecticide resistant insects compared with their susceptible counterparts (reviewed by Enayati et al., 2005). Elevated GST activity has been associated with gene amplification or more commonly through increases in transcription rather than changes in the enzymes (reviewed by Ranson and Hemingway, 2005).

Due to the broad substrate specificities of individual GSTs, these enzymes perform a wide range of functions including metabolism of different classes of insecticides. High enzyme activity was detected in abamectin resistant T. urticae and resistant strains of B. tabaci (Konanz and Nauen, 2004; Rauch and Nauen 2004). Involvement of enhanced GST levels in OP metabolism has also been reported in M. domestica (Wei et al., 2001; Kristensen, 2005), in the chlorfluazuron resistant diamondback moth Plutella xylostella Linnaeus (Lepidoptera: Plutellidae) (Sonoda and Tsumuki, 2005) and the malathion resistant tarnished plant bug Lygus lineolaris Palisot de Beauvois (Hemiptera: Miridae) (Zhu et al., 2007). Additionally, it has been suggested that elevated GSTs in pyrethroid resistant insects may protect the insects.
by sequestering the insecticide (Kostaropoulos et al., 2001). Insecticide resistance associated with enhanced levels of GST has also been reported in various mosquito species (Lumjuan et al., 2007; reviewed by Che-Mandoza, et al., 2009).

1.3 Target site resistance

Target site resistance occurs when genes encoding the proteins on which the insecticides act mutate, resulting in insensitive proteins. Modified acetylcholinesterase and knockdown resistance (kdr) are two resistance mechanisms which involve modifications to the target site of an insecticide and result in insensitivity to that insecticide (Feyereisen 1995).

Other known target site resistance mechanisms are the “Rdl” mutation(s) in the γ-aminobutyric acid (GABA) receptor, conferring resistance to cyclodiene insecticides such as dieldrin (not discussed in the present work) (ffrench-Constant et al., 1993) and mutation(s) in the nicotinic acetylcholine receptor (nAChR), the target for neonicotinoid insecticides (not discussed in the present work) (Liu et al., 2005).

1.3.1 Modified acetylcholinesterase

Acetylcholinesterase (AChE) is the key enzyme of the cholinergic system that terminates nerve impulses in the nervous system by catalysing the hydrolysis of the neurotransmitter acetylcholine (ACh) (Eldefrawi, 1985). AChE, a serine hydrolase, is the target of carbamate and OP insecticides (Casida and Quistad, 2005). Carbamates and OPs exert their toxicity by inhibiting AChE, thereby impairing the transmission of nerve impulses across cholinergic synapses, resulting in death of the insect (Fournier and Mutero, 1994).

Alterations in the primary structure of AChE reduce sensitivity to OPs and carbamates leading to the development of insecticide resistance to these compounds (Fournier, 2005). Initially, mutant forms of AChE were characterised, in vitro, by a lack of inhibition in the presence of a diagnostic concentration of insecticide. Such mutations usually result in a less active enzyme, but insensitive mutations can also result in a more active enzyme (Devonshire and Moores, 1984; Moores et al., 1996; Byrne and Devonshire, 1997). Sequencing of insensitive AChE genes has revealed several point mutations, most of which encode amino acids in the active site gorge (Fournier, 2005).
In the dipteran order of the Cyclorrhapha (true flies), only one AChE gene is present, *ace* (Huchard *et al.*, 2006). However in many other insect species, it has been shown that there are at least two genes, *ace-1* and *ace-2*, encoding AChE1 and AChE2, respectively (Fournier, 2005). In *M. persicae* and *A. gossypii*, it has been shown that although the *ace-2* gene has high similarity with the *ace* of *D. meganogaster*, it is mutations in *ace-1* that correlate with insensitivity to insecticides (Li and Han, 2002; Andrews *et al.*, 2002, 2004).

Identification and characterisation of AChE in resistant *M. domestica* strains revealed five amino acid substitutions that either singly, or in combination, conferred different spectra of insecticide resistance. All mutations were located within the active site of the enzyme, close to the catalytic triad (Walsh *et al.*, 2001). Recently, it has been shown that point mutations in the AChE gene of *T. urticae* strains correlate with OP and carbamate resistance (Khajehali *et al.*, 2010). Mutant AChE correlates with reduced insensitivity to insecticides in several other insects including the olive fruit fly *Bactrocera oleae* Rossi (Diptera: Tephritidae) (Vontas *et al.*, 2002), the stored product insect *Liposcelis borychophila* Badonnel (Pscoptera: Liposcelididae) (Chai *et al.*, 2007), the oriental fruit fly *Bactrocera dorsalis* Hendel (Diptera: Tephritidae) (Hsu *et al.*, 2006, 2008), the codling moth *Cydia pomonella* Linnaeus (Lepidoptera: Torticidae) (Cassanelli *et al.*, 2006) and vectors of human diseases such as mosquitoes (reviewed by Hemingway *et al.*, 2004).

### 1.3.2 Knockdown resistance

The voltage-gated sodium channel (VGSC) protein found in nerve-cell membranes plays a critical role in the function of the insect nervous system. The VGSC is responsible for the conduction of sodium ions (Na⁺) across the membrane underlying the propagation of action potentials in the neuronal cells. During an action potential, the VGSC undergoes transitions between closed-resting, activated and inactivated functional states and the normal function of VGSC is essential for normal transmission of nerve impulses (reviewed by Wang and Wang, 2003). The insect VGSC is a large membrane protein that contains four repeating and homologous domains (I-IV) with each domain consisting of six hydrophobic transmembrane segments (S1-S6) (reviewed by Catterall, 2000). DDT, its analogues, pyrethrins and pyrethroid insecticides act on the VGSC, modifying the gating kinetics and resulting in
prolonged opening of individual channels, leading to paralysis and death of the insect (Narahashi, 2000).

Resistance to DDT and pyrethroids was first reported in resistant *M. domestica* by Busvine in 1951 and this type of resistance was termed “knockdown resistance” (kdr). When the VGSC was sequenced from different insects, comparisons between resistant and susceptible insects identified a number of amino acid substitutions that were associated with VGSC insensitivity (Soderlund and Knipple, 2003; Davies *et al.*, 2007a). Two substitutions in the VGSC, Leu1014Phe and Met918Thr were identified in pyrethroid resistant housefly strains and originally named as kdr and super kdr resistance (s-kdr), respectively (Williamson, *et al*., 1996).

The Leu1014Phe mutation within the IIS6 transmembrane segment of VGSC, has been identified in pyrethroid-resistant clones of *M. persicae* (Martinez-Torres *et al*., 1999). Later the Met918Thr mutation (s-kdr), in the domain between segments IIS4 and IIS5 of the channel protein, was also detected together with the Leu1014Phe mutation in some *M. persicae* clones. Presence of both mutations was associated with extreme resistance to different pyrethroids relative to aphids lacking the mutations (Anstead *et al*., 2004; Eleftherianos *et al*., 2008).

Different alterations in the amino acid sequence of the VGSC protein by inherited mutations in the VGSC genes have been identified in several other pyrethroid resistant insect species (reviewed by Davies *et al*., 2007b; reviewed by Soderlund, 2008). For example, changes in the VGSC have also been associated with pyrethroid resistance including the whitefly *B. tabaci* (Roditakis *et al*., 2006), the two spotted mite *T. urticae* (Tsagkarakou *et al*., 2009), the onion thrip *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) (Toda and Morishita 2009), the cattle tick *Rhipicephalus microplus* Boophilus (Morgan *et al*., 2009) and the human head louse *Pediculus humanus capitis* De Geer (Kasai *et al*., 2009). The genes that confer kdr are recessive which means that the trait will only be expressed in homozygous individuals (Daves *et al*., 2008).
1.4 Cross resistance and multiple resistance

Metabolic detoxification, in contrast to target site insensitivity, can often confer resistance to a number of different insecticides belonging to different chemical classes and this phenomenon is known as ‘cross resistance’. Resistance mechanisms in insects may be present individually or in combination. The presence of a number of resistance mechanisms in the same population is known as ‘multifactorial’ or ‘multiple resistance’ (Oppenoorth, 1985).

The occurrence of different resistance mechanisms in insect populations has the potential to present relatively high levels of resistance to one or more insecticides and thus confer cross resistance within or between chemical classes. The presence of such combinations has been demonstrated in many insect populations. For example, in *M. persicae* it has been demonstrated that elevated E4 and insensitive AChE can enhance the resistance level to pirimicarb by up to 600-fold (Moores *et al.*, 1994). Furthermore, a kdr mutation which on its own conferred 35-fold resistance to deltamethrin, was enhanced to 540-fold when present with highly elevated E4 (Martinez-Torres *et al.*, 1999). Several different combinations of elevated E4 activities and target site mutations (AChE, kdr and s-kdr) were also found in Italian strains of *M. persicae* conferring different levels of resistance to various insecticides (Criniti *et al.*, 2008).

Elevated P450 and GST activities were also found in an abamectin selected strain of *B. tabaci* conferring cross resistance to abamectin, imidacloprid and emamectin benzoate (Wang and Wu, 2007). Additionally, elevated P450 and esterase activity were reported to be correlated with resistance to thiamethoxam, imidacloprid, acetamiprid and abamectin in a strain of *B. tabaci* after 36 generations of selection with thiamethoxam (Feng *et al.*, 2010).

1.5 Insecticide synergists

Metcalf (1967) defined synergists as compounds that are non toxic in the dosage used but which serve to enhance the toxicity of a pesticide chemical when they are combined. Insecticide synergists act by inhibiting metabolic resistance mechanism(s) that would otherwise break down insecticide molecules and thus restore the susceptibility of insects to the chemical (Georgiou, 1983; Ishaaya, 1993). Synergists have been used commercially for many
years to enhance the efficacy of insecticides and have contributed significantly to improve insect control, especially where resistance has occurred (Bernard and Philogène, 1993).

An observation in 1938 that specific compounds enhanced insecticidal activity initiated the use of insecticide synergists (Weed, 1938). Many compounds were tested and in 1940 Eagleson described the use of sesame oil as a synergist when combined with insecticides containing pyrethrum or rotenone. Although Eagleson tested a range of different compounds, mostly other plant and animal oils, sesame oil was the most effective synergist (Eagleson, 1942). Later, Haller et al., (1942) fractionated sesame oil and tested the fractions with pyrethrum against houseflies, identifying the main components as sesamin and sesamolin, two methylenedioxyphenyl (MDP) compounds (Figure 1.3). Since then, many MDP compounds have been investigated as insecticide synergists including some compounds with high synergistic effects such as sesamex, tropital, isosafrole, safrole, propyl isome and piperonyl butoxide (PBO) (Figure 1.3). Of all of these synergists, only PBO was the first truly effective and commercially viable synergist (Wachs, 1947; Casida, 1970; Wilkinson, 1976).
1.5.1 Piperonyl butoxide as an insecticide synergist

Piperonyl butoxide was first synthesised by Wachs in 1947 using the raw material safrole, derived from the sassafras tree, which supplied the MDP moiety of the molecule. Nowadays PBO is made synthetically. Naturally occurring MDP compounds such as safrole, isosafrole and myristicin are found in a wide range of plants including those used as human food, such as parsnips, parsley, nutmeg, sesame seeds, pepper etc (Hodgson and Philpot, 1974).

It was initially suggested that PBO inhibited the oxidative metabolism of insecticides based on in vivo tests using houseflies (Sun and Johnson, 1960). Subsequent in vitro studies demonstrated that PBO and sesamex inhibited the oxidative detoxification of carbamates by rat liver microsomes (Hodgson and Casida 1960, 1961). In vitro studies using houseflies and rat liver further demonstrated that microsomal oxidation inhibition was due to the direct effects of P450 with MDP compounds. These studies demonstrated an apparent reduction in P450 levels in microsomes prepared from houseflies and mice treated with PBO (Perry and Bucknor, 1970; Matthews et al., 1970).

It was suggested that PBO inhibited P450s by the formation of a complex between the haem iron of the P450 and the carbene formed by either direct hydrogen abstraction from the methylene carbon or possibly by elimination of water from a hydroxylated methylene carbon of the MDP compound (Figure 1.4) (Dahl and Hodgson, 1979). Although the carbene, a short lived reactive intermediate, has never been demonstrated directly, this mode of action of the MDP compounds upon P450 receives the widest support and no evidence has been put forward to shed doubt on its validity (Wilkinson et al., 1984; Correia and Ortiz de Montellano, 2005).

Although PBO was generally considered to be a specific inhibitor of microsomal oxidases (Casida, 1970), it has been shown to inhibit resistance associated-esterases in some agriculturally important pests and also enhance insecticide efficacy against insects where esterase resistance mechanisms are present (Gunning et al., 1998; Moores et al., 1998; Young et al., 2005, 2006). Nevertheless the mechanism(s) by which PBO interacts with resistance-associated esterases remains unclear. The ability to interact with esterases means that using
PBO to identify the presence of metabolic resistance due to oxidases should be viewed with caution.

![Diagram of P450 enzymes inactivation](image)

Figure 1.4 The inactivation of P450 enzymes by methylenedioxyphenyl compounds involves oxidation of the methylene bridge to a species that forms a tight complex with the haem iron atom. As shown, the coordinating species is probably a carbene (adapted from Correia and Ortiz de Montellano, 2005).

1.5.2 Use of piperonyl butoxide

Piperonyl butoxide has been used to enhance the potency of many classes of insecticides including pyrethroids, OPs and carbamates (Casida, 1970) and more recently it has been shown to exhibit synergistic effects when used with neonicotinoid insecticides (Bingham et al., 2008).

The synergistic effects of PBO have been demonstrated in many cases where insecticide resistance is present and it has been suggested as a solution to control insect infestations including vectors of diseases such as mosquitoes (Kumar et al., 2002; Vijayan et al., 2007; Fakoorziba et al., 2009; Yadav, et al., 2009), scabies mites Sarcoptes scabiei De Geer (Astigmata: Sarcoptidae) (Pasay et al., 2009), horn fly Haematobia irritans irritans (Diptera: Muscidae) (Li et al., 2008) and agricultural insect pests such as B. tabaci and M. persicae (Bingham, et al., 2007, 2008). Recently, PBO has been shown to provide synergy with pyrethrins against different Liposcclidid psocid species for stored-grain protection and it has...
been suggested that PBO be recommended for protection and disinfestation in storage (Nayak, 2010).

In agriculture, PBO has been used as a mixture with pyrethroids to improve their efficacy against *H. armigera* in Australian cotton fields since the early 1990s (McCaffery, 1998; Forrester *et al.*, 1993). Young *et al.*, (2005) demonstrated that following topical application PBO required around 5 h to inhibit the resistance-associated esterases in *H. armigera*. After this time esterases gradually recovered until full activity was regained after 24 h. In *B. tabaci* similar studies revealed that PBO required around 10 h to inhibit the resistance-associated esterases (Young *et al.*, 2006). These findings show that the success of PBO as an insecticide synergist can be enhanced by using an appropriate pre-treatment time. Treatment with PBO prior to the insecticide application allows time for the metabolic enzymes to be fully inhibited before the insecticidal component is applied. Such a technique is known as ‘temporal synergy’ (Moores *et al.*, 2005). The ideal pre-treatment time is dependent on the insect species (Young *et al.*, 2006). However, pre-spraying crops with PBO several hours before spraying with the insecticide is clearly unsuitable for large areas and therefore novel formulations consisting of a microencapsulation of PBO and an active ingredient have been designed. These microencapsulated formulations give a burst release of PBO several hours before the burst release of the insecticidal component such as pyrethroids, carbamates or neonicotinoids. Such formulations have been used successfully to overcome metabolic resistance in *B. tabaci, H. armigera, A. gossypii* and *M. persicae* (Bingham *et al.*, 2007, 2008).

Studies on PBO usage alone showed that PBO exhibits a range of different effects on several insect species. The toxicity varies greatly between and within insect and mite species (Devine and Denholm, 1998a). In relatively high concentrations (>300 ppm), PBO was found to be lethal to all stages of the cattle tick *Boophilus microplus* Canestrini (Acari: Ixodidae) (Schuntner *et al.*, 1974). Furthermore, it has been found that at higher concentrations (>1000 ppm), PBO had insecticidal effects against all stages of the woolly whitefly *Aleurothrixus floccosus* Maskell (Hemiptera: Aleyrodidae) except eggs (Castener *et al.*, 1989; Garrido *et al.*, 1990). In laboratory experiments using strains of *B. tabaci* with different resistant backgrounds and in field trials, it was shown that PBO has little lethal effect upon adults at
concentrations around 2000 ppm resulting in insufficient control. The slight mortality observed was not correlated with the resistance mechanism or enzyme banding patterns exhibited by the whitefly strains (Devine and Denholm, 1998b; Devine et al., 1998). However, bioassays using a leaf dip method and field trials showed that PBO was an effective nymphicide against early instars of susceptible and insecticide-resistance whiteflies (Devine and Denholm, 1998b; Devine et al., 1998). In *M. domestica*, it was found that in artificial diets of 100-5000 ppm, PBO increased 11-fold the microsomal oxidase activity in 3rd instar larvae and inhibited 15-100% pupation and emergence. Additionally a concentration of 10000 ppm PBO caused approximately 50% decrease in egg production (Yu and Terriere, 1974). Field and laboratory experiments also demonstrated that treatment with PBO can cause slow growth in the mealworm *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), the milkweed bug *Oncopeltus fasciatus* Dallas (Hemiptera: Lygaeae) (Bowers, 1968), *A. gossypii* (Satoh et al., 1995), *L. curpina* (Kotze and Sales, 1994) and *B. tabaci* (Devine and Denholm, 1998b).

It has been shown that an selected insecticide resistant *P. xylostella* strain developed resistance to PBO after larvae were selected with PBO alone or with a mixture of PBO and fenvalerate for 5 and 6 generations, respectively (Chen and Sun, 1986; Hung and Sun 1989). In the mixture selected strain, the resistance to PBO and to PBO plus fenvalerate was unstable and reverted within five generations to those levels prior to selection. In the same report, Chen and Sun (1986) suggested that the strain selected with the mixture of PBO and fenvalerate may develop cross resistance to other MDP synergists, although the same strain remained sensitive to the synergistic action of another microsomal oxidase inhibitor, MGK 264. Considering the PBO selected strain, although Hung and Sun (1989) suggested that the resistance to PBO may develop due to qualitative or quantitative changes of the microsomal monooxygenases, the mechanism(s) were never resolved.

There are a few reports where the toxicity and/or the synergistic effects of PBO against beneficial insects have been studied. Contact bioassays with surfaces treated with 10000 ppm did not cause mortality to adults of the parasitoid wasp, *Diglyphus begini* Ashmead (Hymenoptera: Eulophidae) (Rathaman et al., 1992). However, a low concentration (1200 ppm) killed 74% of the pupated parasitoid wasp, *Cales noacki* Howard (Hymenoptera,
Aphelinidae) developing in *A. floccosus* hosts (Castener *et al*., 1989; Garrido *et al*., 1990) probably as a result of toxicity to the host (Devin and Denholm, 1998a). Synergistic effects of PBO with different classes of insecticides have also been reported in other parasitoid wasps such as *Diaeretiella rapae* M’Intosh (Hymenoptera: Aphidiidae) (Wu and Jiang, 2003), *Pteromalus puparum* Linnaeus (Hymenoptera: Pteromalidae) and *Diadromus collaris* Gravenhorst (Hymenoptera: Ichneumonidae) (Wu and Jiang, 2005). There were also high synergistic effects of PBO when used with different classes of insecticides (OPs, carbamates, pyrethroids), against the *P. xylostella* parasitoid, *Cotesia plutellae* Kurdj (Hymenoptera: Braconidae), although no synergism was reported when PBO was used with the chitin synthesis inhibitor chlorfluazuron (Wu and Jiang, 2004). Investigations of insecticide toxicity and synergistic effects of different enzymatic inhibitors including those caused by PBO against 18 different species of insects and their natural enemies have shown that PBO exhibited the highest synergism. The authors speculated that this resulted from PBO having multiple effects on the activity of esterases, GSTs and AChE (Wu and Miyata, 2005; Wu *et al*., 2007). Based on the high levels of synergism seen when PBO was used with various insecticides in *B. tabaci*, a similar assumption was made for the inhibitory effect of PBO on esterases and AChE (Kang *et al*., 2006). Finally, it has been observed that PBO inhibits AChE in carbamate resistant *H. armigera* (Gunning 2002, 2006).

### 1.5.3 Biphasic response of piperonyl butoxide

Many studies have shown that MDP compounds and some other xenobiotics can act as inducers as well as inhibitors of P450 activity and thus the effect on the enzyme activity is biphasic. The time course of these two activities (induction and inhibition) differs, with inhibition being relatively rapid and induction being relatively slow. It has been observed, mostly in mammalian P450s, that following a single dose of compound there is initially a decrease of P450 activity below control levels, followed by an increase above control levels with, ultimately, a return to the control level (Hodgson *et al*., 1995b). Studies have shown that MDP compounds including isosafrole and PBO are capable of inducing mammalian P450s and that both inhibition and induction may be isozyme-specific and different isozymes may be involved in the two activities of the same chemical (Adams *et al*., 1993; Hodgson *et al*., 1995b). Induction of P450 activity by isosafrole in non–mammalian species such as the
southern armyworm *Spodoptera eridania* Stoll (Lepidoptera: Noctuidae) has also been demonstrated (Marcus et al., 1986). In another study it was reported that PBO was capable of inducing the expression of P450 and GST genes in *D. melanogaster* (Willoughby et al., 2007). More recently, induction of P450 activity in a susceptible strain of *T. urticae* was investigated using a range of known P450 inducers including the MDP compound isosafrole in a series of *in vivo* and *in vitro* assays. Although many of the tested compounds induced P450 activity, 24 h treatment of mites with isosafrole did not influence the P450 O-deethylation activity (Van Pottelberge et al., 2008).
1.6 Aim and objectives of the project

The overall aim of this project was to investigate the interactions of the insecticide synergist PBO and its analogues with metabolic enzymes conferring insecticide resistance in the peach-potato aphid *M. persicae* and the pollen beetle *Meligethes aeneus* Fabricius (Coleoptera: Nitidulidae) by employing a combination of biochemical techniques and *in vivo* assays. The overall objective of the project is to identify more specific and potent insecticide synergists. The hypothesis tested is that by altering the chemical moieties of the PBO molecule, more specific and potent insecticide synergists can be designed.

The specific objectives of the project were:

- To investigate the interaction between PBO and esterases. Biochemical assays were performed using purified E4, a carboxylesterase derived from *M. persicae* conferring broad spectrum insecticide resistance. *In vitro* investigations included a structure activity relationship (SAR) study using a series of structurally similar compounds to PBO (analalogues) and the differences in their binding affinities towards E4 were assessed (Chapter 3).

- To characterise the metabolic resistance mechanism(s) in a Greek *M. persicae* clone (clone 5191A) to imidacloprid and the metabolic factors conferring differential response to α-cypermethrin in four *M. aeneus* populations using PBO and the analogue, EN 16/5-1. The two insect species were examined in a series of *in vitro* and *in vivo* assays incorporating the analogue in conjunction with PBO and results compared with those from insects with previously characterised resistance mechanisms (Chapter 4).

- To determine the synergistic effects of selected PBO analogues against four *M. aeneus* populations by comparison with PBO in a series of *in vivo* and *in vitro* assays (Chapter 5).

- To examine the synergistic effects of selected PBO analogues *in vivo* against two *M. persicae* clones with different resistance mechanism profiles using two different classes of insecticides (Chapter 6).
Chapter 2

General Materials and Methods

2.1 Insects

2.1.1 *Myzus persicae* rearing

Aphids for bioassays were reared on Chinese cabbage leaves (*Brassica rapa* L. var. Pekinensis c.v Wong Bok) (Brassicaceae) in small plastic box-cages (Blackman boxes) (Stewart Plastics Ltd, Surrey, UK) maintained at 18 ± 2°C with a 16 h light: 8 h dark photoperiod (Blackman, 1971). Each box contained two adult aphids placed on a leaf and left for 5 days to produce nymphs. After the 5th day the adults were removed and their esterase levels determined to ensure that no contamination or loss of esterase expression (reversion) among the different clones had occurred during the rearing process (Sawicki et al., 1980; ffrench-Constant et al., 1988; Field et al., 1989). After approximately 13-15 days adults were used for bioassays.

*Myzus persicae* clones, 5191A and 794JZ used for esterase purification were reared on 2 week-old Chinese cabbage plants, as above, in fine-netted cages under conditions described above. Cultures in cages were left for 3-4 weeks and then the aphids were collected and frozen at -20°C until required for esterase purification.

2.1.2 *Myzus persicae* clones

Five different *M. persicae* clones were used in this study: 4106A, TIV, 794JZ, 926B and 5191A. Clone 4106A is a laboratory insecticide-susceptible standard. Clones T1V and 794JZ are laboratory standard resistant variants possessing R₂ and R₃ levels of E4, respectively (Foster et al., 2005). Clone 794JZ additionally carries a kdr mutation conferring resistance to pyrethroid insecticides (Martinez-Torres et al., 1999). Clone 926B is a highly resistant (R₃) clone possessing FE4 variant of enhanced esterase (Moores et al., 1994). Clone 5191A was collected from tobacco in Greece in 2007. All clones have been established from single parthenogenetic females (Foster et al., 2005). All of the parthenogenetic stock cultures were provided by the Plant and Invertebrate Ecology Division, Rothamsted Research (for more details see Appendix 1.)
2.1.3 Meligethes aeneus populations

Five pollen beetle *M. aeneus* samples were collected throughout the spring and summer (May until July) 2008 from oilseed rape fields. A Swiss population, (Stein), was collected from Stein, Switzerland, in May 2008. Three populations originating from different locations in Poland (Rogalin, Lebork and Leszno) were kindly supplied by Dr Pawel Wegorek (Institute of Plant Protection, National Research Institute, Poland) in July 2008. Polish samples were immediately sent to Rothamsted in fine-netted bags for testing. All samples arrived in good condition and were tested (*in vivo* and *in vitro*) within 2-3 days. The sampling locations were regions of intensive rapeseed cultivation and had been sprayed heavily with many classes of insecticides, especially pyrethroids (personal communication Dr Pawel Wegorek). A putative susceptible population (UK) was collected in July 2008 from Hertfordshire, UK. All populations contained adults only and were kept at room temperature (+ 20°C) prior to assay.

All *in vivo* and *in vitro* assays with pollen beetles (non-sexed adults) were carried out at Rothamsted Research with the exception of the *in vivo* assays with the Stein population Stein population, which I carried out in the laboratory of Syngenta (Stein, Switzerland).

2.1.4 Musca domestica strain

A standard susceptible *M. domestica* strain, (WHO) was obtained from Dr Michael Kristensen (Danish Pest Infestation Laboratory, Denmark) and maintained at 28 ± 2°C with a 16 h light:8 h dark photoperiod according to Basden, (1947). Housefly heads were supplied by Dr. Graham Moores, Rothamsted Research.

2.2 Synergists, insecticides and other chemicals

Technical PBO (‘Ultra’, 94%) and PBO analogues (>90%) were supplied by Endura SpA, Italy. The analogue structures are presented in relevant experimental chapters and in Appendix 2 (in numerical order). Design of analogue structures were proposed jointly between myself, Dr Moores (Rothamsted Research, UK) and Endura SpA (Italy) as an ongoing process following preliminary results. Technical imidacloprid (analytical standard 99.7%) was obtained from Promochem Ltd, Welwyn Garden City, UK; technical azamethiphos (analytical standard 99%) was from Riedel-de Haen, UK and technical α-cypermethrin (analytical
standard 98%) was from Sigma-Aldrich, UK, as were other chemicals, e.g. 1-naphthyl acetate (1-NA), 7-ethoxycoumarin (7-EC), NADPH, EDTA, DTT, PTU, PMSF, DTNB, ATChI and Fast Blue RR. Triton X-100 (specially purified for membrane research) was from Roche, UK.

2.3 Biochemical methods
For the recipes and preparations of buffers, gels and substrates see Appendix 3.

2.3.1 Polyacrylamide gel electrophoresis (PAGE)
Native gel electrophoresis was carried out as described by Devonshire and Moores (1982). Briefly, individual apterous aphids of each *M. persicae* clone were homogenised in 1.6 % v/v Triton X-100, in 0.02 M phosphate buffer, pH 7.0 with sucrose, containing a few grains of bromocresol purple as indicator. Following homogenisation, 10 µL samples (from each aphid homogenate) were analysed by electrophoresis on a 7.5% polyacrylamide gel using barbitone buffer, based on the method described by Williams and Reisfeld (1964). After 1.5 h electrophoresis at 150V, gels were rinsed in distilled water, stained in 100 mL 0.2 M phosphate buffer, pH 6.0 containing (0.06%) Fast Blue RR, to which 1 mL of 30 mM 1-NA in acetone (100% AR) was added and stored in the dark. After 20 min, the esterase banding patterns were clearly visible on the gels. The stained gels were then washed with distilled water and 7% acetic acid added to stop the reaction.

2.3.2 Enzyme assays
2.3.2.1 Determination of *Myzus persicae* total esterase activity
Aphid esterase activity was measured using a colourimetric assay modified from Grant *et al.*, 1989. Briefly, single apterous aphids from each clone were homogenised in a total volume of 250 µL 0.02 M phosphate buffer, pH 7.0 with 0.1 % v/v Triton X-100 in separate wells of a NUNC microplate (Fisher Scientific) using a multiple homogeniser (Burkhard Scientific, Middlesex, UK), (ffrench-Constant and Devonshire, 1988). Aphid homogenate (25 µL) was transferred into each well of a new microplate and esterase activity determined by measuring the rate of hydrolysis of the model substrate, 1-NA, to 1-naphthol and acetic acid in the presence of Fast Blue RR (200 µL/well). Concentrations of substrate and Fast Blue RR before addition to the plate were 0.58 mM and 15.46 mM, respectively. The assay was performed
Chapter 2  General Materials and Methods

using a Tmax kinetic microplate reader (Molecular Devices, Corporation; Menlo Park, California) at 450 nm for 10 min, taking readings every 10 sec. The integrated software programme Softmax Pro version 4.6 was used to fit linear regressions, the slopes of which were summarised as a rate of milli optical density per minute at 450 nm (mOD₄₅₀ min⁻¹).

Aphid clones were categorised as S/R₁ (susceptible), R₂ (high level) or R₃ (extreme high) according to the total esterase activity (personal communication Dr Graham Moores).

2.3.2.2 Direct determination of *Myzus persicae* esterase inhibition by PBO and analogues (conventional spectrophotometric assay)

Stock solutions of PBO and analogues (10 mM) were prepared in acetone. Aliquots (10 µL) of purified E4 diluted in 0.02 M phosphate buffer, pH 7.0 (total volume 35 µL) were incubated for 10 minutes with 2-fold serial dilutions of PBO or analogues starting from 0.428 to 8.36x10⁻⁴ mM (concentrations before addition of substrate) in a NUNC microplate. Enzyme lacking synergist was used as the control in the 11th well of the microplate. Enzyme incubated with a serial dilution of acetone was used as an acetone control. Esterase activity was measured as described in section 2.3.2.1.

2.3.2.3 Determination of AChE activity

*Musca domestica* head homogenate was used as the source of AChE. Approximately 10 frozen housefly heads were homogenised in a total volume of 1 mL 0.02 M phosphate buffer, pH 7.0, in an Eppendorf tube. The homogenate was centrifuged at 10 000 g for 1 min and the supernatant used for the assays described below.

*Musca domestica* AChE activity was measured using a colourimetric assay according to Devonshire and Moores, (1984) and Moores et al., (1996) and described initially by Ellman et al., (1961). Housefly homogenate (25 µL) was loaded into separate wells of a NUNC microplate followed by the addition of 75 µL 0.02 M phosphate buffer, pH 7.0, 100 µL dithionitrobenzoate (DTNB) and 100 µL of the model substrate for AChE, acetylthiocholine iodide (ATChI). Final concentrations of ATChI and DTNB were 0.5 mM. The product, 2-nitro-5-thiobenzoate, was determined colourimetrically using a Tmax kinetic microplate.
reader (Molecular Devices, Corporation; Menlo Park, California) at 405 nm for 10 min, taking readings every 10 sec. The integrated software programme Softmax Pro version 4.6 was used to fit linear regressions, the slopes of which were summarised as a rate of mOD$_{405}$ min$^{-1}$.

2.3.3 Reactivation of *Myzus persicae* esterase, E4, following inhibition by azamethiphos

Hydrolysis of azamethiphos by E4 was performed according to Devonshire and Moores (1982) with some modifications. Briefly, the kinetic reaction of esterases with OP compounds is the same process that occurs when the enzyme catalyses the hydrolysis of its substrate (Aldridge and Reiner, 1972) and it is described by the following equations:

$$
\begin{align*}
    &k_1
    \text{EH} + \text{AB} \quad \text{Ehab} \quad \text{EA} + \text{BH} \\
    &k_2
    \text{EA} \quad \text{EH} + \text{AOH} \\
    &k_3
    \text{H}_2\text{O}
\end{align*}
$$

The active enzyme (EH) combines with the organophosphate (AB) to form an enzyme-substrate complex (Ehab) followed by the rapid formation of the phosphorylated enzyme (EA). The final step of the reaction is the hydrolysis of EA and generation of the free enzyme EH. The rate constants $k_1, k_2$ and $k_3$ describe the rates associated with each step in the catalytic process. The rate constant $k_3$ represents the catalytic centre activity (or turnover number) of the enzyme for a particular type of substrate or inhibitor.

To calculate $k_3$ for the hydrolysis of azamethiphos the following assay was performed. Enzyme, E4, (1 mL) in 0.02 M phosphate buffer, pH 7.0 containing 10 µL of insecticide (10$^{-3}$ M) in acetone was incubated at room temperature for 15 min. Enzyme activity before and after incubation was measured using the model substrate 1-NA (section 2.3.2.1) and the enzyme inhibition (%) was recorded. The excess of unbound azamethiphos was removed by passing the sample through a column of Sephadex TM G-25 (Fine) (Amersham Biosciences, Buckinghashire, UK) (d:2 x h:10 cm) and eluted using 0.02 M phosphate buffer, pH 7.0. Fractions (1 mL) were collected and enzyme reactivation was evaluated by taking 10 µL aliquots from each fraction and measuring esterase activity (section 2.3.2.1). Aliquots were taken approximately every 5 min, for approximately 1.5 h. A further enzyme sample (1 mL)
incubated under the same conditions with 10 µL of acetone served as a control. The rate constant $k_3$ was calculated using a single exponential decay (Grafit 3.0, Leatherbarrow, R.J., Erithacus Software).

2.3.4 Enzyme purification

2.3.4.1 Purification of *Myzus persicae* esterase, E4

The resistance-associated esterase, E4, from *M. persicae* clone 794JZ was purified as previously described by Devonshire (1977) with some modifications. Briefly, 5 g aphids (stored at -20°C) were homogenised on ice, in 10 mL 0.02 M phosphate buffer pH 7.0 and centrifuged at 20 000 g for 10 min. The supernatants were filtered using cheese-cloth and passed through a column of SephadexTM G-25 (Amersham Biosciences) (d:4 x h:15 cm) which separated low molecular weight material. The proteins were eluted using 0.02 M Tris/HCl, pH 8.5 buffer. Unretarded fractions were collected and 10 µL aliquots assayed for total esterase activity as described in section 2.3.2.1. Fractions with the highest activity were pooled and loaded onto an ion exchange column (DEAE SepharoseTM Fast Flow, Amersham Biosciences) (d:4 x h:15 cm) and then eluted using a linear 0-0.35 M NaCl gradient in 500 mL of 0.02 M Tris/HCl pH 8.5, as described above. Fractions (5 mL) were collected and tested for total esterase activity and those fractions with the highest activity were pooled. The samples were de-salted, concentrated to approximately 15 mL in a filtration concentrator (Amicon, Hertfordshire, UK) and re-chromatographed on the DEAE SepharoseTM ion exchange column. Samples were eluted with a linear 0-0.35 M NaCl gradient in 500 mL of 0.02 M Tris/HCl pH 8.5 as described above. The purified E4 was stored at -20°C after exchanging the 0.02 M Tris/HCl pH 8.5 buffer for 0.02M phosphate buffer, pH 7.0.

2.3.4.2 Semi-purification of *Myzus persicae* and *Meligethes aeneus* esterases

Esterase from the *M. persicae* clone 5191A and non-specific esterase(s) from the Lebork *M. aeneus* population were semi-purified using the purification method described in section 2.3.4.1 with some modifications. Briefly, aphids or pollen beetles (stored at -20°C) were homogenised in 0.02 M phosphate buffer, pH 7.0. Homogenates were centrifuged at 20 000 g for 5 min and the supernatants filtered, passed through a SephadexTM G-25 column and the proteins eluted using 0.02 M Tris/HCl, pH 8.5 buffer. Unretarded fractions were collected and
tested for total esterase activity according to the method described in section 2.3.2.1. Fractions with the highest activity were pooled and loaded onto a DEAE ion exchange column. Aphid samples were eluted with a linear 0-0.35 M NaCl gradient in 500 ml of 0.02 M Tris/HCl pH 8.5 as described in section 2.3.4.1. Pollen beetle samples were eluted with a linear 0-1 M NaCl gradient in 500 mL of 0.02 M Tris/HCl, pH 8.5. Fractions (5 mL) were collected and tested for total esterase activity (section 2.3.2.1) and those fractions with the highest activity were pooled and used immediately in the esterase interference assay.

2.3.5 Indirect determination of esterase binding (esterase interference assay)

The esterase interference assay indirectly measures the binding affinities of inhibitors (e.g. PBO, analogues and insecticides) to the esterase(s). Esterase(s) was incubated with and without the inhibitor and binding affinities monitored by the inhibition of housefly AChE using azamethiphos (Khot et al., 2008).

2.3.5.1 Preparation of AChE

Homogenates of M. domestica heads were prepared according to the method described in section 2.3.2.3.

2.3.5.2 Incubation of esterases with synergists and insecticides

Stock solutions of technical synergists (PBO or analogues) and insecticides (α-cypermethrin or imidacloprid) in acetone were pre-incubated in Eppendorf tubes for 16 h at 4°C with either purified esterase from M. persicae clone 794ZJ (E4) or semi-purified esterases from clone 5191A or semi-purified esterases from M. aeneus (Epb). Esterase(s) incubated in acetone was used as control.

2.3.5.3 Indirect determination of esterase binding

To the wells of a 96-well NUNC microplate, 25 µL 0.02 M phosphate buffer, pH 7.0 was added, followed by an additional 22 µL of the same buffer in the first well of each row. A two-fold serial dilution of azamethiphos (10^{-6} M, dissolved in acetone) was prepared across the plate starting with the addition of 3 µL in the first column. The dilution was made by taking 25 µL from the first column to the next until the 11th column of the row, from which the 25 µL
was discarded. The last column (12th, control) contained 25 µL of phosphate buffer. The rest of the microplate contained the following: row A: 50 µL per well of phosphate buffer (‘no esterase’); row B: 15 µL per well of esterase pre-incubated overnight with acetone (‘esterase’ e.g E4) followed by 35 µL of phosphate buffer; row C and thereafter: 15 µL per well of esterase pre-incubated overnight with samples (e.g E4+PBO) as described in section 2.3.5.2 and 35 µL of phosphate buffer. After 1 h of incubation at room temperature, 25 µL housefly head homogenate, as a source of AChE (section 2.3.2.3), was added to each well and the plate incubated for a further 15 min at room temperature. Finally 100 µL of DTNB followed by an equal volume of ATChI was added to every well to give a final concentration of 0.5 mM. AChE activity was then determined by the hydrolysis of ATChI and detection of the released thiocholine colourimetrically, at 405 nm, by its reaction with DTNB as described in section 2.3.2.3. Buffer only (row A) and esterase(s) pre-incubated in acetone (row B) served as the negative and the positive controls, respectively. All treatments were performed at least in duplicate.

2.3.6 O-deethylation of 7-ethoxycoumarin (ECOD assay)

2.3.6.1 Enzyme preparations

Microsomal preparations of rabbit liver and Myzus persicae

Fresh rabbit liver (1 g) was diced and homogenised on ice, in 1 mL homogenisation buffer (0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF and 1.46 M sucrose) and diluted with the same buffer lacking sucrose to give a final 10% w/v homogenate. This was centrifuged at 20 000 g for 5 min. The resulting supernatant was centrifuged for a further 1 h at 105 000 g and 4°C. The pellet was resuspended in homogenisation buffer without sucrose (1 mL) and used immediately as a microsomal enzyme source for O-deethylation assays.

Fresh aphids (0.16 g) derived from M. persicae clone 5191A, were homogenised on ice, in 0.5 mL homogenisation buffer and diluted in the same buffer to give a final 10% w/v homogenate as described above. This was centrifuged at 20 000 g for 5 min. Supernatants were further centrifuged at 105 000 g for 1 h at 4°C. The resulting pellet was resuspended in 300 µL
homogenisation buffer and aliquots used directly for the determination of aphid microsomal oxidase activity.

*Meligethes aeneus* homogenisation
Approximately 55 adult pollen beetles of mixed sexes from each population were homogenised on ice in 1 mL 0.1 M sodium phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF and centrifuged at 20 000 g for 5 min. The resulting supernatant was used directly as the source of pollen beetle oxidase.

### 2.3.6.2 Determination of O-deethylation activity

O-deethylation of 7-ethoxycoumarin (7-EC) was measured according to Ullrich and Weber (1972) and adapted to the microplate format, as described by DeSousa et al., 1995. Briefly, 7-EC was dissolved in ethanol (absolute high, 100% AR) to give a 20 mM stock solution and diluted by the addition of 0.1 M, sodium phosphate buffer pH 7.8 to give a concentration of 0.5 mM. Enzyme samples (50 µL final volume) were added to the wells of a microplate (OptiPlate™ Perkin Elmer) followed by the addition of 80 µL of 0.5 mM 7-EC to every well. The plate was incubated for 5 min at 30°C and the reaction initiated by the addition of 10 µL 9.6 mM NADPH in 0.1 M sodium phosphate buffer, pH 7.8. Enzyme activity was measured using a Victor2 1420 multilabel counter (Wallac, Milton Keynes, UK) for 1 h with readings taken every 2 min, using an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Linear regressions of fluorometric units over time were calculated to provide the rate of O-deethylation activity. All treatments were performed in triplicate.

### 2.3.6.3 Determination of O-deethylation inhibition in *Meligethes aeneus* homogenate by PBO and analogues

Pollen beetle enzyme (50 µL) was incubated with 3 µL of 10 mM inhibitor (PBO or analogues) stock solutions in acetone. Enzyme (50 µL) with 3 µL of acetone was used as a control (uninhibited). After 10 min incubation at room temperature, 80 µL of 0.5 mM 7-EC was added and oxidase activity monitored as described in section 2.3.6.2. Wells including homogenisation buffer, inhibitor diluted in acetone, 7-EC and NADPH were used to measure the ‘background’ activity. All treatments were performed in triplicate.
2.4 Insect Bioassays

2.4.1 Full dose response bioassays with *Myzus persicae* clones

Apterous aphids were transferred from Blackman boxes (section 2.1.1) to the abaxial surface of Chinese cabbage leaf discs (approximately 10 adults per leaf disc) held on 1% agar in plastic containers (4 cm in diameter, A.W. Gregory & Co Limited, Kent, UK). The upper edge of each container was coated with Fluon® (Whitford Plastics, Cheshire, UK) to prevent aphid escape from the leaf surface. Netted lids were added to ensure apterous aphids could not escape. After allowing 30 min for the aphids to settle, each was dosed with 0.25 µL of 1g L⁻¹ synergist dissolved in acetone or acetone only using a Burkard microapplicator (Burkhard Scientific, Middx, UK). Synergist concentrations chosen conferred no significant mortality compared to the acetone control treatment in preliminary bioassays. Five hours later, aphids were further treated with 0.25 µL insecticide (imidacloprid or α-cypermethrin) in acetone at a range of concentrations (0.1-3000 ppm, at least 3 replicates per concentration per bioassay). Controls were treated with 0.25 µL of acetone or 1g L⁻¹ synergist in acetone and after 5 h with a further 0.25 µL of acetone. This 5 h pre-treatment time was found to be optimal for *M. persicae* (Khot, 2009).

Treated aphids were kept at 18 ± 2°C and 16 h light: 8 h dark photoperiod and scored 72 h after treatment. Aphids incapable of co-ordinated movement (after gentle touching with a paint brush if necessary) were scored as dead. All bioassays were performed a minimum of three times.

2.4.2 Discriminating dose bioassays with *Myzus persicae* clones

Topical application bioassays using synergists (1 g L⁻¹ PBO or 1 g L⁻¹ EN 16/5-1) and a single diagnostic concentration of an insecticide were carried out against *M. persicae* clones 5191A and 794JZ, as described in section 2.4.1. Insecticide discriminating doses were derived from the insecticide full dose response bioassays (section 2.4.1) and were chosen to give low mortality (below 25%) in the absence of a synergist. Discriminating dose bioassays were performed a minimum of three times.
2.4.3 Full dose response bioassays with *Meligethes aeneus* populations

The bioassay method for pollen beetle bioassays was based on that recommended by the Insecticide Resistance Action Committee (IRAC, method no 11, 2008) and was used to investigate responses to $\alpha$-cypermethrin. Glass vials (d:2.3 x h:5.7 cm) (S Murray & Co, Surrey, UK) were coated with 0.5 mL solution of $\alpha$-cypermethrin dissolved in acetone using a blood-roller (Luckham Multimix Major, Denley Instruments Ltd) at room temperature until the test solution had completely evaporated. Five to 15 adults of mixed sexes were placed into the vials and the top closed loosely with a screw-cap. Three replicates of each insecticide concentration (0.01 ppm-100 ppm) were used. Vials treated with acetone only were used as controls.

Treated vials were stored at 18 ± 2°C and 16 h light: 8 h dark photoperiod. All bioassays were scored 24 h after exposure to the insecticide. Pollen beetles were considered dead if inactive after gentle prodding.

2.4.4 Discriminating dose bioassays with *Meligethes aeneus* populations

Due to limited numbers of pollen beetles, no synergist studies were carried out using full dose response bioassays. For the single dose response bioassays, glass vials were coated with 0.5 mL solution of a mixture of a synergist (0.01g L$^{-1}$) and a single diagnostic concentration of $\alpha$-cypermethrin as described in section 2.4.3. Previous studies had shown that, with pollen beetle, a synergist pre-treatment had no advantage over application of a mixture (personal communication Dr G Moores). Insecticide discriminating doses used in this section were derived from the full dose response bioassays (section 2.4.3) and were chosen to give low mortality in the absence of a synergist. Vials treated with 0.01g L$^{-1}$ synergist and acetone only were used as controls. Five to 15 replicates were used for each treatment depending on insect numbers available. Synergist concentrations chosen conferred no mortality in preliminary bioassays. Treated vials including the pollen beetles were stored and scored as described in section 2.4.3.
2.5 Analysis of data

2.5.1 Interference assay data
Concentrations of azamethiphos required to inhibit 50% of the AChE activity, (IC\textsubscript{50}), were calculated by fitting 4-parameter logistic curves (Grafit 3.0, Leatherbarrow, R.J., Erithacus Software). Details of the statistical analysis are in the relevant experimental chapters.

2.5.2 O-deethylation of 7-ethoxycoumarin (ECOD assay)
Oxidase activity was measured by the rate of production of 7-hydroxycoumarin (7-OH) and expressed as Fluorometric Units per minute (FU\textsubscript{m} min\textsuperscript{-1}) following subtraction of ‘background’ activity. After stability of the reaction was established, the mean absorbance of three replicates at each time point was calculated and a linear regression plotted. Oxidase activity was represented by the slopes (rates) of these regression lines. The efficacy of the inhibitors (e.g. PBO) was expressed as a percentage of the control slope (enzyme + acetone). Details of the statistical analysis are in the relevant experimental chapters.

2.5.3 Full dose response bioassay data
Pooled raw data from at least three separate bioassays were analysed by probit analysis (Busvine, 1951; Finney, 1972) using the statistical programme PC Polo Plus (LeOra, Software, Berkeley, USA). Polo Plus programme calculated the concentrations required to kill 50% of the population (LC\textsubscript{50}), 95% confidence limits (CL\textsubscript{95%}), slopes with standard errors (SE), chi-square (\chi^2) and degrees of freedom (df) (see Tables in relevant experimental chapters and full data analysis outputs can be found in Appendix 4). Natural response was also estimated when control mortality occurred. If no mortality was observed in the controls then the natural response was estimated as zero (Robertson \textit{et al.}, 2007). Comparisons using LC\textsubscript{50} values were based on non-overlap of 95% confidence intervals.

The LC\textsubscript{50} values were used to generate a ‘Resistance factor’ (RF) and a ‘Synergistic factor’ (SF) as follow:

\[
\text{Resistance factor (RF)} = \frac{\text{LC}_{50} \text{ for resistant population}}{\text{LC}_{50} \text{ for susceptible population}}
\]
The resistance factor (RF) estimates the effect of a certain treatment on a population of insects if tested against both resistant and susceptible populations. Generally, RF indicates how much more insecticide is required to provide equal control against a resistant population compared to a susceptible population.

\[
\text{Synergistic factor (SF)} = \frac{\text{LC}_{50} \text{ insecticide for population}}{\text{LC}_{50} \text{ synergised insecticide for population}}
\]

The Synergistic factor (SF) estimates the effect of a synergist when used in conjunction with an insecticide on a particular insect population (either resistant or susceptible) (Metcalf, 1967). The toxicity of the insecticide normally increases with the relative amount of synergist in the synergist/insecticide treatment (Brindley and Selim, 1984; Bingham et al., 2007).

**2.5.4 Discriminating dose bioassay data**

For discriminating dose response bioassays, a Generalized Linear Model (GLM) was fitted to the data calculated as proportions (number of dead aphids out of the total) assuming a Binomial distribution for the probability of death and using a logit link function (for model equation see Appendix 5).
Chapter 3

Use of piperonyl butoxide analogues to investigate the interactions between piperonyl butoxide and E4, a resistance-associated esterase from the peach-potato aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae)

3.1 Introduction

It has been shown previously that PBO exhibits synergistic effects against insects with phase 1 metabolic enzyme systems that confer insecticide resistance, namely esterases and oxidases (Casida, 1970; Wilkinson *et al.*, 1984; Gunning *et al.*, 1998; Moores *et al.*, 1998; Young *et al.*, 2005, 2006). Although the mechanism by which PBO inhibits P450s is well-documented (Dahl and Hodgson, 1979), the mechanism by which PBO exhibits its inhibitory effects on resistance-associated esterases remains unclear. In an attempt to elucidate the mechanism(s) involved in these interaction(s), biochemical assays were performed using purified E4, a carboxylesterase derived from *M. persicae* which confers broad spectrum insecticide resistance (Devonshire and Moores, 1982).

The carboxylesterase E4, first described in 1977 (Devonshire, 1977) is a classic example of the important contribution of this class of metabolic enzymes to insecticide resistance. It has been shown that E4 can detoxify a significant proportion of a toxic dose by hydrolysis and also by sequestration (Devonshire and Moores, 1982). Despite the critical role that E4 plays in insecticide detoxification and the cloning of the gene encoding E4 (Field *et al.*, 1993), the crystal structure of the protein is still unknown. This clearly limits understanding of the interaction(s) between PBO and E4. However, the structure of E4 can be predicted from other closely related serine hydrolases such as other carboxylesterases and AChEs with known crystal structures, since they share a similar structural framework (Xie *et al.*, 2002).

To investigate PBO and E4 interactions, a structure activity relationship (SAR) study using a series of structurally similar compounds to PBO (analogues) was carried out, measuring the
differences in binding affinities to E4 and including theoretical aspects of molecular recognition by these molecules. The crucial role of molecular recognition between a protein and a ligand was first highlighted by the Dutch chemist, Emil Fischer in 1894 who suggested a simple and very comprehensive model that “the enzyme and the substrate must fit together like a lock and key” (Fischer, 1894). Although Fisher’s “Lock–and–Key” model is still valid, a recent and expanded “hand and a glove” model includes those cases where macromolecule enzymes and ligands are flexible and adopt their final shapes during the recognition process (Harmat and Naray-Szabi, 2009). These approaches have been used to study possible aspects of the interaction between PBO and E4.

In the present work extensive use was made of the esterase interference assay initially described by Khot et al., (2008). This indirect assay was developed to overcome the lack of observed inhibition of esterase activity in the presence of PBO when using a conventional spectrophotometric assay. This biochemical assay was used to study the interactions/binding between purified esterase (E4) derived from *M. persicae* (clone 794JZ) and synergists (PBO and its analogues).

The overall aim of this chapter is to give insights into the possible mechanism(s) by which PBO interacts with E4 through a series of biochemical assays including a SAR study with PBO analogues. Conclusions regarding the potency and possible specificity of the analogues are also discussed.
3.2 Materials and Methods

3.2.1 Piperonyl butoxide and analogues
Analogues of PBO with modifications in the MDP moiety, alkyl and polyether side chains were synthesised and used (section 2.2). The structures of analogues are given in Appendix 2.

3.2.2 Purification of E4
Purified E4 derived from *M. persicae* (clone 794JZ) was used in all biochemical assays. E4 was purified as described in section 2.3.4.1.

3.2.3 Direct determination of E4 inhibition by PBO and analogues
Direct determination of E4 inhibition by PBO and analogues was performed as described in section 2.3.2.2.

3.2.4 Reactivation of E4 following inhibition by azamethiphos
Inhibition of E4 activity by azamethiphos and reactivation was performed as described in section 2.3.3.

3.2.5 Indirect determination of E4 binding using different concentrations of PBO
Different stock concentrations (0.3, 3 and 30 mM) of technical PBO diluted in acetone, were pre-incubated in Eppendorf tubes with purified E4 (16 h at 4°C), to give final concentrations of 0.003, 0.03 and 0.3 mM, respectively. Purified E4 incubated with acetone was used as control. Indirect determination of esterase activity inhibition was performed as described in section 2.3.5.3.

3.2.6 Indirect determination of E4 binding using different incubation intervals
Stock solutions (3 mM) of technical PBO in acetone were pre-incubated in Eppendorf tubes with purified E4 for 1, 2, 4 and 24 h at 4°C. Purified E4 incubated with acetone at the same incubation intervals was used as controls. The assay was performed as described in section 2.3.5.3.
3.2.7 Structure–activity relationship study using the esterase interference assay

Stock solutions of technical PBO and analogues (3 mM) in acetone were pre-incubated in Eppendorf tubes with purified E4 for 16 h at 4°C. Purified E4 incubated with acetone was used as control. Indirect determination of E4 inhibition by PBO and analogues was performed as described in section 2.3.5.3.

3.2.8 Analysis of data

3.2.8.1 Direct determination of E4 activity inhibition by PBO and analogues

The mean activity ($\text{mOD}_{450 \text{min}^{-1}}$) of two replicates was calculated and plotted against inhibitor concentrations (concentrations before the addition of the substrate). Slopes were calculated using Grafit 3.0 (Leatherbarrow, R.J., Erithacus Software).

3.2.8.2 Indirect determination of E4 binding

Concentrations of azamethiphos required to inhibit 50% of the AChE activity, ($\text{IC}_{50}$), were initially calculated in Grafit 3.0 (section 2.5.1). Then, $\text{IC}_{50}$ values were converted into percentages giving the “Index” (I) value. The higher the I value, the lower the binding affinity of the compound to the enzyme (E4). Confidence limits (CL) 95% for the I value were also calculated and treatments considered significantly different if the CL 95% did not overlap (see Appendix 6 for details).

3.2.8.3 Correlation between results obtained from the direct and indirect esterase assays for the inhibitory potency of PBO/analogues to E4

Simple linear regression analysis was used to describe the correlation between the results obtained from the direct (conventional spectrophotometric assay) and the indirect (esterase interference assay) esterase assays for the inhibitory potency of PBO/analogues to E4 (GenStat 12$^{\text{th}}$ Edition, VSN International).
3.3 Results

3.3.1 Direct determination of E4 inhibition by PBO and analogues
Ten representative analogues of PBO containing alterations in the MDP ring and/or side chains were tested for the ability to inhibit E4 activity using the conventional esterase assay. Results (change in hydrolysis rate versus inhibitor concentrations-slopes) are given in Figure 3.1 and Table 3.1. Results showed that analogues gave different responses after incubation with the enzyme, but no evidence of inhibition was detected. In fact, EN 14-05 and PBO gave highly increased catalytic centre activity of the enzyme for its model substrate (activation). Three other analogues, EN 1-179, EN 16/5-1 and EN 1-42, gave an intermediate level of increased activity whilst activity with the other analogues did not differ from the control.

Figure 3.1 Activity (mOD$_{450}$min$^{-1}$) of the aphid esterase (E4) versus inhibitor concentrations. Data are means ± SE, n=2.
Table 3.1 Slopes (mOD\textsubscript{\textit{450}}min\textsuperscript{-1}mM\textsuperscript{-1}) corresponding to PBO and analogues (refer to Figure 3.1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slope ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (acetone)</td>
<td>0.45 ± 1.25</td>
</tr>
<tr>
<td>PBO</td>
<td>124 ± 3.08</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>68.2 ± 2.03</td>
</tr>
<tr>
<td>EN 1-40</td>
<td>9.28 ± 0.55</td>
</tr>
<tr>
<td>EN 1-42</td>
<td>68.9 ± 1.51</td>
</tr>
<tr>
<td>EN 1-129</td>
<td>1.68 ± 1.91</td>
</tr>
<tr>
<td>EN 25-35</td>
<td>24.0 ± 0.85</td>
</tr>
<tr>
<td>EN 1-183</td>
<td>3.08 ± 2.89</td>
</tr>
<tr>
<td>EN 16-17</td>
<td>0.44 ± 2.26</td>
</tr>
<tr>
<td>EN 25-10</td>
<td>15.5 ± 0.82</td>
</tr>
<tr>
<td>EN 14-05</td>
<td>167 ± 6.95</td>
</tr>
<tr>
<td>EN 1-164</td>
<td>10.5 ± 3.73</td>
</tr>
<tr>
<td>EN 1-179</td>
<td>75.1 ± 3.43</td>
</tr>
</tbody>
</table>

3.3.2 Reactivation of E4 activity following inhibition by azamethiphos

Incubation of E4 with azamethiphos (10\textsuperscript{-3}M) for 15 min gave ~77% inhibition of activity (Table 3.2).

Table 3.2 Activity (mOD\textsubscript{\textit{450}}min\textsuperscript{-1}) before and after 15 min incubation of E4 with azamethiphos (10\textsuperscript{-3}M). Data are means ± SE, n=2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity ± SE</th>
<th>% Remaining activity ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before incubation</td>
<td>81.1 ± 2.89</td>
<td>22.7 ± 0.41</td>
</tr>
<tr>
<td>After incubation</td>
<td>18.4 ± 0.33</td>
<td></td>
</tr>
</tbody>
</table>

Reactivation rate ($k_3$) of E4, after monitoring at intervals for approximately 1.5 h, was found to be 1.63 (± 0.16) h\textsuperscript{-1} indicating that the enzyme had significant phosphatase activity (Figure 3.2).
3.3.3 Indirect determination of E4 binding using different concentrations of PBO

The esterase interference assay depends upon the action of unsequestered azamethiphos against housefly AChE, so it was necessary to test if PBO or analogues inhibited housefly AChE directly using the standard Ellman assay (Ellman et al., 1961), with ATChI as the substrate for AChE. No inhibition was observed (data not shown).

The principle behind the esterase interference assay is that in the absence of esterase, housefly AChE activity is inhibited by azamethiphos and this is detected as low AChE activity (in Table 3.3 and Figure 3.3 indicated as no E4). In the presence of E4 (E4 only) where sequestration may remove the azamethiphos before it reaches the AChE (i.e. E4 ‘protects’ the AChE), higher concentrations of azamethiphos are needed to inhibit the AChE. When E4 is pre-incubated with PBO prior to exposure to AChE, (E4 + PBO), if there is an interaction between the E4 and the PBO, the E4 will no longer ‘protect’ the AChE from the azamethiphos and AChE inhibition will be similar to that seen in the absence of E4. Hence this would indicate indirectly if E4 has interacted with (blockaded) PBO.
The IC$_{50}$ values of the AChE activity, after incubation of E4 with three different concentrations of PBO, are presented in Table 3.3 and Figure 3.3. The I values, represent the binding affinity of PBO to E4 as a percentage (Figure 3.4). The lower the I value, the higher the binding affinity (where the IC$_{50}$ value for E4=100% and for no E4=0% see Appendix 6 for details). Results showed that binding potencies exhibited by 3 mM PBO and 30 mM PBO were very similar, whilst 0.3 mM PBO gave the lowest binding potency. Based on these results, 3 mM PBO was the concentration used in the next assays (see sections 3.3.4 and 3.3.5) to allow evaluation of the differential binding affinities when a range of PBO analogues were tested in the SAR study (section 3.3.5).

Table 3.3 Concentrations of azamethiphos required for 50% inhibition of AChE activity, (IC$_{50}$), after incubation of E4 with different concentrations of PBO with the Index values, (I), calculated as described in the text (see also Appendix 6). Data are means ± SE, n=3.

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC$_{50}$ (ηM)</th>
<th>SE ±</th>
<th>I (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE only (no E4)</td>
<td>0.12</td>
<td>0.004</td>
<td>0</td>
</tr>
<tr>
<td>E4 only</td>
<td>1.93</td>
<td>0.027</td>
<td>100</td>
</tr>
<tr>
<td>E4 + 0.3 mM PBO</td>
<td>0.73</td>
<td>0.032</td>
<td>48.8</td>
</tr>
<tr>
<td>E4 + 3 mM PBO</td>
<td>0.17</td>
<td>0.008</td>
<td>4.14</td>
</tr>
<tr>
<td>E4 + 30 mM PBO</td>
<td>0.15</td>
<td>0.003</td>
<td>2.51</td>
</tr>
</tbody>
</table>

Figure 3.3 AChE activity (%) after incubation of E4 with different concentrations of PBO (refer to IC$_{50}$ values from Table 3.3).
3.3.4 Indirect determination of E4 binding using different incubation times

The results presented in Figure 3.5 show that the binding of 3 mM PBO to E4 did not change when the incubation time was increased from 1 to 24 h. This suggests that PBO acts as a reversible inhibitor for the protein.

Figure 3.5 Graphical representation of Index values of binding affinities of PBO to E4 using different incubation times. Data are means ± SE, n=3.
Chapter 3

3.3.5 Structure activity relationship study for the interactions between analogues and E4

Results from the interference assay were grouped into three categories according to the modifications to the structure of the analogues compared to PBO. Data in Table 3.4, Table 3.5 and Table 3.6 show the structures and the corresponding I values with the lower and the upper CL (95%). Analogues, such as PBO, EN 1-126 and EN 1-180 are repeated in Tables and Figures for easier comparison.

3.3.5.1 Analogues with general modifications in the alkyl and polyether side chains (aromatic ring type A)

Results in Table 3.4 and Figure 3.6 show significant differences in the analogues’ ability to blockade E4. The highest binding affinity of analogues to E4 was observed with an increasing length of the alkyl chain. Hence, EN 1-16 containing a hexyl side chain showed the highest binding affinity compared to the other analogues. However, the butyl chain in EN 1-14 did not increase the binding affinity to E4 compared to PBO. Removal of the propyl side chain in EN 1-40 resulted in a significantly lower binding than PBO. Introduction of a second polyether chain in EN 1-42, restored the ability to bind a significantly higher amount of E4 than EN 1-40 but less than PBO. Alterations at the end of the polyether chain by the addition of either a carboxyl (-COOH) or a sodium carboxylate (-COONa) group, as shown in EN 1-101 and EN 1-162 respectively, resulted in failure of these analogues to blockade E4. A similar response was observed when the long polyether side chain was replaced by a carboxyl group (EN 1-93). Replacement of the polyether chain by a dodecyl chain and absence of the propyl alkyl chain in EN 1-129, resulted in a significant reduction in binding affinity, although its binding affinity was higher than EN 1-101, EN 1-162 and EN 1-93. EN 1-126, where the polyether chain was replaced by an alkynyl ether chain, showed a high ability to blockade E4 and restored the inhibitory failure of EN 1-101, EN 1-162, EN 1-93 and EN 1-129, although the EN 1-126 binding affinity was significantly lower than PBO, EN 1-14 and EN 1-16. EN 1-180 which is structurally similar to PBO but has an oxygen atom (from the polyether chain) bound directly to the aromatic ring by removal of the methylene bridge group, -CH₂-, (changing from a phenyl methyl structure to a phenyl structure i.e. from aromatic type A to aromatic type B) did not show any significant difference in binding potency compared to PBO. The last two
analogues in Table 3.4, EN 1-126 and EN 1-180, were used to generate further analogues presented in the next two sections 3.3.5.2 and 3.3.5.3.

Table 3.4 Analogues with general modifications in the alkyl and polyether side chain (aromatic ring type A) and corresponding Index values, (I) with lower and upper 95% CL.

<table>
<thead>
<tr>
<th>Name</th>
<th>Aromatic ring</th>
<th>R₁</th>
<th>R₂</th>
<th>I (%)</th>
<th>Lower 95% CL</th>
<th>Upper 95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO</td>
<td>A</td>
<td>CH₃CH₂CH₃</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>5.86</td>
<td>5.14</td>
<td>6.60</td>
</tr>
<tr>
<td>EN 1-14</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>4.82</td>
<td>4.27</td>
<td>5.34</td>
</tr>
<tr>
<td>EN 1-16</td>
<td>A</td>
<td>CH₃CH₂CH₂CH₂CH₃</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>2.17</td>
<td>1.70</td>
<td>2.65</td>
</tr>
<tr>
<td>EN 1-40</td>
<td>A</td>
<td>H</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>28.1</td>
<td>26.1</td>
<td>30.1</td>
</tr>
<tr>
<td>EN 1-42</td>
<td>A</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>10.3</td>
<td>9.11</td>
<td>11.5</td>
</tr>
<tr>
<td>EN 1-101</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>(CH₂OCH₂)₂COOH</td>
<td>85.9</td>
<td>77.8</td>
<td>94.0</td>
</tr>
<tr>
<td>EN 1-162</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>(CH₂OCH₂)₂COONa</td>
<td>84.0</td>
<td>78.2</td>
<td>89.8</td>
</tr>
<tr>
<td>EN 1-93</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>COOH</td>
<td>89.8</td>
<td>80.8</td>
<td>98.8</td>
</tr>
<tr>
<td>EN 1-129</td>
<td>A</td>
<td>H</td>
<td>(CH₃)₂CH₃</td>
<td>61.1</td>
<td>56.2</td>
<td>66.0</td>
</tr>
<tr>
<td>EN 1-126</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>CH₂OCH₂C≡CH₃</td>
<td>12.8</td>
<td>10.9</td>
<td>14.7</td>
</tr>
<tr>
<td>EN 1-180*</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>(OCH₂CH₂)₂CH₂CH₃</td>
<td>4.67</td>
<td>4.07</td>
<td>5.27</td>
</tr>
</tbody>
</table>

* EN 1-180 is represented as an analogue having an aromatic ring type B in the following Tables and Figures.
Figure 3.6 Graphical representation of Index values of analogues with general modifications in the alkyl and polyether side chains (aromatic ring type A). Data are means ± SE, n=3 (refer to Table 3.4).

### 3.3.5.2 Phenyl methyl and phenyl analogues (aromatic ring type A and B)

When phenyl methyl and phenyl analogues (aromatic ring type A and B, respectively) were tested, EN 1-164 exhibited the highest binding affinity (Table 3.5 and Figure 3.7). Thus, it seems that an oxygen atom bound directly to the aromatic ring (by removing the -CH$_2$- group) increases the binding affinity of EN 1-164 compared with EN 1-126 which retains the -CH$_2$- group (aromatic ring type A). Lack of an alkyl chain in EN 1-163 gave lower binding affinity than EN 1-164. Furthermore, reduced binding potency was also observed with EN 1-125, which is structurally similar to EN 1-126 but which also lacks the alkyl side chain. A similar trend was observed with EN 1-175 compared to EN 1-180. An analogue having an alkynyl moiety with the triple bond at the end of the side chain, as in EN 1-183, displayed high binding affinity and was similar to the binding potency of EN 1-164. However, when the alkyl side chain was absent as in EN 1-181 the affinity was reduced. EN 1-179, a compound having a polyether and an alkynyl ether chain, with both chains bound directly to the aromatic ring, showed higher binding potency than PBO or EN 1-180. Interestingly, EN 1-186 having the shortest polyether chain and a propyl side chain revealed higher binding potency than PBO,
EN 1-180 or EN 1-179. Finally EN 1-182, an analogue containing an alkynyl ether moiety and a branched methyl group (aromatic ring type A), exhibited slightly different binding affinity to EN 1-126.

Table 3.5 Analogues with phenyl methyl or phenyl structure (aromatic ring type A and B, respectively) and corresponding Index values, (I) with lower and upper 95% CL.

<table>
<thead>
<tr>
<th>Name</th>
<th>Aromatic ring</th>
<th>R₁</th>
<th>R₂</th>
<th>I (%)</th>
<th>Lower 95 % CL</th>
<th>Upper 95 % CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>(CH₂OCH₃)₂CH₂CH₂CH₃</td>
<td>5.86</td>
<td>5.14</td>
<td>6.60</td>
</tr>
<tr>
<td>EN 1-180</td>
<td>B</td>
<td>CH₂CH₂CH₃</td>
<td>(CH₂CH₂O)₂CH₂CH₂CH₂CH₃</td>
<td>4.67</td>
<td>4.07</td>
<td>5.27</td>
</tr>
<tr>
<td>EN 1-186</td>
<td>B</td>
<td>CH₂CH₂CH₃</td>
<td>CH₂CH₂OCH₃</td>
<td>2.31</td>
<td>1.78</td>
<td>2.84</td>
</tr>
<tr>
<td>EN 1-175</td>
<td>B</td>
<td>H</td>
<td>(CH₂CH₂O)₂CH₂CH₂CH₂CH₃</td>
<td>26.5</td>
<td>24.3</td>
<td>28.7</td>
</tr>
<tr>
<td>EN 1-179</td>
<td>B</td>
<td>OCH₂C≡CCH₃</td>
<td>(CH₂CH₂O)₂CH₂CH₂CH₂CH₃</td>
<td>2.98</td>
<td>2.47</td>
<td>3.50</td>
</tr>
<tr>
<td>EN 1-126</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>CH₂O CH₂C≡CCH₃</td>
<td>12.8</td>
<td>10.9</td>
<td>14.7</td>
</tr>
<tr>
<td>EN 1-164</td>
<td>B</td>
<td>CH₂CH₂CH₃</td>
<td>CH₂C≡CCH₃</td>
<td>0.98</td>
<td>0.65</td>
<td>1.30</td>
</tr>
<tr>
<td>EN 1-125</td>
<td>A</td>
<td>H</td>
<td>CH₂O CH₂C≡CCH₃</td>
<td>37.1</td>
<td>31.7</td>
<td>42.6</td>
</tr>
<tr>
<td>EN 1-163</td>
<td>B</td>
<td>H</td>
<td>CH₂C≡CCH₃</td>
<td>10.3</td>
<td>9.3</td>
<td>11.4</td>
</tr>
<tr>
<td>EN 1-183</td>
<td>B</td>
<td>CH₂CH₂CH₃</td>
<td>CH₂C≡CH</td>
<td>2.06</td>
<td>1.67</td>
<td>2.44</td>
</tr>
<tr>
<td>EN 1-181</td>
<td>B</td>
<td>H</td>
<td>CH₂C≡CH</td>
<td>63.9</td>
<td>60.3</td>
<td>67.5</td>
</tr>
<tr>
<td>EN 1-182</td>
<td>A</td>
<td>H</td>
<td>CH₂ CH (CH₂)CH₂OCH₂C≡CCH₃</td>
<td>9.62</td>
<td>8.84</td>
<td>10.4</td>
</tr>
</tbody>
</table>
3.3.5.3 Analogues with modifications in the MDP moiety and side chains (aromatic ring type C, D, and E)

The absence of one oxygen from the MDP moiety, as in EN 16/5-1 and EN 16-06, (Table 3.6 and Figure 3.8) did not affect their binding affinities compared to PBO. In contrast, absence of both oxygen atoms, as in EN 14-05, resulted in reduced binding affinity. EN 16-17 containing a propyl side chain and an alkynyl ether moiety showed the highest binding affinity to E4. Nevertheless, an absence of the alkyl chain in EN 16-18 resulted in a significantly reduced binding affinity compared to EN 16-17.

Verbutin, (common name for EN 25-10, Pap et al., 2001) showed lower binding affinity than PBO although a structurally similar compound, EN 25-35, gave a similar affinity as PBO. The absence of the propyl side chain in EN 25-37 could be correlated with low binding to E4 in the same way as previously seen for the presence/absence of the alkyl moiety. Similar to previous observations (Table 3.5 and Figure 3.7), a triple bond at the terminal position of the alkynyl

Figure 3.7 Graphical representation of Index values of analogues with phenyl methyl and phenyl structure (aromatic ring type A and B, respectively). Data are means ± SE, n=3 (refer to Table 3.5).
moiety affected the binding affinity and hence, EN 25-36 showed lower binding affinity than PBO.

Table 3.6 Analogues with modifications in the MDP moiety, the side chains (aromatic ring type C, D, E) and PBO (aromatic ring type A) and corresponding Index values, (I), with lower and upper 95% CL.

<table>
<thead>
<tr>
<th>Name</th>
<th>Aromatic ring</th>
<th>R₁</th>
<th>R₂</th>
<th>I (%)</th>
<th>Lower 95 % CL</th>
<th>Upper 95 % CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO</td>
<td>A</td>
<td>CH₂CH₂CH₃</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>5.86</td>
<td>5.14</td>
<td>6.60</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>C</td>
<td>CH₂CH₂CH₃</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>7.63</td>
<td>6.94</td>
<td>8.33</td>
</tr>
<tr>
<td>EN 16-06</td>
<td>C</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>H</td>
<td>6.89</td>
<td>6.13</td>
<td>7.64</td>
</tr>
<tr>
<td>EN 14-05</td>
<td>D</td>
<td>CH₂CH₂CH₃</td>
<td>(CH₂OCH₂)₂CH₂CH₂CH₃</td>
<td>21.6</td>
<td>20.2</td>
<td>23.1</td>
</tr>
<tr>
<td>EN 16-17</td>
<td>C</td>
<td>CH₂CH₂CH₃</td>
<td>OCH₂C≡CCH₃</td>
<td>-1.17</td>
<td>-1.42</td>
<td>-0.94</td>
</tr>
<tr>
<td>EN 16-18</td>
<td>C</td>
<td>OCH₂C≡CCH₃</td>
<td>H</td>
<td>28.6</td>
<td>24.7</td>
<td>32.4</td>
</tr>
<tr>
<td>EN 25-10</td>
<td>E</td>
<td>H</td>
<td>CH (CH₂)OCH₂C≡CCH₃</td>
<td>28.4</td>
<td>23.6</td>
<td>33.2</td>
</tr>
<tr>
<td>EN 25-35</td>
<td>E</td>
<td>CH₂CH₂CH₃</td>
<td>OCH₂C≡CCH₃</td>
<td>5.10</td>
<td>4.09</td>
<td>6.10</td>
</tr>
<tr>
<td>EN 25-37</td>
<td>E</td>
<td>H</td>
<td>OCH₂C≡CCH₃</td>
<td>39.1</td>
<td>36.1</td>
<td>42.1</td>
</tr>
<tr>
<td>EN 25-36</td>
<td>E</td>
<td>CH₂CH₂CH₃</td>
<td>OCH₂C≡CH</td>
<td>15.1</td>
<td>13.0</td>
<td>17.3</td>
</tr>
</tbody>
</table>
3.3.6 Correlation between results obtained from the direct and indirect esterase assays for the inhibitory potency of PBO/analogues to E4

The response (slopes) from the direct determination of E4 inhibition by PBO and analogues (conventional esterase assay, section 3.3.1) were plotted against the I values obtained from the esterase interference assay (Table 3.6, Table 3.7 and Table 3.8). The correlation of the results between the two biochemical assays is shown in Figure 3.9. There is a positive correlation between the slopes and the I values revealing that the smaller the I values (stronger the binding affinity) the less elevated the catalytic centre activity of the enzyme for the 1-NA (less activation). Statistical analysis showed that there was a significant regression ($p=0.005$) (the model being: $\text{Slope} = 16.6 + 7.09 \times \text{Index}$, SEs 15.7 and 1.76, respectively). However, three analogues EN 25-10, EN 1-40 and EN 1-129 do not fit this correlation and the common characteristic of these three analogues is the lack of the alkyl side chain.
Figure 3.9 Correlation between the slopes and Index values derived from the two biochemical assays (standard conventional assay and the esterase interference assay excluding EN 25-10, EN 1-40 and EN 1-129).
3.4 Discussion

The mechanism by which PBO interacts with P450s is well-known (Dahl and Hodgson 1979; Wilkinson et al., 1984). In contrast, the mechanism by which PBO interacts with resistance-associated esterases has not been elucidated. In the present work, a series of biochemical assays including a SAR study was undertaken to determine the interaction(s) between PBO and E4, an esterase derived from the peach-potato aphid *M. persicae*, which is known to confer broad spectrum insecticide resistance (Devonshire and Moores, 1982).

It has been observed that although PBO can act as an esterase inhibitor, it does not interact directly with the active site serine of the protein (Khot et al., 2008), as commonly reported for other esterase inhibitors such as PMSF (Fahrney and Gold, 1963). This was demonstrated by standard spectrophotometric assays using the model substrate 1-NA where homogenates derived from *M. persicae, M. domestica* and *B. tabaci* (Q-biotype) still hydrolysed 1-NA after incubation with PBO showing no detectable inhibition (Khot et al., 2008). In the present study, results from the conventional esterase assay with purified E4 in the presence of PBO/analogues and using the model substrate 1-NA, showed that PBO/analogues either did not affect substrate hydrolysis or increased the catalytic center activity of E4 (Figure 3.1 and Table 3.1). The former reaction where no inhibition was detected is in agreement with previous observations (Khot et al., 2008) and implies that these analogues do not react with the enzyme catalytic centre. However, in the same assay some analogues increased the catalytic activity and this implies that compounds such as EN 14-05 and PBO and to a lesser extent compounds such as EN 1-179, EN 16/5-1 and EN 1-42 are actually “helping” or “guiding” the substrate to reach and act at the catalytic centre at a higher rate. Nevertheless, it confirmed that the conventional assay fails to demonstrate inhibition of E4 activity by PBO/analogues.

Results also show that the organophosphate ester, azamethiphos, acts directly on the E4 active site serine by forming initially a phosphoserine ester bond followed by enzyme dephosphorylation and finally recovery of E4 activity (Table 3.2 and Figure 3.2). However, the esterase interference assay showed that in the presence of PBO, azamethiphos is not
sequestered by E4. This suggests that PBO is preventing the azamethiphos reacting with the active site of the protein. Given that PBO prevents azamethiphos from interacting with the catalytic centre but allows 1-NA to reach and act on the active site, it seems that PBO is binding to the protein at an area that prevents the substrate reaching the active site.

Carboxylesterases such as E4, AChE and butyrylcholinesterases (BuChE) are classified within the super family of serine esterases and into the B-esterase group (Aldridge, 1993). These esterases share a common structural framework with the active site containing a serine hydrolase catalytic triad which is composed of a serine (Ser), a histidine (His) and either an aspartate (Asp) or a glutamate (Glu) residue (Wheelock et al., 2005). For example E4’s catalytic triad consists of Ser191, His440 and Glu316 residues (Field et al., 1993). The catalytic triad of Torpedo californica AChE (TcAChE), one of the most extensively studied examples of a serine hydrolase, is composed of Ser200, His440 and Glu327 (TcAChE numbering). X-ray crystallography of the structure of TcAChE has also shown that its catalytic triad is located at the bottom of a deep and narrow cavity - the active site gorge (or aromatic gorge). The active site gorge is lined by 14 conserved aromatic residues and there is a site located close to the rim of the active site gorge, the so-called peripheral anionic site (PAS) (Sussman et al., 1991). The contribution of the 14 conserved aromatic amino acids in the active site gorge is fundamental since they provide both a substrate guidance system and flexibility in protein-ligand interactions (Xu et al., 2008). The arrangement of these residues accelerating the passage of substrate accounts for the extremely high turnover number of AChE (Ripoll et al., 1993). In particular, a tryptophan residue (Try279, TcAChE numbering) positioned at the lip of the aromatic gorge is responsible for directing substrate molecules towards the active site and removal of this residue by site-directed mutagenesis results in an enzyme with a decreased catalytic centre activity (Harel et al., 1995; Morel et al., 1999; Xu et al., 2008). Alignment of the amino acid sequences of TcAChE with E4 (23.7% sequence identity) shows that only 7 of the 14 conserved aromatic amino acids present in TcAChE are conserved in E4 (Figure 3.10). Taking account of the similarity between E4 and AChE and the findings in the present study showing that in the presence of the enzyme, PBO allows acylation by 1-NA but prevents phosphorylation by azamethiphos, it can be hypothesised that PBO (and analogues) bind to amino acid residues located near the rim of the active site gorge.
Furthermore, it is possible that analogues showing an increase in the catalytic centre activity of the enzyme in the conventional assay (e.g EN 14-05 and PBO) may bind in such a way that the aromatic moiety of these analogues acts similarly to the Try279 residue of TcAChE, i.e. guiding the substrate down the aromatic gorge. For analogues in which the catalytic centre activity is not increased (e.g EN 1-101, EN 1-162 and EN 1-129), the aromatic moiety is presumably not in the preferred configuration. Thus hindrance of azamethiphos may still be apparent, but passage of 1-NA is unchanged.

Since the conventional spectrophotometric assay could not be used to study the inhibition of esterase activity by PBO and analogues, all further studies investigating the interactions of PBO and E4 were performed using the esterase interference assay.

Results from the esterase interference assay using different incubation intervals of PBO with E4 showed that PBO acts as a reversible inhibitor (Figure 3.5). This suggests that PBO binds to the enzyme with non-covalent bonds such as hydrogen bonds, hydrophobic interactions, steric effects, etc.

In an attempt to elucidate the interactions between PBO and E4, a SAR study was undertaken using a series of PBO analogues. Binding affinities of the analogues to E4 were used to give insights into the structural requirements of PBO to maximise the binding interactions in the protein-ligand complex. The SAR study could provide information about the future design of more potent and selective inhibitors.

Results from the SAR study revealed that the presence of the alkyl chain may be of major importance for binding to the enzyme as shown with the analogues PBO/EN 1-40 (aromatic type A, Figure 3.6) and EN 1-180/EN 1-175 (aromatic type B, Figure 3.7) where the only difference in their structures is the presence or absence of the alkyl chain and the effects seem to be independent of other changes that have occurred to the structure. A similar trend was observed with two analogue groups where the polyether chain was replaced with an alkynyl moiety, EN 1-126/EN 1-125 (aromatic type A, Figure 3.7) and EN 1-164/EN 1-163 (aromatic type B, Figure 3.7). With the triple bond at the end of the alkynyl moiety and the propyl side
Chapter 3  Use of PBO analogues to investigate the interactions between PBO and E4

chain, EN 1-183 bound a greater amount of E4 compared to EN 1-181 (aromatic type B, Figure 3.7). Given the same presence/absence of an alkyl side chain, compounds having a modification in the MDP moiety and an alkynyl moiety, EN 16-17 and EN 25-35 showed higher binding potency than EN 16-18 and EN 25-37, respectively (aromatic type C and D, respectively, Figure 3.8). Additionally, increasing the length of the alkyl chain to a hexyl substituent in EN 1-16 is correlated with an increase of the binding potency to the esterase. These observations are in line with previous findings showing a positive correlation between the inhibitory potency of a series of compounds to mammalian carboxylesterases and an increased length in the alkyl chain (Wadkins et al., 2007). Results from the present study suggest that the presence of the hydrophobic alkyl substituent is of considerable importance and may interact with a corresponding hydrophobic region of the protein by hydrophobic effects. Assuming that E4 has a similar structure to TcAChE and possesses an aromatic gorge, (as described above) this would be relatively hydrophobic and provide a suitable binding area for the hydrophobic substituent of the analogues by hydrophobic interactions. The alkyl side chain is also likely to exert steric effects that may improve interactions with the amino acid residues of the binding area.

The present results also demonstrate a significant contribution of the polyether chain to the binding affinity of the analogues to E4. This is best seen when the polyether chain is replaced with a dodecyl side chain (EN 1-129) resulting in a lower binding affinity compared to PBO (Figure 3.6). In this case, findings imply that the electron-donating effects of the oxygen atoms in the polyether chain play an additional role and affect the binding affinities. This suggests that electrostatic attraction/repulsions are important in improving the interactions with surrounding amino acids of the protein. Additionally, these lone pairs of electrons could support certain types of shapes of the structures or affect bond angles providing a better orientation of the compounds.

A combination of an alkyl hydrophobic substituent and a polyether chain in analogue structure (as in PBO) provides a higher binding affinity than the presence of two polyether chains, as found in EN 1-42 (Figure 3.6). The presence of two long and bulky polyether chains, containing lone pair electrons from oxygen atoms decreases the binding potency of EN 1-42
and this may occur because of extensive steric hindrance effects resulting from the electrostatic repulsions of the two chains. Thus both the alkyl chain and the polyether chain may be deemed necessary for close binding of the ligand to the esterase.

For EN 1-101 and EN 1-93 both having a carboxyl group (-COOH), binding affinities were significantly low (Figure 3.6). Since the carboxyl group is a strong functional group, it may bind rapidly to an area of the protein before these analogues actually reach the appropriate binding area. Alternatively, this carboxyl group may prevent the analogues taking the same orientation as the other structures, even if bound to the same residues. This hypothesis is supported by Rebek (1987) who suggested that “Functional attached groups tend to diverge or become directed away from the cavity and from the substrates that are held inside”. It is also likely that a similar action occurs in the case of the EN 1-162 (Figure 3.6) since in an aquatic environment the -COONa group of EN 1-162 can react to form a –COOH group following the reaction: RCOONa + H₂O → RCOOH − + NaOH.

When the oxygen atom is bound directly to the aromatic ring as in EN 1-180 (aromatic type B, Figure 3.7), results show there was no significant difference in the binding potency compared to PBO. This may imply that the oxygen’s contribution to the binding potency, by the presence of lone pair electrons, is not affected by their position in the polyether chain. Interestingly, EN 1-186 having the shortest polyether chain showed higher binding affinity than EN 1-180 (Figure 3.7). This may suggest that the structure of EN 1-186 is allowing a better configuration of the molecule and thus increasing the interactions in the suitable binding area.

When the polyether chain was replaced by an alkynyl ether moiety with the oxygen bound directly to the aromatic ring (aromatic ring type B), the binding affinity increased dramatically in some analogues compared to PBO. A combination of an alkynyl ether moiety and the propyl chain as in EN 1-164 (aromatic ring type B), increased the binding potency compared to PBO, EN 1-126 or EN 1-125 (aromatic ring type A, Figure 3.7). This increase is possibly due to the delocalized electrons associated with the triple bond and the lone pair electrons spread across the oxygen which is bound directly to the aromatic ring. A combination of
delocalized electrons from the triple bond and those from the aromatic MDP moiety could form a conjugated system that interacts with aromatic rings from surrounding amino acid residues, providing further forces through aromatic stacking interactions. It is possible that these aromatic stacking interactions stimulate the insertion of the aromatic ring moiety into an aromatic pocket/binding area in the protein. This would explain the observation that analogues such as EN 1-164 and EN 1-183 exhibited high binding affinities. Similarly, delocalized electrons from the triple bond could occur in the alkynyl ether moiety of EN 1-179 (Figure 3.7) in a combination with the lone pair electrons of the oxygen atoms in the polyether chain (as discussed above). However, the slightly lower binding affinity of EN 1-179 is likely to be due to the electrostatic effects in both side chains creating repulsion forces, thus contributing to a sub-optimal orientation of the molecule to the binding area.

The presence of the branched methyl group in the alkynyl ether moiety of EN 1-182 (aromatic ring type A, Figure 3.7) is possibly correlated with the relatively high binding affinity to the protein. This structure may allow free rotation of the methyl group that could either provide a closer orientation of the molecule or the branched methyl group “wedged” between hydrophobic groups of the protein and improve the interaction with the binding area. Furthermore, the presence of the branched methyl group in the side chain gives additional hydrophobic properties to the molecule.

Alteration of the MDP moiety by the removal of one oxygen atom in EN 16/5-1 and EN 16-06 (aromatic ring type C) did not affect the binding affinities (Figure 3.8). Nevertheless, removing both oxygen atoms from the MDP moiety in EN 14-05 (aromatic ring type D), resulted in reduction of the binding potency compared to PBO (Figure 3.8). The requirement of an oxygen in the MDP ring to retain a high binding affinity could be explained by the electron donating effects and/or the provision of a bulky structure which could interact with the appropriate binding area. A lack of oxygen atoms in the MDP ring of EN 14-05 provides the molecule with a near-planar structure that is not easily retained within a binding pocket. EN 16-17 and EN 16-18 (aromatic type C, Figure 3.8) both having an alkynyl side chain showed different binding affinities with the former revealing higher than the latter, probably due to the presence of the hydrophobic alkyl side chain (as discussed above).
Compounds where the MDP moiety was replaced by two bulky methoxy groups, similar to the verbutin structure (aromatic ring type E, verbutin analogues) first proposed by Pap *et al.*, (2001), seem to affect the interaction with the protein (Figure 3.8). The two methoxy groups replacing the two oxygen atoms connected with the methylene carbon of the MDP moiety are relatively bulky and probably fit in a binding cavity like the MDP moiety. Although EN 25-10 has an alkynyl ether moiety, the relatively low binding potency observed is possibly related to the absence of the hydrophobic propyl substituent and/or the position of the branched methyl group in the alkynyl ether chain. In contrast to the branched methyl group in EN 1-182, as discussed above (aromatic ring type A, Figure 3.7), where the methyl group is positioned between two -CH$_2$- groups, the branched methyl group in EN 25-10 is between the oxygen of the alkynyl ether chain and the aromatic ring. This probably restricts its free rotation because of the free lone pair of electrons of the neighbouring oxygen atom (of the alkynyl ether moiety) and the delocalised electrons of the aromatic ring. EN 25-35 showed the highest binding potency compared to the rest of the verbutin analogues and it is likely to be correlated with the presence of the hydrophobic alkyl chain and the triple bond (as discussed above). The lower binding potency of EN 25-36 compared to EN 25-35 is probably due to the position of the triple bond, which in the former is located at the end of the chain (RC≡H$^+$) and the active proton H$^+$ (electric charge of +1) is acting as an attached functional group, giving acidic properties to the molecule. Subsequently, EN 25-36 could interact with surrounding amino acid residues before actually reaching the appropriate binding site or may settle in a different orientation.

A final observation is that there is a positive correlation between the increased rate of the hydrolysis of 1-NA in the conventional assay (slopes) and the I values obtained from the interference assay (Figure 3.9). This suggests that the lower the binding affinity obtained from the interference assay the higher the increase of the catalytic centre activity. Furthermore, it is suggested that analogues such as EN 25-10, EN 1-40 and EN 1-129 lacking the propyl chain do not follow the above trend and are giving low binding affinities (high I values) with no increase of the catalytic centre activity. This again suggests that the alkyl side chain is important in the final orientation of the analogues.
3.5 Conclusions

Results presented in this chapter suggest that the interactions between PBO/analogues and E4 are based on a balance of non-covalent interactions. Several factors may contribute to the relative binding strength of inhibitors to the protein depending on the chemical and structural properties of both the ligands and the protein. Since the crystal structure of E4 is unknown, the binding properties with PBO/analogues can only be hypothesised. Considering that E4 has similarities with other carboxylesterases, AChE and BuChE (Xie et al., 2002; Imai, 2006), it is hypothesised that PBO and analogues are binding to a location near the active site gorge. Hypothetical aspects of the ligand-protein interaction have been discussed, involving conformation and flexibility of the inhibitors with amino acid residues of the binding site due to a combination of steric effects, hydrophobic bindings and aromatic stacking interactions.

Using PBO analogues with specific organic groups such as alkyl and polyether chains, alkynyl moieties and functional groups such as -COOH and -COONa, some insights into esterase-PBO interactions have been suggested. Sizes and shapes of molecules, electrical charge distribution and geometry of bond angles of these organic groups seem to affect the properties of the interaction with the protein. Preliminary results revealed a significant contribution of the alkyl chain, suggesting that the presence of this hydrophobic substituent is orientating the ligand towards a hydrophobic area, possibly equivalent to the aromatic gorge of Tc:AChE (Sussman et al., 1991), by hydrophobic interactions and thus increasing the binding affinity. Furthermore, results suggest that increasing hydrophobicity by increasing the length of the alkyl side chain, leads to an increase in the binding affinity, as seen in EN 1-16. The length of the alkyl chain could also produce steric effects leading to a better orientation of the molecules in the binding area. The polyether chain is contributing to the binding affinity possibly by the lone pair of electrons of the oxygen atoms which are acting as electron-donating substituents. It may be that the polyether chain is also helping the molecule to a preferable orientation in the binding area. Additionally, replacement of the polyether chain with an alkynyl moiety suggests that the contribution of the delocalised electrons around a triple bond is also an important factor. Thus, either the charge derived from lone pairs of electrons on the oxygen
atoms of the polyether chain and/or delocalised electrons from the triple bond are likely to be contributing to the high binding affinity seen between the ligands and the protein.

Overall, this study has identified possible parameters (such as the length of the alkyl chain and presence of the polyether chain) that are important for the binding potencies of PBO/analognes to E4. Additionally, replacement of the polyether chain by an alkynyl chain in some cases increases the binding affinity of the analogues. Although conclusions at this stage can only be speculative, in the long term such study may contribute to the design of novel esterase inhibitors. Furthermore, since E4 can interact with several compounds, it is possible to search for a specific inhibitor for this protein. This would help in the characterisation of metabolic resistance which is not possible with the dual inhibitory ability of PBO on oxidases and esterases. Certainly, a crystal structure of E4 would contribute dramatically to the design of more potent and specific esterase inhibitors to overcome insecticide resistance problems arising from these metabolic enzymes.
Chapter 3
Use of PBO analogues to investigate the interactions between PBO and E4

$T_c \text{ AChE}$

1 10 20 30 40 50

---DDHSELLVNTKSGKMGTRPVVLSS-HIS

MKNTCGILLNLFLIGCFLTCSASNTPKVQHSGELAGGFEFYTYNGRKLY

51 60 70 80 90 100

$T_c \text{ AChE}$

AFGLIPAPAEPVGMRFPPPEPKKPVSGVWNASTYPNNNQYVDEQFPGF

$T_c \text{ AChE}$

SFLGIPYASPPVQNNFKEFQYQPWLGVWNATVPGSACLGIEFGSGSKI

101 110 120 130 140 150

$T_c \text{ AChE}$

SSEMWNPNEMSEDCLVLNVLWSPRKS------TTVNGWVTGGGFGSG

IC-----------QEDCLVNVTKLPQENSAGDLMNTVHIGGXYFG

151 160 170 180 190 200

$T_c \text{ AChE}$

SSTLDVNGKYLATBVEVLVSLSYVMGAFGFLAHGSEQAPGNVGLLDQ

---EGILVGYPHYLLNNFVYVSNYRGLVGEASTGDG--VLTQNNGLKDQ

201 210 220 230 240 250

$T_c \text{ AChE}$

RMALQWHDNIQPFGDPTKLIFGESACGASVGMHILSPGSDLFRAI

VAIKNQQNVFVAFGDPNSMTITGMACASVHNHLSPMSTAGLFNRAI

251 260 270 280 290 300

$T_c \text{ AChE}$

LQSGSPNCPWSVSAEVGRRAVEGLRNCNLNSDEELELHCLEEKPEQE

IQSGSAFCHWTAENVA---QHTYLANMCPTNNSVECLRSNPAKA

301 310 320 330 340 350

$T_c \text{ AChE}$

LIDVEWNLPSDFISFRFSVFPVFPVIGEPFPTIESLNSGNFKTQVLGIV

IAKSYLNFMPFRRNFPTPGTVVAGYEKLFIPDPEKLVPHDIPVLI SI
Use of PBO analogues to investigate the interactions between PBO and E4

Figure 3.10 Alignment of the *Myzus persicae* esterase (E4) and *Torpedo californica* AChE (TcAChE) showing the 14 conserved residues in TcAChE active site gorge (→) and the 7 common conserved in E4 (←) [Using the Programme: AlignX (Vector NTI Advance 10.1.1 2005 Invitrogen Corporation)]
Chapter 4

Use of a piperonyl butoxide analogue, EN 16/5-1, to characterise metabolic resistance in two agricultural insect pests

4.1 Introduction

Metabolic resistance has often been inferred from correlations between resistance factors and enhanced enzyme activity when test insects are compared with their susceptible counterparts (Brown and Brogdon, 1987; Hemingway et al., 1996). The alternative has been to use a synergist and examine reductions in resistance factors due to inhibition of the enzymes conferring metabolic resistance (Raffa and Priester, 1985; Metcalf, 1989).

Piperonyl butoxide, a MDP synergist was used for many years as a research tool to identify metabolic resistance resulting from elevated P450 activity (Sun and Johnson, 1960; Casida, 1970). However, it was later reported that PBO not only inhibits microsomal oxidases, but also resistance-associated esterases in many insect pests (Gunning et al., 1998; Moores et al., 1998; Young et al., 2005, 2006). The ability to inhibit both major metabolic enzyme systems makes PBO an ideal synergist for agricultural use, but negates its use as an indicator of resistance by P450 metabolism.

Methylenedioxyphenyl compounds, such as PBO, exert their inhibitory effects on P450 monooxygenases through the formation of a P450-MDP complex. This stable complex results from the interaction of the carbene with the haem iron of the cytochrome P450 (Dahl and Hodgson, 1979). Theoretically by altering the MDP ring, such a reaction could no longer take place due to the failure of carbene formation. An analogue of PBO, EN 16/5-1 (Figure 4.1), containing a modification in the MDP moiety, was synthesised. This analogue should no longer have the ability to form the P450-MDP inhibitory complex but retains its ability to inhibit esterases (as shown in the SAR study, Chapter 3). However, the analogue and PBO itself may well be able to inhibit oxidases other than the P450s.
The aims of the work reported in this chapter are to characterise the metabolic resistance mechanism(s) in a Greek *M. persicae* clone (clone 5191A) that exhibited resistance to imidacloprid and also to investigate the metabolic factor(s) conferring differential insensitivity to α-cypermethrin in four *M. aeneus* populations. The two insect species were examined in a series of *in vitro* and *in vivo* assays using PBO and the analogue (EN 16/5-1) and compared with insects possessing well-characterised resistance mechanisms.

The *in vitro* assays described include the indirect determination of esterase binding using the esterase interference assay (Khot *et al.*, 2008) and the ECOD assay (Ullrich and Weber, 1972). The esterase interference assay was used to study the interactions of semi-purified esterases derived from *M. persicae* clone 5191A and *M. aeneus* with two synergists, (PBO and EN 16/5-1) and two insecticides (α-cypermethrin and imidacloprid). The ECOD assay was performed to measure the ability of the two synergists to inhibit *O*-deethylation activity in *M. aeneus* homogenates derived from the four populations with different resistance levels to α-cypermethrin.
4.2 Materials and Methods

4.2.1 Insects
Five different *M. persicae* clones were used 4106A, TIV, 794JZ, 926B and 5191A (section 2.1.2 and Appendix 1) and four *M. aeneus* populations UK, Stein, Rogalin and Lebork (section 2.1.3). The susceptible *M. domestica* strain, WHO, (section 2.1.4) was used as the AChE source for the esterase interference assay.

4.2.2 Insecticides and synergists
Two technical insecticides were used, imidacloprid and α-cypermethrin and two synergists, technical PBO (‘Ultra’, 94%) and its analogue, EN 16/5-1 (98%) (Figure 4.1).

![PBO and EN 16/5-1 structures](image)

Figure 4.1 Structures of PBO and its analogue EN 16/5-1.

4.2.3 Polyacrylamide gel electrophoresis
Individual apterous *M. persicae* from each clone T1V, 926B, 5191A and 794JZ were homogenised in 25 µL of 0.02 M phosphate buffer, pH 7.0 and analysed by electrophoresis as described in section 2.3.1.

4.2.4 Determination of *Myzus persicae* total esterase activity
Esterase activity of aphids from clone 5191A was measured alongside four well-established aphid clones, 4106A, TIV, 794JZ and 926B using a colourimetric assay as described in section 2.3.2.1.
4.2.5 Semi-purification of *Myzus persicae* and *Meligethes aeneus* esterases

Aphids from clone 5191A (5 g) and pollen beetles from Lebork (1.5 g) (stored at -20°C) were homogenised in 10 mL and 15 mL 0.02 M phosphate buffer, pH 7.0, respectively. The homogenates were used for the semi-purification of relevant esterases, as described in section 2.3.4.2.

4.2.6 Indirect determination of esterase binding

Semi-purified esterase from *M. persicae* clone 5191A, (FE4), was pre-incubated with stock solutions of PBO, EN 16/5-1, α-cypermethrin and imidacloprid to give final concentrations of 1 mM of synergists and insecticides (section 2.3.5.2). Aliquots (15 µL) of FE4, FE4+PBO, FE4+EN 16/5-1, FE4+α-cypermethrin and FE4+imidacloprid were used in the esterase interference assay as described in section 2.3.5.3.

Semi-purified esterase(s) from *M. aeneus* (E\textsubscript{pb}) was pre-incubated with PBO, EN 16/5-1 and α-cypermethrin to give final concentrations of 1 mM of synergists and insecticide (section 2.3.5.2). Aliquots (15 µL) of E\textsubscript{pb}, E\textsubscript{pb}+PBO, E\textsubscript{pb}+EN 16/5-1 and E\textsubscript{pb}+α-cypermethrin were used in the esterase interference assay as described in section 2.3.5.3. For this study, esterase(s) from the Lebork population was used as this population displayed the highest level of resistance to pyrethroid.

4.2.7 Determination of *O*-deethylation activity

4.2.7.1 Enzyme preparation

Microsomal preparations from fresh rabbit liver and *M. persicae* aphid clone 5191A and *M. aeneus* homogenates were prepared as described in section 2.3.6.1.

4.2.7.2 Determination of *O*-deethylation activity using microsomal enzymes from rabbit liver and *Myzus persicae*

Aliquots (25 µL) of either liver or aphid microsomes were added to separate wells of a microplate followed by 25 µL 0.1 M sodium phosphate buffer, pH 7.6. Then, a mixture of liver and aphid microsomes (1:1, total volume 50 µL) was added to separate wells of the same plate and the assay performed as described in section 2.3.6.2.
4.2.7.3 Determination of O-deethylation inhibition in *Meligethes aeneus* homogenates by PBO and EN 16/5-1

The anti-oxidase potency of PBO and EN 16/5-1 (10 mM stock solution) against pollen beetle homogenates was compared by measuring ECOD activity as described in section 2.3.6.3.

4.2.8 Insect bioassays

4.2.8.1 Full dose response and discriminating dose response bioassays (*Myzus persicae*)

Apterous adults were individually dosed with either PBO or EN 16/5-1 in acetone. After 5 h the aphids were further treated with either imidacloprid or α-cypermethrin in acetone as described in section 2.4.1.

To investigate response (mortality) to PBO alone, aperous aphids were topically treated with 0.25 µL aliquots of PBO from stock solutions of 0.3 – 100 g L\(^{-1}\) in acetone. Controls were treated with acetone only.

Discriminating dose response bioassays were carried out using the two synergists (PBO or EN 16/5-1) and a single diagnostic concentration of either 1 ppm for imidacloprid against clone 5191A or 100 ppm for α-cypermethrin against clone 794JZ as described in 2.4.2. Treated aphids were stored and scored as described in section 2.4.1.

4.2.8.2 Full dose response and discriminating dose response bioassays (*Meligethes aeneus*)

Full dose response bioassays were performed as described in section 2.4.3. Discriminating dose response bioassays using the two synergists (PBO or EN 16/5-1) and a single diagnostic concentration of 0.01 ppm for UK, 0.3 ppm for Stein and 1 ppm for Rogalin and Lebork α-cypermethrin were carried out as described in section 2.4.4.

4.2.9 Analysis of data

4.2.9.1 Indirect determination of esterase binding by PBO and EN 16/5-1

Concentrations of azamethiphos sufficient to inhibit 50% of AChE activity, (IC\(_{50}\) values, section 2.5.1) were tested for significance using one-way ANOVA analysis (GenStat 12\(^{th}\)
Chapter 4 Use of a PBO analogue, EN 16/5-1, to characterise metabolic resistance in two agricultural insect pests

Edition, VSN International). Following the ANOVA analysis, significance of the difference between selected pairs of means for treatments (IC_{50} mean of two or three replicates within each treatment) was assessed using the Least Significant Difference (LSD) at the 5% level.

**4.2.9.2 Determination of O-deethylation inhibition in *Meligethes aeneus* homogenates by PBO and EN 16/5-1**

The mean absorbance of three replicates at each time point was calculated and linear regressions plotted using Grafit 3.0 (Leatherbarrow, R.J., Erithacus Software). Then the efficacy of the inhibitors (PBO and EN 16/5-1) was estimated (section 2.5.2.). Standard errors for the ratios were estimated using the formula for the variance of a ratio (see Appendix 7).

**4.2.9.3 Insect bioassay data**

Full dose response and discriminating dose bioassay data were analysed as described in sections 2.5.3 and 2.5.4, respectively.
4.3. Results

4.3.1 Polyacrylamide gel electrophoresis and total esterase activity

The enhanced resistance-associated esterase present in clone 5191A was of the FE4 variant, commonly found in *M. persicae* samples from Mediterranean countries (Figure 4.2.) (Field and Devonshire, 1998). Both the mobility and intensity of staining of the esterase bands of clone 5191A were identical to those found in clone 926B. Additionally, clone 5191A had total esterase activity corresponding to category R$_3$ (extremely high) similar to clones 926B and 794JZ (Table 4.1). Aphid clones 794JZ (E4, R$_3$) and T1V (E4, R$_2$) were included to provide a reference for esterase mobility and intensities.

![Polyacrylamide gel electrophoresis showing presence of E4 in Myzus persicae clones T1V and 794JZ and FE4 in 926B and 5191A.](image)

Table 4.1 *Myzus persicae* esterase levels: absorbance values in mOD$_{450}$min$^{-1}$.

<table>
<thead>
<tr>
<th><em>Myzus persicae</em> clone</th>
<th>Esterase level*</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>926B</td>
<td>R$_3$</td>
<td>133 ± 7.13</td>
</tr>
<tr>
<td>794JZ</td>
<td>R$_3$</td>
<td>113 ± 0.53</td>
</tr>
<tr>
<td>T1V</td>
<td>R$_2$</td>
<td>34.1 ± 4.21</td>
</tr>
<tr>
<td>4106A</td>
<td>S</td>
<td>6.40 ± 3.35</td>
</tr>
<tr>
<td>5191A</td>
<td>R$_3$</td>
<td>96.0 ± 2.41</td>
</tr>
</tbody>
</table>

*S (susceptible), R$_2$ (high), R$_3$ (extremely high), n=19
4.3.2 Indirect determination of esterase binding

Since the interference assay depends on the action of unsequestered azamethiphos against housefly AChE, prior to the assays being performed the two insecticides (imidacloprid and α-cypermethrin) were tested for direct inhibition of housefly AChE using the standard Ellman assay (Ellman et al., 1961). No inhibition was observed (data not shown).

4.3.2.1 Indirect determination of esterase binding (*Myzus persicae*)

The reduced protection of AChE against azamethiphos provided by FE4 following pre-incubation with synergists or insecticides, is presented in Figure 4.3. Corresponding IC₅₀ values are presented in Table 4.2.

Results of ANOVA analysis reveal significant differences among the treatments (*p*<0.001). The synergists, PBO and EN 16/5-1, were not significantly different in their action (*p>*0.05) and both bound greater amounts of FE4 than the insecticides (*p*<0.05). There was a significant difference in the esterase blockaded by the two insecticides, with α-cypermethrin binding more esterase than imidacloprid (*p*<0.05).

![Figure 4.3](image-url)

Figure 4.3 Indirect determination of *Myzus persicae* clone 5191A esterase (FE4) binding by synergists (PBO and EN 16/5-1) and insecticides (α-cypermethrin and imidacloprid). Data are means of triplicates ± SE, LSD (95%)=0.2301, df=20.
Table 4.2 Concentrations of azamethiphos required to give 50% inhibition of AChE activity (IC$_{50}$) after pre-incubation of synergists (PBO and EN 16/5-1) and insecticides ($\alpha$-cypermethrin and imidaclorpid) with FE4 from *Myzus persicae* clone 5191A (refer to Figure 4.3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC$_{50}$ (nM)</th>
<th>SE ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE only (no FE4)</td>
<td>0.165</td>
<td>0.006</td>
</tr>
<tr>
<td>FE4 only (FE4)</td>
<td>3.88</td>
<td>0.516</td>
</tr>
<tr>
<td>FE4 + PBO</td>
<td>0.215</td>
<td>0.016</td>
</tr>
<tr>
<td>FE4 + EN 16/5-1</td>
<td>0.197</td>
<td>0.007</td>
</tr>
<tr>
<td>FE4 + $\alpha$-cypermethrin</td>
<td>0.437</td>
<td>0.034</td>
</tr>
<tr>
<td>FE4 + imidaclorpid</td>
<td>0.908</td>
<td>0.087</td>
</tr>
</tbody>
</table>

4.3.2.2 Indirect determination of esterase binding (*Meligethes aeneus*)

Using the semi-purified esterases, E$_{pb}$, from the Lebork population, results showed that PBO, EN 16/5-1 and $\alpha$-cypermethrin were not significantly different in their action and bound similar amounts of semi-purified esterase ($p>0.05$) (Figure 4.4 and Table 4.3).

Figure 4.4 Indirect determination of *Meligethes aeneus* (Lebork population) semi-purified esterase (E$_{pb}$) binding by synergists (PBO and EN 16/5-1) and insecticide ($\alpha$-cypermethrin). Data are means of duplicates ± SE, LSD (95%)=0.04482, df=5.
Chapter 4  Use of a PBO analogue, EN 16/5-1, to characterise metabolic resistance in two agricultural insect pests

Table 4.3 Concentrations of azamethiphos required to give 50% inhibition of AChE activity (IC\(_{50}\)) after pre-incubation of synergists (PBO and EN 16/5-1) and insecticide (\(\alpha\)-cypermethrin) with semi-purified esterases \(E_{pb}\) from *Meligethes aeneus* (Lebork population) (refer to Figure 4.4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC(_{50}) (nM)</th>
<th>SE ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE only (no (E_{pb}))</td>
<td>0.10</td>
<td>0.0024</td>
</tr>
<tr>
<td>(E_{pb}) only ((E_{pb}))</td>
<td>0.42</td>
<td>0.0095</td>
</tr>
<tr>
<td>(E_{pb}) + PBO</td>
<td>0.31</td>
<td>0.006</td>
</tr>
<tr>
<td>(E_{pb}) + EN 16/5-1</td>
<td>0.26</td>
<td>0.0035</td>
</tr>
<tr>
<td>(E_{pb}) + (\alpha)-cypermethrin</td>
<td>0.33</td>
<td>0.0147</td>
</tr>
</tbody>
</table>

4.3.3 Determination of \(O\)-deethylation activity using microsomal enzymes from rabbit liver and *Myzus persicae*

Microsomal preparations from the aphid clone 5191A failed to exhibit any \(O\)-deethylation activity (Figure 4.5). Furthermore, when mixed with liver microsomes, which gave the expected high oxidase activity (Adams *et al.*, 1993), the aphid microsomes inhibited the \(O\)-deethylation activity of the liver.

![Figure 4.5 O-deethylation activity of the microsomal preparations from rabbit liver, *Myzus persicae* (clone 5191A) and liver + *M. persicae* (clone 5191A) mixture. Data are means of triplicates ± SE.](image-url)
4.3.4 Determination of $O$-deethylation inhibition in *Meligethes aeneus* homogenates by PBO and EN 16/5-1

Pollen beetle homogenate was used in a fluorometric assay to measure the ability of PBO and EN 16/5-1 to inhibit the oxidation of 7-EC. The rate of 7-OH production reflects the activity of the oxidases (Table 4.4). Piperonyl butoxide was found to be a more potent inhibitor than EN 16/5-1 in all cases. The activity remaining (%) after inhibition by PBO varied according to the population. Thus, Lebork had the lowest oxidase activity remaining after inhibition by PBO (~19%) whilst the Rogalin population had the highest oxidase activity remaining (~35%). The percentage activity remaining after inhibition by the analogue was, in all cases, at least twice that after inhibition by PBO.

Table 4.4 $O$-deethylation by *Meligethes aeneus* homogenates in the presence/absence of the two synergists, PBO and EN 16/5-1.

<table>
<thead>
<tr>
<th>Population</th>
<th>Enzyme activity (FUs min$^{-1}$) ± SE</th>
<th>(% Activity remaining PBO ± SE)</th>
<th>(% Activity remaining EN 16/5-1 ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>250 ± 8.21</td>
<td>29.8 ± 3.61</td>
<td>99.7 ± 4.12</td>
</tr>
<tr>
<td>Rogalin</td>
<td>147 ± 5.55</td>
<td>35.3 ± 7.59</td>
<td>74.7 ± 6.99</td>
</tr>
<tr>
<td>Lebork</td>
<td>138 ± 6.17</td>
<td>19.1 ± 4.76</td>
<td>73.4 ± 5.65</td>
</tr>
<tr>
<td>Stein</td>
<td>90.0 ± 11.9</td>
<td>22.9 ± 11.1</td>
<td>61.5 ± 8.16</td>
</tr>
</tbody>
</table>

$n=3$

4.3.5 Full dose response bioassays with *Myzus persicae* clones

The results of the full dose response bioassays of *M. persicae* clone 5191A, together with other clones in which the resistance status has been characterised previously, are presented in Table 4.5 and Table 4.6 (see also Appendix 4). Considering the imidacloprid bioassay data (Table 4.5), the Greek clone 5191A had a considerably higher resistance factor (RF 56.5) than clone 926B (RF 4). The mechanism increasing the resistance of clone 5191A to imidacloprid did not confer cross-resistance to $\alpha$-cypermethrin (Table 4.6) thus clones 5191A and 926B both had a similar response to the pyrethroid (RF 13 and RF 16.3, respectively). When PBO was used as a pre-treatment on clone 5191A, the LC$_{50}$ was reduced substantially, indicating that the resistance is, at least partially, due to a metabolic mechanism. When EN 16/5-1 was used as the pre-treatment on clone 5191A, the LC$_{50}$ was again reduced, although to a lesser
extent than with PBO (Table 4.5). Both clones 926B and 794JZ with esterase-based metabolic mechanisms (R3), were synergised by PBO and EN 16/5-1 resulting in similar SFs with both synergists (Table 4.6). As expected, 794JZ showed very high resistance against \( \alpha \)-cypermethrin due to the presence of target-site resistance, kdr (Martinez-Torres et al., 1999). However, the SF for this clone was found to be similar to 926B, illustrating that the metabolic resistance is comparable in both clones (Table 4.6).

Table 4.5 Full dose response bioassays with *Myzus persicae* clones against imidacloprid using a 5h pre-treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aphid Clone</th>
<th>(^a\text{LC}_{50}) ppm</th>
<th>(^b\text{CL}) 95%</th>
<th>(^c\text{RF})</th>
<th>(^d\text{SF})</th>
</tr>
</thead>
<tbody>
<tr>
<td>imidacloprid</td>
<td>4106A</td>
<td>1.55</td>
<td>1.18-1.99</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>926B</td>
<td>6.53</td>
<td>5.32-8.03</td>
<td>4.21</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>5191A</td>
<td>90.4</td>
<td>51.0-185</td>
<td>56.5</td>
<td>--</td>
</tr>
<tr>
<td>+ 1g L(^-1) PBO</td>
<td>5191A</td>
<td>6.22</td>
<td>4.04-8.70</td>
<td>--</td>
<td>14.5</td>
</tr>
<tr>
<td>+ 1g L(^-1) EN 16/5-1</td>
<td>5191A</td>
<td>12.2</td>
<td>8.80-16.4</td>
<td>--</td>
<td>7.41</td>
</tr>
</tbody>
</table>

\(^a\text{LC}_{50}\)=Lethal concentration to kill 50% of the clone, \(^b\text{CL}\)= Confidence limits, \(^c\text{RF}\)= resistance factor (\( \text{LC}_{50} \) resistant clone /\( \text{LC}_{50} \) susceptible clone), \(^d\text{SF}\)= synergism factor (\( \text{LC}_{50} \) unsynergised clone /\( \text{LC}_{50} \) synergised clone)

Table 4.6 Full dose response bioassays with *Myzus persicae* clones against \( \alpha \)-cypermethrin using a 5h pre-treatment.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aphid Clone</th>
<th>(^a\text{LC}_{50}) ppm</th>
<th>(^b\text{CL}) 95%</th>
<th>(^c\text{RF})</th>
<th>(^d\text{SF})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-cypermethrin</td>
<td>4106A</td>
<td>0.43</td>
<td>0.29-0.56</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>5191A</td>
<td>5.60</td>
<td>5.94-6.33</td>
<td>13.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>926B</td>
<td>7.08</td>
<td>5.63-8.70</td>
<td>16.3</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>794JZ</td>
<td>1303</td>
<td>1060-1632</td>
<td>3030</td>
<td>--</td>
</tr>
<tr>
<td>+ 1g L(^-1) PBO</td>
<td>926B</td>
<td>2.10</td>
<td>1.24-2.88</td>
<td>--</td>
<td>3.37</td>
</tr>
<tr>
<td>+ 1g L(^-1) EN 16/5-1</td>
<td>926B</td>
<td>1.48</td>
<td>0.79-2.23</td>
<td>--</td>
<td>4.78</td>
</tr>
<tr>
<td>+ 1g L(^-1) PBO</td>
<td>794JZ</td>
<td>271</td>
<td>220-327</td>
<td>--</td>
<td>4.80</td>
</tr>
<tr>
<td>+ 1g L(^-1) EN 16/5-1</td>
<td>794JZ</td>
<td>264</td>
<td>215-321</td>
<td>--</td>
<td>4.94</td>
</tr>
</tbody>
</table>

* \(a, b, c, d, \) as per Table 4.5
4.3.6 Discriminating dose bioassays with *Myzus persicae* clones

The effects of a 5 h pre-treatment with either PBO or EN 16/5-1 on clones 794JZ and 5191A, prior to exposure to a discriminating dose of insecticide, are presented in Figure 4.6 and Figure 4.7, respectively. Bioassays with clone 794JZ against α-cypermethrin revealed that PBO and EN 16/5-1 were not significantly different in their synergistic action (*p*>0.05) (Moores *et al*., 2009). The equal synergism was expected since it is known that the sole metabolic insecticide resistance mechanism in clone 794JZ is the enhanced esterase activity (Martine-Torres *et al*., 1999).

![Figure 4.6 Synergistic effects of PBO and EN 16/5-1 (1 g L⁻¹) on mortality against *Myzus persicae* clone 794JZ using a discriminating dose of α-cypermethrin (100 ppm) and a 5h pre-treatment. Data are means ± SE, n=3 for acetone, EN 16/5-1 and PBO, n=15 to 17 for acetone/α-cypermethrin, EN 16/5-1/α-cypermethrin and PBO/α-cypermethrin, N=56, LSD (5%)=0.1188, df=44.](image)

Results from bioassays with clone 5191A against imidacloprid reveal that PBO and EN 16/5-1 were significantly different in their synergistic action with PBO giving higher synergism (*p*<0.001). EN 16/5-1 gave a significant increase in mortality in comparison with insecticide alone (*p*<0.05). Results from the discriminating dose bioassays were therefore in accordance with the results obtained from full dose response bioassays (section 4.3.5).
Chapter 4  

Use of a PBO analogue, EN 16/5-1, to characterise metabolic resistance in two agricultural insect pests

Use of a PBO analogue, EN 16/5-1, to characterise metabolic resistance in two agricultural insect pests

Figure 4.7 Synergistic effects of PBO and EN 16/5-1 (1 g L$^{-1}$) on mortality against *Myzus persicae* clone 5191A using a discriminating dose of imidacloprid (1 ppm) and a 5h pre-treatment. Data are means ± SE, n=9 to 12 for acetone, EN 16/5-1 and PBO, n=24 to 30 for acetone/imidacloprid, EN 16/5-1/imidacloprid and PBO/imidacloprid, N=116, LSD (5%)=0.08065, df=98.

4.3.7 Full dose response bioassays with *Myzus persicae* clones against PBO

To investigate the response (mortality) to PBO only, high concentrations of the synergist were required. Three standard *M. persicae* clones were tested alongside clone 5191A. The LC$_{50}$ values obtained for clones 4106A, 794JZ and 926B were similar, however clone 5191A required approximately twice the dose of PBO to achieve equivalent mortality (Table 4.7, see also Appendix 4).

Table 4.7 Full dose response bioassays with *Myzus persicae* clones against PBO.

<table>
<thead>
<tr>
<th>Aphid Clone</th>
<th>Esterase level</th>
<th>LC$_{50}$ (ppm)</th>
<th>CL 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4106A</td>
<td>S</td>
<td>14892</td>
<td>12527-18041</td>
</tr>
<tr>
<td>794JZ</td>
<td>R$_3$</td>
<td>12196</td>
<td>8485-18788</td>
</tr>
<tr>
<td>926B</td>
<td>R$_2$</td>
<td>10540</td>
<td>6865-17325</td>
</tr>
<tr>
<td>5191A</td>
<td>R$_3$</td>
<td>25738</td>
<td>21570-30947</td>
</tr>
</tbody>
</table>

$^a$LC$_{50}$=Lethal concentration of PBO to kill 50% of the clone, $^b$CL=Confidence limits.
4.3.8 Full dose response bioassays with *Meligethes aeneus* populations

The response of the four pollen beetle populations to α-cypermethrin are presented in Table 4.8 (see also Appendix 4). The LC₅₀ value obtained from the UK population was considered to be representative of a susceptible population since it was found to be the most sensitive in comparison with the other populations. Rogalin and Lebork populations gave the highest RFs (~23 and ~34, respectively). The resistance level for Stein was lower with a RF of ~3.6.

<table>
<thead>
<tr>
<th>Population</th>
<th>a LC₅₀ (ng/cm²)</th>
<th>b CL 95%</th>
<th>c RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>2.81</td>
<td>0.19-4.52</td>
<td>1</td>
</tr>
<tr>
<td>Stein</td>
<td>10.0</td>
<td>6.01-17.0</td>
<td>3.57</td>
</tr>
<tr>
<td>Rogalin</td>
<td>65.0</td>
<td>40.0-100</td>
<td>23.2</td>
</tr>
<tr>
<td>Lebork</td>
<td>95.0</td>
<td>66.0-140</td>
<td>33.9</td>
</tr>
</tbody>
</table>

a LC₅₀=Lethal concentration to kill 50% of the population, b CL= Confidence limits, c RF =resistance factor (LC₅₀ resistant population /LC₅₀ susceptible population)

4.3.9 Discriminating dose bioassays with *Meligethes aeneus* populations

Different discriminating doses of α-cypermethrin were applied to each population, based on the results of the full-dose bioassay (section 4.3.8) and the effects of the synergists are presented in Figure 4.8. Results showed that PBO and EN 16/5-1 conferred different levels of synergism. In particular, PBO gave significantly higher synergism to α-cypermethrin in all populations (p<0.05), increasing the mortality to around 90%. Even in the susceptible UK population, PBO conferred high synergism to α-cypermethrin. In contrast, EN 16/5-1 did not significantly increase the mortality compared to the insecticide alone (p>0.05). No mortality was observed in treatments with acetone, PBO and EN 16/5-1 alone (data not shown).
Figure 4.8 Synergistic effects of PBO and EN 16/5-1 (0.01 g L\(^{-1}\)) mixed with a discriminating dose of \(\alpha\)-cypermethrin against *Meligethes aeneus* populations. Data are means ± SE. For UK \(n=5\), \(N=15\), LSD (95%)=0.1589 df=11; for Stein \(n=6\), \(N=18\), LSD (95%)=0.1827, df=15; for Rogalin* and Lebork* \(n=5\), \(N=70\). LSD (95%)=0.1507, df=56 [*taken from the 7 treatment analysis (see Chapter 5, Figure 5.3)].
## 4.4 Discussion

Piperonyl butoxide, a well known MDP insecticide synergist, has been used for many years to enhance insecticide efficacy in the presence of resistance due to oxidative mechanisms (Sun and Johnson, 1960; Casida, 1970; Bernard and Philogène, 1999). However, subsequent studies have reported that PBO can also inhibit resistance-associated esterases in many agricultural pests (Gunning et al., 1998; Moores et al., 1998; Young et al., 2005, 2006). This dual ability enables PBO to be an excellent synergist in the field, but negates its ability to be used as a research tool to identify which category of metabolic enzyme is conferring resistance.

In this study, an analogue of PBO, EN 16/5-1, that maintains the ability to inhibit esterases (Chapter 3) but loses potency against P450s (due to a modification of the MDP ring) has been utilised. Using examples of insects where resistance mechanisms have been well-characterised, in combination with resistant insects where their resistance-associated enzymes were under investigation, this analogue facilitated the characterisation of metabolic resistance (Moores et al., 2009). A series of in vivo and in vitro assays were applied to investigate possible contributions of metabolic enzymes conferring resistance. In vivo assays included full and discriminating dose bioassays incorporating the two synergists (PBO and the analogue) and in vitro assays included the esterase interference assay and ECOD assay. It is important to consider, at this stage which oxidative enzymes can contribute to the ECOD conversion. It is reported that some P450 enzymes will deethylate 7-EC (Hogdson, 1983) but other oxidative systems will also be capable of this interaction (Benedetti et al., 2006). The modification of the MDP ring will nullify the formation of the carbene complex and so P450 enzymes will be expected to remain active in the presence of the analogue, although inhibition of non-P450 oxidases remains unclear.

While earlier reports had indicated low levels of imidaclorpid resistance in M. persicae, similar to that observed in clone 926B (Foster et al., 2008), clone 5191A possessed an additional degree of resistance, similar to that found previously in Greece, on tobacco (Margaritopoulos et al., 2007). This enhanced resistance of clone 5191A could be due to target site and/or metabolic resistance.
Synergism by PBO, as revealed in the full dose response (Table 4.5) and discriminating dose bioassays (Figure 4.7), suggested that the additional levels of imidaclorpid resistance exhibited by *M. persicae* clone 5191A are due, at least partly, to metabolic enzyme activity. Since PBO is able to inhibit both esterases and oxidases, differential synergist bioassays were performed with PBO and EN 16/5-1 to identify the enzyme that contributed the major effect. *In vitro* studies were adapted using PBO and its analogue, EN16/5-1, to confirm the specificity of synergist action.

Total esterase assay and PAGE showed that clone 5191A possessed a R₃ esterase level and esterase pattern identical to 926B (FE4), a clone whose resistance profile had already been characterised (Moores *et al.*, 1994). Subsequently, esterase(s) from clone 5191A were purified and used for the esterase interference assay. Results showed that FE4 bound equally to PBO and EN 16/5-1 (Figure 4.3). This equivalent interaction of PBO and its analogue with FE4 is in agreement with the α-cypermethrin bioassay results. These results showed very similar synergism factors (SF ~4) using either PBO or EN 16/5-1, against α-cypermethrin in both clones 926B (FE4) and 794JZ (E4) with full dose response bioassays (Table 4.6) and discriminating dose bioassays (Figure 4.6). Enhanced esterase activity has already been reported to be the metabolic mechanism conferring resistance to a broad spectrum of insecticides (Devonshire and Moores, 1982). The esterase interference assay also demonstrated that FE4 was capable of sequestering imidaclorpid, though to a lesser extent than α-cypermethrin (Figure 4.3). The ability of FE4 to sequester imidaclorpid could confer the 4-fold resistance of clone 926B against imidaclorpid (Table 4.5).

Measurement of *O*-deethylation of 7-EC by *M. persicae* has been recently reported (Kwon *et al.*, 2009) however, there was no appreciable activity found in the clone 5191A microsomal preparation in the present study (Figure 4.5). This failure to measure the oxidase activity could be due to high levels of oxidase inhibitors present in *M. persicae* homogenates (Devonshire, 1973; Devonshire and Moores, 1983). It was therefore impossible to assess the relative *O*-deethylation inhibitory potencies of PBO and EN 16/5-1. However, it was found that EN 16/5-1 had the expected decreased ability to inhibit oxidases using the ECOD assay with imidaclorpid resistant whitefly *B. tabaci* (B-biotype) and this was in agreement with the
differential synergism found between PBO and EN 16/5-1, with the former revealing greater synergism (Moores et al., 2009). Synergism bioassays with clone 5191A against imidacloprid using full dose response (Table 4.5) and discriminating dose bioassays (Figure 4.7) revealed that PBO gave significantly higher synergism than EN 16/5-1. Following correction for control mortality there was a 5-fold increase in mortality with EN 16/5-1 and a 14-fold increase with PBO using the discriminating dose bioassay, showing good agreement with the full dose bioassays. Thus, both synergism bioassays and the esterase interference assay suggest the involvement of P450s as a major resistance mechanism in clone 5191A, similar to results obtained with imidacloprid resistant B. tabaci (Moores et al., 2009). The involvement of P450 in the imidacloprid resistant B. tabaci was also confirmed by recent studies (Karunker et al., 2008, 2009). However in the case of clone 5191A this cannot be absolutely confirmed since the in vitro oxidase assay was unable to validate the effect of PBO and EN 16/5-1. However, it is most likely that PBO inhibits M. persicae P450s whilst EN 16/5-1 does not. The difference in the two structures is unlikely to induce significant changes in other pharmaco-kinetic parameters, such as penetration. The resistance levels to α-cypermethrin in both clones 5191A and 926B (Table 4.6) further support enhanced esterases were not responsible for the additional difference in the imidacloprid response.

An additional observation is the response of clone 5191A to PBO alone. It was found to have an ‘insensitivity’ of ~2-fold when compared to other M. persicae clones. This ‘resistance’ to PBO is not correlated to esterase content; hence the R3 clones 794JZ and 926B have the same response as the susceptible clone 4106A (Table 4.7). Previous reports of PBO resistance have suggested high levels of P450 activity in P. xylostella, although full characterisation of the mechanism was never carried out (Chen and Sun, 1986; Hung and Sun 1989). These same reports also noted a reduction in the ability of PBO to synergise, which could explain why complete susceptibility was not restored when clone 5191A was pre-treated with PBO. However, it is common in bioassay studies not to restore full susceptibility after application of a synergist, since the synergistic effect is correlated with the applied ratio of insecticide and synergist (Brindley and Selom, 1984; Bingham et al., 2007). An alternative explanation could be the possible involvement of another metabolic enzyme and/or target site resistance conferring low resistance levels.
Considering the *M. aeneus* results, *in vivo* synergistic bioassays demonstrated that PBO gave high synergism to α–cypermethrin in all tested populations (even in the most sensitive to the pyrethroid, UK population) (Figure 4.8). This would suggest that the different response to α–cypermethrin in pollen beetles is, at least partially, due to metabolic mechanisms. In contrast, no significant increase in mortality was obtained when EN 16/5-1 was incorporated in a mixture with α-cypermethrin. Loss of synergistic activity following modification of the MDP ring has also been reported by Wilkinson *et al.*, (1966) and more recently by Pap *et al.*, (2001).

To further preclude that *M. aeneus* esterases were not contributing to pyrethroid resistance, the interference assay was adapted using semi-purified esterase from the Lebork population, since this population was found to exhibit the highest resistance level to α-cypermethrin (Table 4.8). The protection afforded to the AChE against azamethiphos by *M. aeneus* esterase was found to be less than that afforded by FE4 derived from *M. persicae* per equivalent activity i.e. the catalytic centre activity of pollen beetle esterase is presumably higher, resulting in less moles of esterase present. Although the protection provided was lower, incubations with PBO and EN 16/5-1 showed an equal reduction of esterase protection.

The ability of PBO and EN 16/5-1 to inhibit ECOD activity from *M. aeneus* was compared. As expected, modification of the MDP ring in EN 16/5-1 resulted in reduced inhibition of oxidase activity compared to PBO. The highest inhibition of ECOD activity by EN 16/5-1 was observed in the Stein population, followed to a lesser extent by Rogalin and Lebork (Table 4.4). The *O*-deethylation inhibition by EN 16/5-1 must be presumed to be on non-P450 enzymes present in the insect homogenates. Since EN 16/5-1 did not synergise insecticides *in vivo* (Figure 4.8), these enzymes are presumably not contributing to α-cypermethrin resistance.

What might be considered surprising in this study is the observation that the susceptible UK *M. aeneus* population showed the highest ECOD activity. This finding is in contrast to most cases where enhanced enzyme activities using model substrates are correlated with resistance factors. However, it must be emphasised that oxidases are a large family of enzymes which can metabolise, to a different extent, an array of substrates (Wilkinson, 1983) and
consequently it is possible oxidases other than P450s are able to \( O \)-deethylate 7-EC (Benedetti \textit{et al.}, 2006). This example reinforces the necessity to fully characterise the contribution of enzyme systems conferring resistance, rather than simply looking for an increase in activity using a model substrate. Scott, (1999) stated that “it is possible that a similar phenomenon to the ‘mutant ali –esterase theory’ (Oppenoorth, 1985) could arise in the case of MFOs”. This statement is supported further since it has been shown that a single change in P450 amino acid sequence by site-directed mutagenesis can alter the substrate specificity of a P450 (Lindberg and Negishi, 1989). Qualitatively different P450s have also been reported in some housefly strains exhibiting diazinon and dimethoate resistance and this did not correlate to enhanced oxidase activity (Hogdson, 1983).

Since the purpose of the present study was not to identify, or characterise, individual cytochrome P450s or specific oxidases involved in \( \alpha \)-cypermethrin resistance, 7-EC was used as a model substrate, using whole insect homogenates. Results were interpreted following the hypothesis that an oxidative pyrethroid detoxification would be the result of a de -alkylation reaction similar to the \( O \)-deethylation of 7-EC by P450 (Roberts and Hutson, 1999; Hodgson, 1983) and this hypothesis was supported by the correlation of the \textit{in vitro} and the \textit{in vivo} results. Pollen beetle homogenates were used in the ECOD assay, rather than microsomal preparations, to include the possible contribution of all oxidases that contributed to \( \alpha \)-cypermethrin resistance, not only membrane-bound examples such as microsomal P450s (Benedetti \textit{et al.}, 2006).

\section*{4.5 Conclusions}

The results reported here demonstrate that PBO and its close analogue EN 16/5-1 may be used in parallel to characterise metabolic insecticide resistance. If both synergists show equal effect, it is likely that either esterases are the major metabolic resistance mechanism, or oxidases other than P450s are responsible. For this reason, the esterase interference assay should also be carried out to aid diagnosis. This is supported by the results with \textit{M. persicae} clones in which esterase (E4/FE4) is known to confer resistance to a broad spectrum of insecticides and supports the findings of Moores \textit{et al.}, (2009). If PBO shows greater synergism than EN 16/5-1, it suggests that the difference between the two SFs is due to the
contribution of P450s. This premise is strengthened by the *in vivo* and *in vitro* assays presented here with clone 5191A against imidacloprid, where *in vivo* studies showed that PBO synergised to a greater extent than EN 16/5-1 whilst results of *in vitro* studies (esterase interference assay) showed both synergists bound equally with esterases. The latter results showed an esterase contribution in the imidacloprid resistant clone, 5191A. This esterase contribution is demonstrated when imidacloprid bound slightly to FE4 and conferred a RF of around 4 in clone 926B. In the case of *M. aeneus* investigations, synergism was achieved with PBO but not with EN 16/5-1 whilst *in vitro* both synergists bound equally with esterases (esterase interference assay), but the inhibitory potency of PBO upon oxidase was greater than the analogue (ECOD assay). This is in line with previous reports that imidacloprid resistance in *B. tabaci* is also synergised more strongly by PBO than the analogue, implying the contribution of P450s as the main metabolic enzyme conferring resistance (Moores *et al.*, 2009). Whitefly imidacloprid resistance due to an oxidative mechanism has also been supported by other reports based on correlations between enhanced ECOD activity and resistance factors (Rauch and Nauen, 2003; Roditakis, *et al.*, 2009; Wang *et al.*, 2009). However, recently it has been reported that overexpression of cytochrome P450 CYP6CM1 in B-biotype *B. tabaci* was associated with high level of imidacloprid resistance (Karunker *et al.*, 2008, 2009). Clearly, the analogous CYP6 in imidacloprod-resistant *M. persicae* would be worthy of further investigation.
Chapter 5

In vivo and in vitro studies of piperonyl butoxide and analogues against four pollen beetle Meligethes aeneus Fabricius (Coleoptera: Nitidulidae) populations

5.1 Introduction

Insecticide synergists have been used for many years and have contributed significantly to improve the efficacy of insecticides, particularly when problems of resistance have arisen (Berhard and Philogènes, 1993). Although several chemicals have been produced, in earlier studies, for the specific purpose of enhancing the insecticidal properties of various toxicants (Beroza and Barthel, 1957; Moorefield, 1958; Metcalf and Fukuto, 1965), MDP compounds have been the most extensively studied due to their inhibitory effects against enzymes capable of oxidising insecticides (Casida, 1970). Piperonyl butoxide and a few closely related MDP compounds such as sesamex, sesame oil, isosafrole and safrole have demonstrated potent synergistic effects. However, only PBO is in commercial use and is the leading insecticide synergist (Jones, 1998; Keserú et al., 1999). Sesamex has a structure similar to PBO (Chapter 1, Figure 1.3) and although it was one of the first potent synergists reported (Eldefrawi et al., 1960; Farnham, 1971, 1973, 1974), it failed to achieve commercial use because in the presence of sunlight and moisture it decomposes to yield objectionably coloured products (Casida, 1970).

In this chapter different PBO analogues have been examined for their ability to synergise the effects of α-cypermethrin against four M. aeneus populations that exhibited different sensitivity to this insecticide. The analogue structures studied in this chapter have modifications in the MDP moiety and/or the side chains and include phenyl methyl and phenyl ethers.
Of particular interest were four MDP analogues; two phenyl methyl but-2-ynyl ethers, EN 1-126 and EN 1-125 and two phenyl but-2-ynyl ethers, EN 1-164 and EN 1-163 that were tested in conjunction with PBO (Figure 5.1). These two sets of analogues differed only in the presence/absence of the methylene bridge (–CH₂-) between the aromatic ring and the oxygen atom of the alkynyl ether moiety. EN 1-164 and EN 1-163 lack the methylene bridge and the oxygen atom is bound directly to the aromatic ring (aromatic ring type B in Chapter 3) i.e in a similar position to sesamex, in an attempt to replicate its high synergism (the structures of EN 1-164 and EN 1-163 have been presented in an European Patent Application, No 09168083.5, under the title “Substituted Alkynyl phenoxy compounds and their uses”, see Appendix 8). EN 1-126 and EN 1-125 retain the methylene bridge between the aromatic ring and the oxygen atom of the alkynyl ether moiety (aromatic ring type A in Chapter 3). Structurally similar phenyl ethers have been reported in early studies for their synergistic effects with carbamate insecticides against susceptible houseflies (Barnes and Fellig, 1969; Fellig et al., 1970) and more recently phenyl methyl ethers (equivalent to EN 1-126 and EN 1-125) showed high synergism against pyrethroid and carbamate resistant houseflies (Pap et al., 2001; Bertók et al., 2003). Other compounds examined for their synergistic potency are compounds having alterations either in the MDP moiety (EN 16/5-1 and EN 14-05, aromatic ring type C and D respectively in Chapter 3) or in the side chains (EN 1-129 and EN 1-40 aromatic ring type A in Chapter 3).

The aim of this chapter is to investigate the synergistic effects of these analogues and their ability to inhibit O-deethylation of 7-EC in comparison to PBO using four M. aeneus populations. Correlations between in vivo/in vitro investigations and analogue structures are discussed.
5.2 Materials and Methods

5.2.1 Insects
Four *M. aeneus* populations were used: Lebork, Leszno, Rogalin and UK (section 2.1.3). Due to insufficient insect numbers not all the analogues were tested with all populations.

5.2.2 Insecticide and synergists
Technical α-cypermethrin was used as the insecticide. Analytical grade PBO and analogues were used as synergists (Figure 5.1).

Figure 5.1 Structures of PBO and analogues
5.2.3 Insect bioassays
Different discriminating concentrations of α-cypermethrin were incorporated with 0.01g L\(^{-1}\) synergists in a mixture. Concentrations of insecticide used in the discriminating bioassays were derived from full dose response bioassays (Chapter 4, section 4.3.8, Table 4.8). Treatments including acetone and 0.01g L\(^{-1}\) of synergists alone were used as controls.

5.2.4 Determination of O-deethylation inhibition by PBO and analogues
Pollen beetle homogenates were prepared as described in section 2.3.6.1 and O-deethylation inhibition by PBO and analogues was performed as described in section 2.3.6.3.

5.2.5 Analysis of data
5.2.5.1 Insect bioassay data
Results from full dose and discriminating dose bioassays incorporating synergists were analysed as described in sections 2.5.3 and 2.5.4, respectively.

5.2.5.2 Determination of O-deethylation inhibition by PBO and analogues
The mean absorbance at each time point was calculated as described in section 2.5.2. Data were analysed using linear regression with correlated errors (GenStat 12\(^{th}\) Edition, VSN International). O-deethylation inhibition by analogues was considered significantly different if the CL95% of the remaining activity (ratio) did not overlap.
5.3. Results

5.3.1 Insect bioassays

Full dose response bioassays with \( \alpha \)-cypermethrin against the four \textit{M. aeneus} populations are presented in Table 5.1 (see also Appendix 4). Data in Table 5.1 also includes LC\(_{50}\) values presented in Chapter 4 (section 4.3.8, Table 4.8) and repeated here for convenience. The additional data in Table 5.1 show the resistance level for the Leszno population which had a RF of 8 and therefore was less resistant than Rogalin and Lebork.

Table 5.1 Full dose response bioassays with \textit{Meligethes aeneus} populations against \( \alpha \)-cypermethrin (including data from Chapter 4, Table 4.8).

<table>
<thead>
<tr>
<th>Population</th>
<th>( ^a \text{LC}_{50} ) (( \eta \text{g/cm}^2 ))</th>
<th>( ^b \text{CL} \text{ 95%} )</th>
<th>( ^c \text{RF} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>2.81</td>
<td>0.19-4.52</td>
<td>1</td>
</tr>
<tr>
<td>Leszno</td>
<td>22.5</td>
<td>13.2-35.0</td>
<td>8.00</td>
</tr>
<tr>
<td>Rogalin</td>
<td>65.0</td>
<td>40.0-100</td>
<td>23.2</td>
</tr>
<tr>
<td>Lebork</td>
<td>95.0</td>
<td>66.0-140</td>
<td>33.9</td>
</tr>
</tbody>
</table>

\( ^a \text{LC}_{50}=\text{Lethal concentration to kill 50\% of the population}, \quad ^b \text{CL}=\text{Confidence limits}, \quad ^c \text{RF}=\text{resistance factor (LC}_{50} \text{ resistant population/LC}_{50} \text{ susceptible population)} \)

In general, discriminating dose bioassays incorporating synergists showed that MDP compounds with the exception of EN 1-129 (Figure 5.2, Figure 5.3 and Figure 5.4) exhibited high synergistic activity against all studied pollen beetle populations (UK, Rogalin and Lebork). In contrast, analogues with alterations in the MDP ring (EN 16/5-1 and EN 14-05) did not show high synergism with any of the populations (see also Chapter 4 for synergism bioassays with PBO and EN 16/5-1). Treatments with acetone and 0.01 g L\(^{-1}\) of synergists alone did not confer mortality (data not shown).

Considering firstly the susceptible UK population (Figure 5.2), EN 1-126 was significantly the most potent synergist compared to the other MDP compounds \( (p<0.05) \). Synergistic effects exhibited by PBO were not significantly different from EN 1-164, EN 1-163, EN 1-40 and EN
1-125 ($p>0.05$). Additionally, there was no significant difference in the synergistic effects between EN 16/5-1 and EN 1-129 ($p>0.05$) and both analogues were not significantly different from the insecticide alone ($p>0.05$).

![Figure 5.2 Synergistic effects of PBO and analogues (0.01 g L$^{-1}$) mixed with a discriminating dose of α-cypermethrin (0.3 ppm) against the UK population. Data are means ±SE, n=10 to 15, N=106, LSD (95%)=0.2012, df=97.](image)

Overall, the synergistic effects shown from the most resistant populations, Rogalin and Lebork, were not significantly different ($p=0.520$) (Figure 5.3). EN 1-126 and EN 1-164 exhibited no significant differences in their potency ($p>0.05$) and both were significantly more potent than PBO ($p<0.05$).
In order to distinguish clearly differences in the synergistic ability of PBO and analogues, a relatively low concentration of α-cypermethrin (0.3 ppm), sufficient to confer less than 10% mortality in the absence of synergist, was incorporated in a mixture with 0.01g L\(^{-1}\) of synergists and tested against the two most resistant populations, Rogalin and Lebork (Figure 5.4). Overall, there was no statistically significant difference in response between the two populations \((p=0.697)\). Analogues containing an alkynyl ether moiety showed higher synergistic activities than PBO. Overall, EN 1-126 was the more potent analogue when compared to the remaining analogues. The two phenyl ethers, EN 1-163 and EN 1-164, exhibited no significant difference in their synergistic effects \((p>0.05)\) and both were more potent than PBO \((p<0.05)\). Analogues with modifications in the MDP moiety, EN 16/5-1 and EN 14-05, showed no significant synergism when compared to insecticide only \((p>0.05)\).
5.3.2 Determination of $O$-deethylation inhibition by PBO and its analogues

The inhibition of ECOD activity by PBO and its analogues was measured in pollen beetle homogenates. The rate of the reaction product, 7-OH, reflects the oxidase activity. The ECOD activity remaining (%) after inhibition by PBO and its analogues with upper and lower limits for each population is presented in Table 5.2, Table 5.3, Table 5.4 and Table 5.5. It is important to consider at this stage that NADPH has a similar fluorometric profile to 7-OH (Chauret et al., 1999), thus if the amount of NADPH is reduced during the assay the fluorometric emission is reduced. Normally this reduction is ‘hidden’ by conversion of 7-EC to 7-OH, but if $O$-deethylation activity is completely inhibited the overall slope can be negative i.e. indicative of very high oxidase inhibition. A more appropriate ‘background’ activity to subtract would have been homogenate in the presence of NADPH and analogue, but absence of 7-EC. However, limitations on the amount of homogenate available precluded this procedure. The results are graphically presented in Figure 5.5.
In vivo and in vitro studies of PBO analogues against four pollen beetle populations

Table 5.2 Inhibition of O-deethylation of 7-ethoxycoumarin activity (ECOD) by PBO and analogues in the UK population.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Remaining activity (%) ± SE</th>
<th>Lower CL 95 %</th>
<th>Upper CL 95 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO</td>
<td>26.8 ± 4.38</td>
<td>18.2</td>
<td>35.3</td>
</tr>
<tr>
<td>EN 1-126</td>
<td>1.15 ± 3.49</td>
<td>-5.69</td>
<td>8.00</td>
</tr>
<tr>
<td>EN 1-164</td>
<td>10.4 ± 3.82</td>
<td>2.93</td>
<td>17.8</td>
</tr>
<tr>
<td>EN 1-163</td>
<td>-9.30 ± 3.13</td>
<td>-15.4</td>
<td>-3.16</td>
</tr>
<tr>
<td>EN 1-129</td>
<td>129 ± 7.90</td>
<td>113</td>
<td>144</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>101 ± 6.94</td>
<td>87.2</td>
<td>114</td>
</tr>
<tr>
<td>EN 14-05</td>
<td>101 ± 6.93</td>
<td>87.0</td>
<td>114</td>
</tr>
<tr>
<td>EN 1-40</td>
<td>41.0 ± 4.87</td>
<td>31.5</td>
<td>50.6</td>
</tr>
<tr>
<td>EN 1-125</td>
<td>-1.04 ± 3.41</td>
<td>-7.73</td>
<td>5.66</td>
</tr>
</tbody>
</table>

Table 5.3 Inhibition of O-deethylation of 7-ethoxycoumarin activity (ECOD) by PBO and analogues in the Leszno population.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Remaining activity (%) ± SE</th>
<th>Lower CL 95 %</th>
<th>Upper CL 95 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO</td>
<td>39.3 ± 8.64</td>
<td>22.3</td>
<td>56.2</td>
</tr>
<tr>
<td>EN 1-126</td>
<td>23.7 ± 7.67</td>
<td>8.68</td>
<td>38.7</td>
</tr>
<tr>
<td>EN 1-164</td>
<td>40.2 ± 8.70</td>
<td>23.1</td>
<td>57.2</td>
</tr>
<tr>
<td>EN 1-163</td>
<td>19.9 ± 7.44</td>
<td>5.32</td>
<td>34.4</td>
</tr>
<tr>
<td>EN 1-129</td>
<td>90.6 ± 11.8</td>
<td>67.4</td>
<td>114</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>85.6 ± 11.5</td>
<td>63.0</td>
<td>108</td>
</tr>
</tbody>
</table>
Table 5.4 Inhibition of $O$-deethylation of 7-ethoxycoumarin activity (ECOD) by PBO and analogues in the Rogalin population.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Remaining activity (%) ± SE</th>
<th>Lower CL 95 %</th>
<th>Upper CL 95 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO</td>
<td>20.4 ± 7.78</td>
<td>5.17</td>
<td>35.6</td>
</tr>
<tr>
<td>EN 1-126</td>
<td>-6.76 ± 6.76</td>
<td>-18.5</td>
<td>5.05</td>
</tr>
<tr>
<td>EN 1-164</td>
<td>27.5 ± 8.24</td>
<td>11.4</td>
<td>43.7</td>
</tr>
<tr>
<td>EN 1-163</td>
<td>-11.2 ± 5.73</td>
<td>-22.4</td>
<td>0.00</td>
</tr>
<tr>
<td>EN 1-129</td>
<td>114 ± 13.8</td>
<td>87.1</td>
<td>141</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>66.3 ± 10.7</td>
<td>45.2</td>
<td>87.4</td>
</tr>
<tr>
<td>EN 14-05</td>
<td>88.2 ± 12.1</td>
<td>64.4</td>
<td>112</td>
</tr>
</tbody>
</table>

Table 5.5 Inhibition of $O$-deethylation of 7-ethoxycoumarin activity (ECOD) by PBO and analogues in the Lebork population.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Remaining activity (%) ± SE</th>
<th>Lower CL 95 %</th>
<th>Upper CL 95 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO</td>
<td>11.0 ± 5.94</td>
<td>-0.64</td>
<td>22.6</td>
</tr>
<tr>
<td>EN 1-126</td>
<td>-15.4 ± 4.52</td>
<td>-24.2</td>
<td>-6.53</td>
</tr>
<tr>
<td>EN 1-164</td>
<td>-2.24 ± 5.23</td>
<td>-12.5</td>
<td>8.02</td>
</tr>
<tr>
<td>EN 1-163</td>
<td>-38.9 ± 3.26</td>
<td>-45.3</td>
<td>-32.5</td>
</tr>
<tr>
<td>EN 1-129</td>
<td>90.9 ± 10.2</td>
<td>70.9</td>
<td>111</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>70.3 ± 9.12</td>
<td>52.4</td>
<td>88.2</td>
</tr>
<tr>
<td>EN 14-05</td>
<td>71.4 ± 9.17</td>
<td>53.4</td>
<td>89.4</td>
</tr>
</tbody>
</table>
In general terms, the inhibition of ECOD activity varied among the tested compounds but results showed a consistent trend. Although widely used, PBO did not exhibit the highest inhibition of ECOD activity against any of the four pollen beetle homogenates. In contrast, MDP compounds having an alkynyl ether moiety in the side chain were found to be the most potent \( O \)-deethylation inhibitors, with EN 1-163 conferring the highest inhibition in all four populations.

In the Lebork population, EN 1-163 was significantly the most potent \( O \)-deethylation inhibitor when compared to the rest of the analogues, followed by EN 1-126. Furthermore, EN 1-163 and EN 1-126 inhibited ECOD activity in both the UK and Rogalin populations equally. EN 1-125, an analogue having an alkynyl ether moiety but lacking the alkyl side chain, conferred equal inhibition as the above compounds (EN 1-126, EN 1-163) when tested against the UK population. Furthermore, EN 1-40 (an analogue lacking the alkyl chain) showed similar \( O \)-deethylation inhibition to PBO against the UK population, indicating that an alkyl side chain may not be crucial for inhibiting ECOD activity in the MDP polyether compounds. EN 1-164 was a relatively potent inhibitor of ECOD activity exhibiting similar inhibition to EN 1-126 and PBO in UK and Rogalin/Lebork populations, respectively. Considering the intermediate resistant population, Leszno, analogues having the MDP moiety exhibited similar \( O \)-deethylation inhibition except EN 1-129. Indeed, EN 1-129 despite having the MDP moiety, failed to inhibit ECOD activity to the same extent as the other MDP compounds and its potency appeared to be similar to EN 16/5-1 and EN 14-05 in all tested populations. EN 16/5-1 and EN 14-05 are two analogues having either one or both oxygen atoms removed from their MDP rings, respectively.
Figure 5.5 O-deethylation activity remaining after inhibition by PBO and analogues in four *Meligethes aeneus* populations. Error bars represent ± SE (refer to Tables 5.2, 5.3, 5.4 and 5.5).
5.4 Discussion

Pyrethroid insecticides have been widely used because of their effectiveness and low mammalian toxicity (Casida et al., 1983). Subsequently, the extensive exposure of insects to pyrethroids has selected for resistance in many crop pest populations and human disease vectors (Liu et al. 2006; Jacobson et al., 2009; Kaufman et al., 2010; Komagata et al., 2010). There is now the threat of widespread pollen beetle populations resistant to pyrethroids, highlighting the danger of the frequent use of this, or any single class, of insecticides (Hansen, 2003; Tiilikaninen and Hokkanen, 2008; Wegorek et al., 2009).

In this chapter the enhancement of pyrethroid insecticide potency against different pollen beetle populations using synergists such as PBO and its analogues was investigated using a combination of in vivo and in vitro assays. The mechanism conferring resistance to α-cypermethrin in these pollen beetle populations has been proposed to be oxidase-based (Chapter 4). For the in vitro assays whole insect homogenates were used to ensure inclusion of all oxidases that may contribute to resistance to α-cypermethrin and could be potentially inhibited by the analogues. To interpret the in vivo results, ECOD activity was assayed as a measure of the dealkylation reaction often observed for pyrethroid detoxification (Roberts and Hutson, 1999) and because this assay has been widely used for biochemical characterisation of oxidative resistance (Yang et al., 2004; Audino et al., 2005; Marcombe et al., 2009; Wang et al., 2009).

Inhibition of ECOD activity by PBO and its analogues generally correlated with results of the in vivo investigations. Results from both assays indicated that, at least in some cases, the classic MDP inhibitor, PBO, is not the most potent oxidase inhibitor and that analogues possessing a combination of the MDP ring and an alkynyl ether moiety exhibit the greatest inhibitory activities. This is best demonstrated in the case of the Rogalin and Lebork populations where a low concentration of pyrethroid insecticide was applied to separate inhibitors with the greatest potency (Figure 5.4).
Chapter 5  In vivo and in vitro studies of PBO analogues against four pollen beetle populations

Results of the present study show that replacement of the polyether side chain with an alkynyl ether moiety increases the inhibition of ECOD activity and synergism in the in vivo assays. These findings support previous in vivo investigations where structures equivalent to PBO analogues EN 1-125 and EN 1-126 were 6-fold and 4-fold respectively, more potent than PBO when used as synergists with carbofuran against resistant houseflies (Pap et al., 2001). The oxidative inhibitory potency of alkynyl groups was also evaluated in a series of alkylarenes against methoxyresorufin O-demethylation activity (MROD) using CYP6D1, a P450 isoform that has been found to be responsible for monooxygenase-mediated pyrethroid resistance in a housefly strain (Scott et al., 2000).

Although it was shown previously that removal of the propyl side chain resulted in considerably lower inhibition of ECOD and MROD activity compared to PBO inhibition (Scott 1996; Scott et al., 2000), results in the present study showed that removal of the propyl chain in EN 1-40 had no effect upon the inhibition of ECOD activity (Figure 5.5) and this is supported further by the bioassay results with the UK population (Figure 5.2). Results, here are also consistent with previous in vivo investigations with permethrin against houseflies (Pap et al., 2001).

Sesamex (Figure 1. 3, Chapter 1) was one of the first potent synergists reported (Eldefrawi et al., 1960; Farnham, 1971, 1973, 1974). Its structure contains a MDP ring and a polyether side chain similar to the structure of PBO, but having the first oxygen atom of the polyether chain bound directly to the aromatic ring instead of via a methylene bridge. In view of this, two phenyl but-2-ynyl ethers (EN 1-163 and EN 1-164) that had the oxygen atom bound directly to the aromatic ring (similar to sesemex) and also carried a triple bond, were synthesised. Bioassays and ECOD assays incorporating EN 1-163 and EN 1-164 showed, in some cases, significantly greater inhibition than PBO, similar to EN 1-126, a phenyl methyl but-2-ynyl ether compound which retains a methylene bridge between the aromatic ring and the oxygen atom of the alkynyl ether chain. A similar improvement of MDP alkynyl ethers over PBO was demonstrated in an earlier study where a phenyl prop-2-ynyl ether similar to EN 1-163 showed 2-3 fold greater synergism than PBO when tested with carbaryl against houseflies (Barnes and Fellig, 1969). An additional observation is that EN 1-126 showed greater
synergistic effects with all populations, although in some cases EN 1-163 was a significantly more potent inhibitor of ECOD activity. This may be the result of EN 1-126 being a more lipophilic compound and thus able to penetrate the insect cuticle more rapidly when applied as a mixture with the insecticide.

It was initially surprising that the MDP compound EN 1-129 was unable to synergise α-cypermethrin in vivo or exhibit inhibitory potency in the ECOD assay. This finding suggests that the absence of a polyether chain, in contrast to the absence of an alkyl chain, within MDP compounds, is critical for synergism and O-deethylation of 7-EC. However, potency can be regained if it is replaced by an alkynyl ether moiety. The contribution of a polyether chain with MDP compounds for synergistic activity has been suggested in an earlier report where it was proposed that “a side chain with at least one or more oxygen atoms incorporated into an ether group is considered of major importance in preventing insecticide detoxification” (Esaac and Casida, 1969). Nonetheless, the presence of polyether and alkyl side chains, but an absence of the MDP ring, is not sufficient to enable synergistic activity in bioassays and inhibition of O-deethylation of 7-EC, as revealed with EN 16/5-1 and EN 14-05. This failure was expected since the fundamental contribution of the MDP ring of (methylenedioxyphenyl) polyether compounds to synergistic activity was established previously (Wilkinson et al., 1966; Casida, 1970).

Compounds with modifications to the MDP ring, such as EN 16/5-1 and EN 14-05, were previously evaluated as synergists with carbamate insecticides. Results revealed a lack of synergism, although replacement of one oxygen with sulphur resulted in only a slight decrease in the synergistic effect (Wilkinson et al., 1966). Furthermore, high synergism was reported following replacement of the MDP ring with a 1,2 dimethoxybenzene group and incorporation of an alkynyl ether side chain (forming the known synergist verbutin) (Pap et al., 2001; Bertok et al., 2003).

Due to the extensive application of many conventional insecticide groups i.e. pyrethroids, OPs, carbamates, many insect pests have developed resistance. Results from the in vivo and in vitro investigations in the present study clearly indicate that it is possible to design new
insecticide synergists that can effectively increase the toxicity to pest insects and prolong the use of existing pesticides (Moores et al., 2005).
Chapter 6

*In vivo* synergistic effects of piperonyl butoxide analogues using two *Myzus persicae* clones with different metabolic resistance mechanisms

6.1 Introduction

Bioassays using live insects play a crucial role in investigating insecticide resistance and such *in vivo* assays are the most reliable method of estimating resistance levels and evaluating the potency of toxicants against resistant insects. Therefore, a complete evaluation of either current insecticide resistance or new strategies for using insecticides and/or synergists must include *in vivo* tests alongside *in vitro* studies. However, difficulties can arise in interpreting comparative results obtained from *in vivo* and *in vitro* studies.

Following *in vitro* investigations discussed in previous chapters, different analogues together with PBO were used as insecticide synergists *in vivo* against two *M. persicae* clones which have different metabolic resistance mechanisms. The analogues have different alterations in the MDP moiety and the side chains. Piperonyl butoxide was used as the standard synergist for comparison. The synergistic efficacy of the analogues derived from *in vivo* studies are compared with the *in vitro* assays described in previous chapters.
Chapter 6  
In vivo synergistic effects of PBO analogues using two *M. persicae* clones with different metabolic resistance mechanisms

6.2 Materials and Methods

6.2.1 Insects
Two *M. persicae* aphid clones were used: 5191A and 794JZ (section 2.1.2 and Appendix 1).

6.2.2 Insecticides and synergists
Two technical insecticides were used: imidacloprid and $\alpha$-cypermethrin. Analogues and PBO were used as synergists (section 2.2 and for analogue’ structures see Appendix 2).

6.2.3 Insect bioassays (full dose response bioassays)
Individual apterous adult aphids were dosed with either PBO or analogues in acetone and were further treated 5 h later with either imidacloprid or $\alpha$-cypermethrin in acetone, as described in section 2.4.1.

6.2.4 Analysis of data
Full dose response bioassay data were analysed as described in section 2.5.3.
Chapter 6  *In vivo synergistic effects of PBO analogues using two *M. persicae *clones with different metabolic resistance mechanisms*

6.3 Results

6.3.1 Full dose response bioassays with imidacloprid and *Myzus persicae* clone 5191A

Results from full dose response bioassays using imidacloprid against the resistant *M. persicae* clone 5191A are presented in Table 6.1 (see also Appendix 4). The results show that the analogues conferred a range of synergistic potencies with EN 1-126 giving the highest SF (292) and EN 14-05 the lowest SF (2.17). The classic MDP synergist PBO gave a SF of 14.5.

Table 6.1 Synergism factors of analogues with imidacloprid in full dose response bioassays against *Myzus persicae* clone 5191A using a 5h pre-treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>aLC50 (ppm)</th>
<th>bCL95%</th>
<th>cSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>imidacloprid*</td>
<td>90.4</td>
<td>51.0-185</td>
<td>--</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ PBO*</td>
<td>6.22</td>
<td>4.04-8.70</td>
<td>14.5</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 16/5-1*</td>
<td>12.2</td>
<td>8.80-16.4</td>
<td>7.41</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 1-164</td>
<td>1.25</td>
<td>0.75-1.97</td>
<td>72.4</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 1-126</td>
<td>0.31</td>
<td>0.21-0.43</td>
<td>292</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 1-163</td>
<td>2.98</td>
<td>1.90-4.51</td>
<td>30.3</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 25-10</td>
<td>4.88</td>
<td>1.20-11.0</td>
<td>18.5</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 1-129</td>
<td>28.4</td>
<td>13.6-45.8</td>
<td>3.18</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 16-17</td>
<td>7.11</td>
<td>5.12-9.53</td>
<td>12.7</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 16-18</td>
<td>18.4</td>
<td>9.69-29.5</td>
<td>4.91</td>
</tr>
<tr>
<td>+ 1 g L⁻¹ EN 14-05</td>
<td>41.5</td>
<td>26.1-62.6</td>
<td>2.17</td>
</tr>
</tbody>
</table>

*Data from Chapter 4

aLC50=Lethal concentration to kill 50% of the clone, bCL= Confidence limits, cSF = synergism factor (LC50 unsynergised clone/ LC50 synergised clone)

Analogues containing the MDP moiety gave different potencies with EN 1-129 showing the lowest SF (3.18) and EN 1-126 showing the highest SF (292). Generally, the highest synergism was observed with analogues having a combination of the MDP ring and an alkynyl
ether chain, EN 1-126, EN 1-164 and EN 1-163. Of these, EN 1-126 had a LC$_{50}$ value of 0.31 ppm which is even lower than the LC$_{50}$ value obtained from the susceptible aphid clone (4106A) treated with imidaclorpid, which had a LC$_{50}$ value of 1.55 ppm (Chapter 4, Table 4.5).

Considering EN 1-126, which has a bridging -CH$_2$- group connecting the aromatic ring and the alkynyl ether side chain, the SF was 4-fold higher than EN 1-164 in which the bridging -CH$_2$- group is absent. The presence of a propyl side chain in EN 1-164 increased the SF 2.38-fold compared to EN 1-163 where the propyl side chain is absent. Similarly, EN 16-17 gave a 2.58-fold higher SF than EN 16-18 which lacks the propyl side chain. Both of these analogues had one oxygen atom removed from the MDP ring and synergism was decreased compared with analogues that retained the complete MDP moiety. The lowest SF was seen for EN 14-05 which had both oxygen atoms removed from the MDP ring. EN 25-10 (verbutin), a compound lacking the MDP ring, gave a SF of 18.54, higher than some MDP compounds including PBO and EN 1-129.

6.3.2 Full dose response bioassays with α-cypermethrin and Myzus persicae clone 794JZ

Results from full dose response bioassays using α-cypermethrin against the resistant M. persicae clone 794JZ are presented in Table 6.2 (see also Appendix 4). Synergistic factors of the analogues fall within a narrow range with only a 4.28-fold difference between the highest (EN 1-16, SF 8.87) and the lowest (EN 1-129, SF 2.07). Piperonyl butoxide showed an intermediate synergistic effect (SF 4.8).

Analogue with an increased length of the alkyl side chain, EN 1-16 and EN 1-14, gave the highest SFs with values of 8.87 and 6.35, respectively followed by the aryl alkynyl ether EN 25-10 with a SF of 6.07. The two other aryl alkynyl ethers with a MDP moiety, EN 1-126 and EN 1-164, gave 1.65-fold difference in their synergistic potency. Compounds such as PBO, EN 16/5-1 and EN 1-40 exhibited similar SFs (~5) and the lowest SFs were obtained with EN 14-05, EN 1-129 and EN 1-93 with SFs between 2 and 3.
Table 6.2 Synergism factors of analogues with α-cypermethrin in full dose response bioassays against *Myzus persicae* clone 794JZ using a 5h pre-treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(^a\text{LC}_{50}) (ppm)</th>
<th>(^b\text{CL}_{95%})</th>
<th>(^c\text{SF})</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-cypermethrin*</td>
<td>1303</td>
<td>1061-1632</td>
<td>----</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) PBO*</td>
<td>271</td>
<td>220-327</td>
<td>4.80</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 16/5-1*</td>
<td>264</td>
<td>215-321</td>
<td>4.94</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 1-14</td>
<td>205</td>
<td>162-258</td>
<td>6.35</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 1-126</td>
<td>218</td>
<td>139-335</td>
<td>5.97</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 1-164</td>
<td>361</td>
<td>211-473</td>
<td>3.60</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 1-93</td>
<td>507</td>
<td>370-675</td>
<td>2.57</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 1-40</td>
<td>259</td>
<td>207-313</td>
<td>5.03</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 25-10</td>
<td>215</td>
<td>157-276</td>
<td>6.07</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 14-05</td>
<td>569</td>
<td>377-898</td>
<td>2.29</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 1-129</td>
<td>629</td>
<td>484-878</td>
<td>2.07</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 1-16</td>
<td>147</td>
<td>101-195</td>
<td>8.87</td>
</tr>
</tbody>
</table>

*Data from Chapter 4  
\(^a\text{LC}_{50}\)=Lethal concentration to kill 50% of the clone, \(^b\text{CL}\)= Confidence limits,  
\(^c\text{SF}\)= synergism factor (\text{LC}_{50} unsynergised clone/ \text{LC}_{50} synergised clone)
6.4 Discussion

*In vivo* synergism with PBO and its analogues was investigated with two insecticides, imidacloprid and α-cypermethrin and two aphid clones, 5191A and 794JZ respectively.

As shown previously, clone 5191A exhibited a 56.5-fold resistance to imidacloprid. *In vivo* and *in vitro* assays suggest that although clone 5191A has high levels of esterase (FE4 variant, R$_3$), imidacloprid resistance was primarily due to an oxidative based mechanism (Chapter 4). The bioassay results reported in this chapter show that MDP compounds with an alkynyl ether side chain, EN 1-126, EN 1-164 and EN 1-163, are the most potent synergists when used with imidacloprid (Table 6.1). Similarly, bioassay results with the pyrethroid resistant pollen beetle populations showed that EN 1-126 was the most potent synergist followed by EN 1-164 and EN 1-163 (Chapter 5, Figure 5.4). It is of note that the oxidative mechanism in clone 5191A does not exhibit enhanced levels of pyrethroid resistance (only a RF of 13, due to FE4 -R$_3$, Chapter 4) compared to the high level of imidacloprid resistance (RF of 56.5, Chapter 4). Furthermore, pollen beetle populations from Europe that have exhibited pyrethroid resistance (as also found in Chapter 5) have not exhibited cross-resistance to imidacloprid (Slater and Nauen, 2007). This implies that the oxidase enzyme systems responsible for imidacloprid resistance in clone 5191A and pyrethroid resistance in pollen beetles have different substrate profiles. This is perhaps unsurprising, given that oxidases show a large variation in substrate specificity (Wilkinson, 1983). However, the three aryl alkynyl ethers (EN 1-126, EN 1-164 and EN 1-163) are potent synergists with both insecticides against the two insect species which exhibited oxidative metabolic resistance. This suggests that these analogues have broad spectrum activity and are capable of inhibiting a variety of oxidase enzymes.

Another observation is that EN 1-129 and EN 14-05 gave low SFs when tested with the imidacloprid resistant aphid clone 5191A (Table 6.1) and also low synergism in the discriminating dose bioassays with pyrethroid resistant pollen beetles (Chapter 5, Figure 5.3 and Figure 5.4). This implies that the presence of only the MDP moiety or only the polyether chain is not sufficient for high synergistic efficacy. This is further supported by the *in vitro*
results with pollen beetle homogenates where EN 1-129 and EN 14-05 failed to inhibit the ECOD activity to the same extent as the other compounds tested.

The significant contribution of the MDP moiety is also demonstrated by the lower synergistic effects of two other analogues with modifications in the MDP moiety (EN 16-17 and EN 16-18) when compared to EN 1-126. The low synergism exhibited by EN 16-17 and EN 16-18 further supports the finding that imidacloprid resistance is associated with an oxidative mechanism (Chapter 4).

Of particular note is the significance of the propyl side chain in the analogue’s structure. EN 1-164 and EN 1-163 both of which contain a MDP moiety and alkynyl side chain differ only in that EN 1-164 has a propyl side chain. Bioassay results revealed that EN 1-164 exhibited a 2.4-fold higher SF than EN 1-163. Furthermore, two other analogues, EN 16-17 and EN 16-18, differ again in that the former carries a propyl side chain and exhibited a 2.6-fold higher SF than EN 16-18. This relatively constant difference between analogues with and without the propyl side chain implies that the presence of the alkyl chain provides specific properties to the synergists. The presence of the alkyl side chain in both cases increases the lipophilicity of the compounds and consequently may help penetration of the synergist through the insect cuticle. Since the optimum pre-treatment times for individual analogues were not defined and all experiments used a 5 h delay (shown to be the optimal for PBO against *M. persicae* – Khot, 2009), it could be that this pre-treatment time is more appropriate for analogues having a propyl side chain, similar to PBO. This would also explain the observation that although EN 1-163 was the significantly more potent inhibitor against ECOD activity, followed by EN 1-126 and EN 1-164, it did not exhibit significantly different synergism from EN 1-164 when both were tested *in vivo* in a mixture with α-cypermethrin against pollen beetles (Chapter 5). Similarly with the imidacloprid resistant clone 5191A, EN 1-163 had a lower synergistic effect than EN 1-164 (Table 6.1). This could be due to the 5 h pre-treatment being insufficient for EN 1-163 and other similar analogues which lack the propyl side chain. However, the inhibitory potency of EN 1-163 upon oxidative systems derived from different insect species remains unclear. The importance of inhibiting the enhanced metabolic enzymes prior to the
active ingredient being applied (‘temporal synergism’) has been demonstrated previously (Young et al., 2005, 2006; Bingham et al., 2007, 2008).

Bioassays with the aphid clone 5191A showed that EN 25-10 (verbutin), a compound with an alkynyl ether moiety and two methoxy groups instead of the MDP moiety, showed synergistic effects similar to PBO. EN 25-10 has previously been reported to synergise pyrethroid and carbamate insecticides against housefly strains possessing enhanced oxidases (Pap et al., 2001; Bertók et al., 2003).

In contrast to the synergism observed for the analogues with the imidacloprid resistant clone 5191A, the bioassay results for the pyrethroid resistant clone 794JZ showed relatively low SFs (Table 6.2). Additionally, results showed that SFs obtained from analogues tested against clone 794JZ were uniform. However, a common observation with both of these resistant aphid clones and with the pyrethroid resistant pollen beetles is the low synergism with EN 1-129, EN 1-93 and EN 14-05. Synergistic effects derived from these analogues also concur with the SAR study results (Chapter 3) where the two MDP compounds EN 1-129 and EN 1-93 exhibited very low binding affinities against E4 (Chapter 3, Figure 3.6) and EN 14-05 had a significantly lower binding affinity than PBO (Chapter 3, Figure 3.8). The highest synergism was observed with EN 1-16 followed by EN 1-14 (Table 6.2). In both these analogues the propyl chain has been replaced by a hexyl or butyl chain, respectively. These two analogues also exhibited high binding affinities in the SAR study, with the former showing a significantly higher binding affinity than the latter (Chapter 3, Figure 3.6). As discussed previously, the presence of the alkyl side chain gives the compounds a more lipophilic profile and may be an important factor for the high synergistic effects exhibited by these analogues. Additionally, as discussed for the in vitro studies, increasing lipophilicity is positively correlated with high binding potency to E4, possibly due to hydrophobic interactions between the alkyl chain and a hydrophobic area of the enzyme (Chapter 3, SAR study).

The relatively low SFs from the pyrethroid resistant aphid clone 794JZ (Table 6.2) compared to the high SFs obtained from the imidacloprid resistant aphid clone 5191A clone (Table 6.1) could result from the different resistance profiles of the two aphid clones. The pyrethroid resistant
794JZ aphid clone possessed enhanced metabolic enzyme (E4 variant, R₃) and a target site mutation (kdr) and this combination is reported to increase insensitivity to pyrethroid compounds (Martinez-Torres et al., 1999). Although not all analogues were tested *in vivo* against clone 794JZ, the SFs obtained were low and not appreciably different. This implies that the target site mutation (kdr) is the major resistance mechanism in clone 794JZ and the contribution of E4 is relatively minor. In contrast, when the same analogues were tested *in vitro* (Chapter 3, SAR study) differences in their binding affinities were clearly evident. The difference in results between the SAR study and the *in vivo* assays may be due to the esterase interference assay being optimised for this purpose and thus being capable of distinguishing any differences in binding affinities towards the enzyme (E4). Since with *in vivo* studies this separation was not possible, the fine differences observed in the SAR study would not be found. However, use of a more potent insecticide with the *in vivo* assays may help to define distinguishable differences among the analogues and thus provide a better correlation with the binding affinities displayed by the SAR study. Nevertheless, the extreme responses (the most potent and the weakest synergists) are still evident in the *in vivo* assays and are in agreement with the binding affinities obtained in the *in vitro* study.
Chapter 7

General discussion

7.1 Summary of the main findings

The main findings from this study have been summarised below:

- The conventional spectrophotometric assay using the model substrate 1-NA, confirmed previous findings that PBO does not inhibit substrate hydrolysis when incubated with insect esterases. Additionally, it has been shown that PBO and some of its analogues increased the catalytic centre activity when incubated with purified E4 (activation). This implies that some compounds are actually “helping” or “guiding” the substrate to reach and act at the catalytic centre at a higher rate (Chapter 3).

- Incubations of PBO with purified E4 at different intervals showed that PBO acts as a reversible inhibitor suggesting that it binds to the enzyme with non-covalent bonds such as hydrogen bonds, hydrophobic interactions, steric effects etc (Chapter 3).

- The alkyl and the polyether side chains of the analogues make significant contributions to their binding affinity to E4 (Chapter 3).

- High binding affinities to E4 were found with analogues where the polyether chain was replaced by an alkynyl ether chain, especially with the oxygen atom bound directly to the aromatic ring (aromatic type B), or where the length of the alkyl chain was increased (Chapter 3).

- Alterations to the MDP moiety, by removing one oxygen atom (aromatic ring type C) did not affect the binding affinities; however, removal of both oxygen atoms (aromatic ring type D) resulted in a reduction of the binding potency of the analogues compared to PBO (Chapter 3).

- There was a positive correlation between the slopes and Index values derived from the two biochemical assays (conventional esterase assay and the interference assay, respectively) with the exception of analogues lacking the alkyl side chain. This correlation shows that the stronger the binding affinity, the less the catalytic centre activity of the enzyme for 1-NA was increased (less activation) (Chapter 3).
• An analogue of PBO, EN 16/5-1, that retains the ability to inhibit esterases (Chapter 3) but loses potency against oxidase activity (due to a modification of the MDP ring), was used in parallel with PBO (using in vivo and in vitro assays) to characterise metabolic enzymes conferring resistance in two different insect species (Chapter 4).

• MDP compounds containing the alkynyl ether side chain conferred high synergism in vivo and were potent inhibitors of ECOD activity when tested against pyrethroid resistant pollen beetles (Chapter 5).

• Synergist bioassays results showed that MDP compounds with the alkynyl ether side chain were the most potent synergists when tested against the imidacloprid resistant aphid clone 5191A. Furthermore, results with clone 5191A suggested a 5 h pre-treatment time to be a more appropriate time delay for analogues containing a propyl side chain, similar to PBO (Chapter 6).

• Synergist bioassays with analogues against the pyrethroid resistant aphid clone 794JZ gave relatively low and uniform SFs (Chapter 6) in contrast to the SAR study where binding affinities were easily distinguishable (Chapter 3). However, analogues with an increased length of the alkyl side chain exhibited high synergism in agreement with findings from the SAR study (Chapter 6).

• Generally, analogues with the MDP and the alkynyl ether moieties exhibited high SFs against both species, aphids and pollen beetles, carrying oxidative resistance mechanisms. If the alkyl side chain is also present the synergistic effects are even greater (Chapter 5 and 6). Similar responses with these compounds were observed with in vitro assays, i.e. the SAR study and the ECOD assay (Chapter 3, 5). In contrast, results from in vivo and in vitro assays with analogues having modifications in the MDP ring, showed that the response depended upon the resistance mechanism present in the insect population studied (Chapter 3, 4, 5 and 6).

7.2 Discussion and future work
The widespread occurrence of insecticide resistance is a serious threat for the control of many important agricultural pests and vectors of human diseases. Resistance to different classes of insecticides has developed and over 500 species of insects and mites are resistant to one or more insecticides. As a result, the destruction and limitation of food supplies caused by the
failure to control insect infestations causes annual economic losses of several billion dollars worldwide (Elzen and Hardee, 2003). Economic losses by insect control failure are further increased by the high cost of inventing and developing new active ingredients with novel modes of action. Since few classes of insecticides are available for insect control and few new insecticides are being produced, increasing our understanding and/or improving the efficacy of existing insecticides is essential. Insecticide synergists such as PBO are capable of prolonging the use of the existing insecticides and overcoming insecticide resistance (Bernard and Philogène, 1993).

In the present study the interactions between PBO/analogues and metabolic enzymes conferring insecticide resistance have been investigated using insect species with different insecticide resistance mechanism profiles. Piperonyl butoxide, probably the most well-known synergist, has been used in the field to reduce resistance factors. Additionally, it has been used for research purposes to characterise metabolic resistance, since it was considered to be a specific inhibitor of MFOs. Subsequent studies, however, showed that PBO can also interact with resistance-associated esterases.

This study supports the previous findings that PBO can inhibit both phase 1 metabolic enzymes, namely oxidases and esterases. In an attempt to gain a better understanding of the mechanism by which PBO interacts with resistance-associated esterases, a SAR study was undertaken using PBO analogues which have different modifications in the MDP moiety and side chains. This SAR study, incorporating the interference assay, showed that different levels of binding affinity to E4 occur when the PBO molecule is modified. The extent of these differences was dependent upon the changes made to the analogue molecule. Although the exact mechanism by which PBO interacts with E4 remains unclear, preliminary results from the present study gave some insights into this interaction. The presence of the alkyl chain and the polyether chain made a significant contribution to the binding affinity of analogues to E4. Furthermore, replacement of the polyether side chain by an alkynyl ether chain with the oxygen bound directly to the aromatic ring and the presence of an alkyl chain, increased the binding affinity to E4. These findings suggest parameters that are fundamental for the high binding potency to E4 and may contribute to the design of potent inhibitors of this esterase.
Although conclusions at this stage can only be speculative, design of specific compounds for inhibiting E4 would prolong the use of existing insecticides controlling aphid populations and eliminate synergistic effects against non-target insects, thus contributing to integrated pest management (IPM) strategies.

Analogues combining the MDP ring and the alkynyl ether chain, such as EN 1-164, EN 1-126, showed high binding affinities to E4, inhibition of ECOD activity from insect homogenates and excellent synergistic effects in *in vivo* tests against both *M. persicae* and *M. aeneus*. These analogues could be used to enhance the efficacy of existing insecticides from different classes and inhibit relevant metabolic enzymes. Such analogues could be used as insecticide synergists in insect resistance management (IRM) programmes in order to maintain utility of current insecticides by slowing, preventing or reversing development of resistance.

Alterations to the MDP moiety, by removal of one oxygen atom, did not affect the binding affinities to esterases from *M. persicae* and *M. aeneus*, but significantly reduced the inhibition of oxidative enzymes in the latter. Unfortunately in this study, assessment of aphid microsomal oxidase activity was not possible and thus *in vitro* investigations for evaluation of analogue potency against oxidative enzymes could not be completed. However, the analogue EN 16/5-1 could be used as a first indication of which metabolic resistance mechanism is present in a resistant population and consequently, the appropriate synergist could be applied to overcome the resistance.

Previous work had shown that the efficacy of PBO as an insecticide synergist can be enhanced by using an appropriate pre-treatment time between the application of the synergist and the insecticide. Additionally, it has been shown that PBO pre-treatment time differs between insect species (Young *et al.*, 2005, 2006). Synergism in the present study was investigated using either a 5 h pre-treatment time (the optimal time delay for PBO against *M. persicae*), or a mixture of the synergist with the insecticide. However, analogues may have a different optimal pre-treatment time compared to PBO. Hence, synergist efficacies could be increased by using the appropriate pre-treatment time for each analogue. Results here indicated that
analogues having the alkyl side chain to increase analogue lipophilicity may have similar pre-
treatment times to PBO.

Microencapsulated formulations have been developed to give a burst release of PBO several
hours prior to a second burst release of the active ingredient (Bingham et al., 2007, 2008). It is
probable that similar microencapsulated formulations containing a PBO analogue could
produce more potent synergistic effects, provided the pre-treatment times were optimised.
Furthermore, by manipulating this parameter it is possible to reduce undesirable effects
against non-targeted insects, such as beneficial insects.

The contribution of metabolic inhibitors such as synergists to reduce resistance factors in the
fields is unquestioned (Bernard and Philogène, 1993). As shown in previous reports and
confirmed in the present study, PBO and analogues could be used to prolong the use of
existing insecticides. However, their use needs to be considered with caution. It is likely that
target insects would resist metabolic inhibitor treatment as they resist other xenobiotics, such
as insecticides and plant toxicants and genetic changes will occur, perhaps with a concomitant
increase in target-site resistance. Careful design for their application is also needed to meet
IPM strategies. The frequency and rate of synergist application in combination with the
ecology and biology of the target insects are fundamental factors for the successful used of
synergists. A similar programme for the control of *H. armigera* in Australian cotton fields
where the use of PBO is restricted to once per season with a single pyrethroid (Forrester et al.,
1993) could be adapted for the long-term successful management of synergists in field
resistance situations contributing to IPM and IRM strategies.

Further investigation of the interactions between metabolic enzymes (esterases and oxidases)
and PBO analogues would help to clarify the role of different chemical moieties and allow the
synthesis of potent synergists, similar to analogues having an alkynyl ether moiety in the
present study. Although investigations were undertaken using purified E4 and semi-purified
esterases from pollen beetles, other enzymes e.g different P450 isozymes need to be evaluated
in the presence of analogues in appropriate assays. Differences in enzyme structures could be
the key to the design of specific synergists for specific insects, thus minimising undesired effects on beneficials such as honeybees.

Preliminary investigations were made to find specific inhibitors to characterise the different metabolic enzymes confirming insecticide resistance, since PBO inhibits both, esterases and oxidases. Although EN 16/5-1 can be used as an esterase inhibitor, the need for a specific oxidase inhibitor still remains. Furthermore, since assessment of aphid microsomal oxidase activity was not possible in the present study, further studies need to be undertaken to obtain information on this enzyme group and clarify its contribution to insecticide resistance.

Design of a systemic synergist is of major importance to contribute further to IPM strategies. In the present study, EN 1-101 was designed specifically for this purpose by adding a -COOH group at the end of the polyether chain to achieve high water solubility. However, EN 1-101 gave low binding affinity to E4 and could be considered an ineffective esterase inhibitor. It would be interesting to evaluate the inhibitory potency of EN 1-101 against ECOD activity, since this analogue retains the MDP moiety and the polyether chain. If it retains inhibitory effects upon ECOD activity, it could be tested further in systemic studies as a specific systemic oxidase inhibitor.

It can be concluded from this study that some analogues of the insecticide synergist PBO have potential to enhance insecticide efficacies. Compounds within a given structural family such as PBO analogues, may exhibit varying biological activities depending on their interactions with different enzymes (esterases and oxidases). By altering chemical moieties or functional groups of a lead compound, in this case PBO, its interactions against target proteins and other properties (e.g physicochemical) can be modified and improved. Further investigations are needed to design analogues to act as insecticide synergists with high potency and specificity. Such compounds will improve crop production and contribute to IPM and IRM strategies.
References


References


References


References


ffrench-Constant, R.H., Steichen, J.C., Rocheleau, T.A., Aronstein, K. and Roush, R.T. 1993. A single-amino acid substitution in a gamma-aminobutyric acid subtype A receptor locus is


References


References


References


Appendices

Appendix 1. Resistance mechanisms and origins of *Myzus persicae* clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>¹Esterase</th>
<th>²kdr</th>
<th>Origin</th>
<th>Date of collection</th>
<th>Crop of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>4106A</td>
<td>S</td>
<td>SS</td>
<td>Scotland, UK</td>
<td>August, 2000</td>
<td>Potato</td>
</tr>
<tr>
<td>T1V</td>
<td>R₂, E4</td>
<td>SS</td>
<td>Beds, UK</td>
<td>June, 1975</td>
<td>Sugar beet</td>
</tr>
<tr>
<td>794JZ</td>
<td>R₃-E4</td>
<td>RR</td>
<td>Evesham, UK</td>
<td>March, 1982</td>
<td>Chrysanth</td>
</tr>
<tr>
<td>926B</td>
<td>R₃-FE4</td>
<td>SS</td>
<td>Greece</td>
<td>July, 1990</td>
<td>Tobacco</td>
</tr>
<tr>
<td>5191A*</td>
<td></td>
<td>Greece</td>
<td>July, 2007</td>
<td>Tobacco</td>
<td></td>
</tr>
</tbody>
</table>

¹Determinate by immunoassay: S/R₁ (susceptible), R₂ (high), R₃ (extreme resistant) (Devonshire *et al*., 1986).

²Based on direct DNA sequencing of PCR-amplified sodium channel gene fragments from aphid genomic DNA (Martinez-Torres *et al*., 1999).

* Resistance mechanisms of clone 5191A were under investigation in the present study.
Appendix 2. Analogue structures (in numerical order)

5-((2-(2-butoxyethoxy)ethoxy)methyl)-6-propylbenzo[d][1,3]dioxole

5-((2-(2-butoxyethoxy)ethoxy)methyl)-6-butybenzo[d][1,3]dioxole

5-((2-(2-butoxyethoxy)ethoxy)methyl)-6-hexylbenzo[d][1,3]dioxole

5-((2-(2-butoxyethoxy)ethoxy)methyl)benzo[d][1,3]dioxole

5,6-bis((2-(2-butoxyethoxy)ethoxy)methyl)benzo[d][1,3]dioxole
6-propylbenzo[\textit{d}][1,3]dioxole-5-carboxylic acid

\{2-\textit{[2-(6-Propyl-benzo[1,3]dioxol-5-ylmethoxy)ethoxy]-ethoxy}\}-acetic acid

5-((but-2-ynyloxy)methyl)benzo[\textit{d}][1,3]dioxole

5-((but-2-ynyloxy)methyl)-6-propylbenzo[\textit{d}][1,3]dioxole

5-dodecylbenzo[\textit{d}][1,3]dioxole
sodium 2-((2-((6-propylbenzo[d][1,3]dioxol-5-yl)methoxy)ethoxy)ethoxy)acetate

5-(but-2-ynyloxy)benzo[d][1,3]dioxole

5-(but-2-ynyloxy)-6-propylbenzo[d][1,3]dioxole

5-(2-(2-butoxyethoxy)ethoxy)benzo[d][1,3]dioxole

5-(but-2-ynyloxy)-6-(2-(2-butoxyethoxy)ethoxy)benzo[d][1,3]dioxole
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EN 1-180
5-(2-(2-butoxyethoxy)ethoxy)-6-propylbenzo[\textit{d}][1,3]\textit{dioxole}

EN 1-181
5-(prop-2-ynyloxy)benzo[\textit{d}][1,3]\textit{dioxole}

EN 1-182
5-(3-But-2-ynyloxy-2-methyl-propyl)-benzo[1,3]\textit{dioxole}

EN 1-183
5-(prop-2-ynyloxy)-6-propylbenzo[\textit{d}][1,3]\textit{dioxole}

EN 1-186
5-(2-methoxyethoxy)-6-propylbenzo[\textit{d}][1,3]\textit{dioxole}
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EN 14-05

6-(2-(2-butoxyethoxy)ethoxy)methyl)-5-propyl-2,3-dihydro-1H-indene

EN 16/5-1

5-((2-(2-butoxyethoxy)ethoxy)methyl)-6-propyl-2,3-dihydrobenzofuran

EN 16-06

5-((2-(2-butoxyethoxy)ethoxy)methyl)-2,3-dihydrobenzofuran

EN 16-17

6-(but-2-ynyloxy)-5-propyl-2,3-dihydrobenzofuran

EN 16-18

5-(but-2-ynyloxy)-2,3-dihydrobenzofuran
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EN 25-10 (Verbutin)

(R)-4-(1-(but-2-ynyloxy)ethyl)-1,2-dimethoxybenzene

EN 25-35

1-(but-2-ynyloxy)-4,5-dimethoxy-2-propylbenzene

EN 25-36

1,2-dimethoxy-4-(prop-2-ynyloxy)-5-propylbenzene

EN 25-37

4-(but-2-ynyloxy)-1,2-dimethoxybenzene
Appendix 3. Buffers, substrates and electrophoresis recipes

Buffers

- **0.02 M phosphate buffer, pH 7.0**
  3.58 g di-sodium hydrogen orthophosphate dodecahydrate (Na$_2$HPO$_4$.12H$_2$O) and 1.36 g (KH$_2$PO$_4$) potassium dihydrogen orthophosphate dissolved in 1 L distilled water (d$_2$H$_2$O).

- **0.1 M sodium phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF and 1.46 mM sucrose**
  6.23 g Na$_2$HPO$_4$.12H$_2$O, 0.406 g KH$_2$PO$_4$, 0.074 g EDTA, 0.03 g DTT, 0.03 g PTU, 0.034 g PMSF and 100 g sucrose were dissolved in 200 mL d$_2$H$_2$O. The PTU and PMSF were firstly dissolved in 1 mL ethanol (absolute high, 100%).

- **0.1 M sodium phosphate buffer, pH 7.8**
  3.3 g Na$_2$HPO$_4$.12H$_2$O and 0.133 g KH$_2$PO$_4$, were dissolved in 100 mL d$_2$H$_2$O.

- **0.02 M Tris/HCl, pH 8.5**
  0.884 g Trizma hydrochloride and 1.74 g Trizma Base were dissolved in 1 L d$_2$H$_2$O

Substrates

- **30 mM 1-naphthyl acetate (MW= 186.21)**
  0.558 g of 1-naphthyl acetate in 100 mL of acetone 100% AR

- **5, 5’ DiThio-bis (2-NitroBenzoic Acid) – DTNB (MW: 396.3)**
  0.015 g DTNB in 25 mL 0.02 M phosphate buffer, pH 7.0 to give a concentration of 1.5 mM

- **AcetylThioCholine Iodide–ATChI– (MW: 289.2)**
  0.022 g ATChI in 50 mL 0.02 M phosphate buffer, pH 7.0 to give a concentration of 1.5 mM
Polyacrylamide Gel Electrophoresis (PAGE)

Small pore gel (for approximately 15 gels)
Solution 1:  Potassium ferricyanide 12 mg
              Acrylamide (40%)  60 mL
              Bis-acrylamide (2%)  32 mL
              Tris-base  534 mg
              Tris-HCl  2.873 mg
              Temed (Electran)  180 µL
Made up to 250 mL with dH₂O
Solution 2: 224 mg ammonium persulphate were dissolved in 30 mL d H₂O
Mix solution 1 and solution 2
Solution 3: 40 mL 1.6% Triton in dH₂O
Pour into moulds and seal with 2-methyl-2-butanol
Allow to 1-2 h to set before adding large pore gel

Large pore gel (for approximately 15 gels)
Solution 1:  Acrylamide (40%)  8 mL
              Bis-acrylamide (2%)  19 mL
              Tris-base  33 mg
              Tris-HCl  180 mg
              Temed (Electran)  35 µL
Made up to 45 mL with dH₂O
Solution 2:  Riboflavin 0.004% in dH₂O  7.5 mL
              1.6% Triton X-100 in dH₂O  7.5 mL
Mix solutions 1 and 2.
Wash 2-methyl-2-butanol off small pore gel with dH₂O
Pour large pore gel and add combs, leave them in front of light to set (1-2h)

Barbitone buffer (Tank buffer)
27.6 g Barbitone and 5g Tris-base were dissolved in 5 L dH₂O
0.2 M phosphate buffer, pH 6.0 (Staining buffer)
8.81 g Na₂HPO₄.12H₂O and 23.85 g KH₂PO₄ were dissolved in 1 L dH₂O

0.2% Fast Blue RR (FBRR)
100 mg FBRR made up to 50 mL with 0.2 M phosphate buffer, pH 6.0 and filtered
## Appendix 4. Polo Plus analysis outputs

Table 7.1 Full dose response bioassays with *Myzus persicae* clones against imidacloprid using a 5h pre-treatment (Chapter 4, refer to Table 4.5).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aphid Clone</th>
<th>aLC₅₀ (ppm)</th>
<th>bCL 95%</th>
<th>Slope ±SE</th>
<th>cdf</th>
<th>dχ²</th>
<th>eRF</th>
<th>fSF</th>
<th>Number of insects</th>
</tr>
</thead>
<tbody>
<tr>
<td>imidacloprid</td>
<td>4106A</td>
<td>1.55</td>
<td>1.18-1.99</td>
<td>1.90 ±0.16</td>
<td>60</td>
<td>82.9</td>
<td>1</td>
<td>--</td>
<td>658</td>
</tr>
<tr>
<td></td>
<td>926B</td>
<td>6.53</td>
<td>5.32-8.03</td>
<td>1.41 ±0.08</td>
<td>82</td>
<td>78.08</td>
<td>4.21</td>
<td>--</td>
<td>877</td>
</tr>
<tr>
<td></td>
<td>5191A</td>
<td>90.4</td>
<td>51.0-185</td>
<td>0.93 ±0.07</td>
<td>58</td>
<td>192.9</td>
<td>56.5</td>
<td>--</td>
<td>667</td>
</tr>
<tr>
<td>+ 1g L⁻¹ PBO</td>
<td>5191A</td>
<td>6.22</td>
<td>4.04-8.70</td>
<td>1.50 ±0.14</td>
<td>86</td>
<td>154.1</td>
<td>--</td>
<td>14.5</td>
<td>980</td>
</tr>
<tr>
<td>+ 1g L⁻¹ EN 16/5-1</td>
<td>5191A</td>
<td>12.2</td>
<td>8.80-16.4</td>
<td>1.24 ±0.09</td>
<td>89</td>
<td>105.4</td>
<td>--</td>
<td>7.41</td>
<td>956</td>
</tr>
</tbody>
</table>

*LC₅₀=Lethal concentration to kill 50% of the population, CL=Confidence limits, df=degrees of freedom, χ²=chi-square, RF=Resistance factor (LC₅₀ resistant strain/LC₅₀ susceptible strain), SF=Synergism factor (LC₅₀ unsynergised/LC₅₀ synergised)
Full dose response bioassays with *Myzus persicae* clones against α-cypermethrin using a 5h pre-treatment (Chapter 4, refer to Table 4.6.).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aphid Clone</th>
<th>$^{a}\text{LC}_{50}$ (ppm)</th>
<th>$^{b}$CL 95%</th>
<th>Slope ±SE</th>
<th>$^d$df</th>
<th>$^d\chi^2$</th>
<th>$^e\text{RF}$</th>
<th>$^f\text{SF}$</th>
<th>Number of insects</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-cypermethrin</td>
<td>4106A</td>
<td>0.43</td>
<td>0.29-0.56</td>
<td>2.98 ±0.40</td>
<td>36</td>
<td>64.4</td>
<td>1</td>
<td>--</td>
<td>408</td>
</tr>
<tr>
<td></td>
<td>5191A</td>
<td>5.60</td>
<td>5.94-6.33</td>
<td>3.14 ±0.24</td>
<td>102</td>
<td>88.2</td>
<td>13.0</td>
<td>--</td>
<td>539</td>
</tr>
<tr>
<td></td>
<td>926B</td>
<td>7.08</td>
<td>5.63-8.70</td>
<td>2.91 ±0.32</td>
<td>65</td>
<td>70.0</td>
<td>16.3</td>
<td>--</td>
<td>731</td>
</tr>
<tr>
<td></td>
<td>794JZ</td>
<td>1303</td>
<td>1060-1632</td>
<td>1.71 ±0.18</td>
<td>63</td>
<td>56.2</td>
<td>30.30</td>
<td>--</td>
<td>760</td>
</tr>
<tr>
<td>+ 1g L$^{-1}$ PBO</td>
<td>926B</td>
<td>2.10</td>
<td>1.24-2.88</td>
<td>2.49 ±0.51</td>
<td>42</td>
<td>21.79</td>
<td>--</td>
<td>3.37</td>
<td>824</td>
</tr>
<tr>
<td>+ 1g L$^{-1}$ EN 16/5-1</td>
<td>926B</td>
<td>1.48</td>
<td>0.79-2.23</td>
<td>1.53 ±0.20</td>
<td>102</td>
<td>117.7</td>
<td>--</td>
<td>4.78</td>
<td>1092</td>
</tr>
<tr>
<td>+ 1g L$^{-1}$ PBO</td>
<td>794JZ</td>
<td>271</td>
<td>220-327</td>
<td>2.26 ±0.24</td>
<td>81</td>
<td>96.02</td>
<td>--</td>
<td>4.80</td>
<td>1106</td>
</tr>
<tr>
<td>+ 1g L$^{-1}$ EN 16/5-1</td>
<td>794JZ</td>
<td>264</td>
<td>215-321</td>
<td>2.16 ±0.21</td>
<td>100</td>
<td>113.7</td>
<td>--</td>
<td>4.94</td>
<td>1131</td>
</tr>
</tbody>
</table>

$^a\text{LC}_{50}$=Lethal concentration to kill 50% of the population, $^b$CL=Confidence limits, $^d$df=degrees of freedom, $^d\chi^2$=chi-square,

$^e\text{RF}$=resistance factor ($\text{LC}_{50}$ resistant strain/$\text{LC}_{50}$ susceptible strain), $^f\text{SF}$=synergism factor ($\text{LC}_{50}$ unsynergised/$\text{LC}_{50}$ synergised)
Full dose response bioassays with *Myzus persicae* clones against PBO (Chapter 4, refer to Table 4.7).

<table>
<thead>
<tr>
<th>Aphid Clone</th>
<th>(^a)LC(_{50}) (ppm)</th>
<th>(^b)CL 95%</th>
<th>Slope ±SE</th>
<th>(^c)df</th>
<th>(^d)(\chi^2)</th>
<th>Number of insects</th>
</tr>
</thead>
<tbody>
<tr>
<td>4106A</td>
<td>14892</td>
<td>12527-18041</td>
<td>2.95 ±0.31</td>
<td>41</td>
<td>41.4</td>
<td>489</td>
</tr>
<tr>
<td>794JZ</td>
<td>12196</td>
<td>8485-18788</td>
<td>1.72 ±0.17</td>
<td>36</td>
<td>75.7</td>
<td>440</td>
</tr>
<tr>
<td>926B</td>
<td>10540</td>
<td>6865-17325</td>
<td>1.42 ±0.15</td>
<td>45</td>
<td>125</td>
<td>512</td>
</tr>
<tr>
<td>5191A</td>
<td>25738</td>
<td>21570-30947</td>
<td>2.25 ±0.20</td>
<td>100</td>
<td>140</td>
<td>838</td>
</tr>
</tbody>
</table>

\(^a\)LC\(_{50}\)=Lethal concentration of PBO to kill 50% of the population, \(^b\)CL=Confidence limits, \(^c\)df=degrees of freedom, \(^d\)\(\chi^2\)=chi-square

Full dose response bioassays with *Meligethes aeneus* against \(\alpha\)-cypermethrin (Chapter 4 and 5, refer to Table 4.8 and 5.1).

<table>
<thead>
<tr>
<th>Population</th>
<th>(^a)LC(_{50}) ((\eta)g/cm(^2))</th>
<th>(^b)CL 95%</th>
<th>Slope ±SE</th>
<th>(^c)df</th>
<th>(^d)(\chi^2)</th>
<th>(^e)RF</th>
<th>Number of insects</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>2.81</td>
<td>0.19-4.52</td>
<td>1.58 ±0.18</td>
<td>26</td>
<td>42.3</td>
<td>1</td>
<td>293</td>
</tr>
<tr>
<td>Stein</td>
<td>10.0</td>
<td>6.01-17.0</td>
<td>0.84 ±0.08</td>
<td>27</td>
<td>14.9</td>
<td>3.57</td>
<td>343</td>
</tr>
<tr>
<td>Rogalin</td>
<td>65.0</td>
<td>40.0-100</td>
<td>1.40 ±0.20</td>
<td>18</td>
<td>11.9</td>
<td>23.2</td>
<td>148</td>
</tr>
<tr>
<td>Lebork</td>
<td>95.0</td>
<td>66.0-140</td>
<td>2.55 ±0.48</td>
<td>18</td>
<td>6.23</td>
<td>33.9</td>
<td>135</td>
</tr>
<tr>
<td>Leszno</td>
<td>22.5</td>
<td>13.2-35.0</td>
<td>1.23 ±0.18</td>
<td>18</td>
<td>17.3</td>
<td>8.00</td>
<td>118</td>
</tr>
</tbody>
</table>

\(^a\)LC\(_{50}\)=Lethal concentration to kill 50% of the population, \(^b\)CL= Confidence limits, \(^c\)df=degrees of freedom, \(^d\)\(\chi^2\)=chi-square

\(^e\)RF=Resistance factor (LC\(_{50}\) resistant strain/LC\(_{50}\) susceptible strain)
Synergism factors of analogues with imidacloprid in full dose response bioassays against *Myzus persicae* clone 5191A using a 5h pre-treatment (Chapter 6, refer to Table 6.1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(^a\text{LC}_{50}) (ppm)</th>
<th>(^b\text{CL} 95%)</th>
<th>Slope ±SE</th>
<th>(^c\text{df})</th>
<th>(^d\chi^2)</th>
<th>(^e\text{SF})</th>
<th>Number of insects</th>
</tr>
</thead>
<tbody>
<tr>
<td>imidacloprid</td>
<td>90.4</td>
<td>51.0-185</td>
<td>0.93 ±0.07</td>
<td>58</td>
<td>193</td>
<td>--</td>
<td>667</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) PBO</td>
<td>6.22</td>
<td>4.04-8.70</td>
<td>1.50 ±0.14</td>
<td>86</td>
<td>154</td>
<td>14.5</td>
<td>980</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 16/5-1</td>
<td>12.2</td>
<td>8.80-16.4</td>
<td>1.24 ±0.09</td>
<td>89</td>
<td>105</td>
<td>7.41</td>
<td>956</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 164</td>
<td>1.25</td>
<td>0.74-1.97</td>
<td>1.23 ±0.08</td>
<td>77</td>
<td>266</td>
<td>72.4</td>
<td>869</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 126</td>
<td>0.31</td>
<td>0.21-0.43</td>
<td>1.69 ±0.14</td>
<td>87</td>
<td>154</td>
<td>292</td>
<td>911</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 163</td>
<td>2.98</td>
<td>1.90-4.51</td>
<td>0.81 ±0.05</td>
<td>80</td>
<td>138</td>
<td>30.3</td>
<td>954</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 25-10</td>
<td>4.88</td>
<td>1.20-11.0</td>
<td>1.13 ±0.15</td>
<td>33</td>
<td>84.8</td>
<td>18.5</td>
<td>389</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 129</td>
<td>28.4</td>
<td>13.6-45.8</td>
<td>1.39 ±0.19</td>
<td>42</td>
<td>70.0</td>
<td>3.18</td>
<td>499</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 16-17</td>
<td>7.11</td>
<td>5.12-9.53</td>
<td>1.13 ±0.06</td>
<td>146</td>
<td>265</td>
<td>12.7</td>
<td>1600</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 16-18</td>
<td>18.4</td>
<td>9.69-29.5</td>
<td>1.14 ±0.11</td>
<td>79</td>
<td>156</td>
<td>4.91</td>
<td>862</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) EN 14-5</td>
<td>41.5</td>
<td>26.1-62.6</td>
<td>1.12 ±0.13</td>
<td>42</td>
<td>42.1</td>
<td>2.17</td>
<td>506</td>
</tr>
</tbody>
</table>

\(^a\text{LC}_{50}\)=Lethal concentration to kill 50% of the population, \(^b\text{CL}\)=Confidence limits, \(^c\text{df}\)=degrees of freedom, \(^d\chi^2\)=chi-square,

\(^e\text{SF}\)=synergism factor (LC\(_{50}\) unsynergised/LC\(_{50}\) synergised)
Synergism factors of analogues with α-cypermethrin in full dose response bioassays against Myzus persicae clone 794JZ using a 5h pre-treatment (Chapter 6, refer to Table 6.2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(^a\text{LC}_{50}) (ppm)</th>
<th>(^b\text{CL} 95%)</th>
<th>Slope ±SE</th>
<th>(^c\text{df})</th>
<th>(^d\chi^2)</th>
<th>(^e\text{SF})</th>
<th>Number of insects</th>
</tr>
</thead>
<tbody>
<tr>
<td>α- cypermethrin</td>
<td>1303</td>
<td>1061-1632</td>
<td>1.70 ±0.20</td>
<td>63</td>
<td>56.2</td>
<td>----</td>
<td>760</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) PBO</td>
<td>271</td>
<td>220-327</td>
<td>2.26 ±0.24</td>
<td>81</td>
<td>96.0</td>
<td>4.81</td>
<td>1106</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 16/5-1</td>
<td>264</td>
<td>215-321</td>
<td>2.17 ±0.21</td>
<td>100</td>
<td>114</td>
<td>4.94</td>
<td>1131</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 1-14</td>
<td>205</td>
<td>162-258</td>
<td>2.67 ±0.33</td>
<td>54</td>
<td>67.5</td>
<td>6.35</td>
<td>684</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 126</td>
<td>218</td>
<td>139-335</td>
<td>1.45 ±0.25</td>
<td>47</td>
<td>67.2</td>
<td>5.97</td>
<td>547</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 164</td>
<td>361</td>
<td>211-473</td>
<td>3.46 ±0.84</td>
<td>43</td>
<td>63.6</td>
<td>3.60</td>
<td>494</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 1-93</td>
<td>507</td>
<td>370-675</td>
<td>2.44 ±0.46</td>
<td>62</td>
<td>80.7</td>
<td>2.57</td>
<td>540</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 1-40</td>
<td>259</td>
<td>207-313</td>
<td>2.18 ±0.31</td>
<td>87</td>
<td>80.3</td>
<td>5.03</td>
<td>971</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 25-10</td>
<td>215</td>
<td>157-276</td>
<td>1.71±0.22</td>
<td>107</td>
<td>155</td>
<td>6.07</td>
<td>1147</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 14-05</td>
<td>569</td>
<td>377-898</td>
<td>2.27 ±0.49</td>
<td>52</td>
<td>92.0</td>
<td>2.29</td>
<td>590</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 129</td>
<td>629</td>
<td>484-878</td>
<td>1.70 ±0.29</td>
<td>76</td>
<td>73.3</td>
<td>2.07</td>
<td>868</td>
</tr>
<tr>
<td>+ 1g L(^{-1}) 1-16</td>
<td>147</td>
<td>101-194</td>
<td>1.74 ±0.25</td>
<td>71</td>
<td>81.2</td>
<td>8.87</td>
<td>806</td>
</tr>
</tbody>
</table>

\(^a\text{LC}_{50}\)=Lethal concentration to kill 50% of the population, \(^b\text{CL}\)=Confidence limits, \(^c\text{df}\)=degrees of freedom, \(^d\chi^2\)=chi-square,

\(^e\text{SF}\)=synergism factor (\text{LC}_{50} \text{ unsynergised}/\text{LC}_{50} \text{ synergised})
Appendix 5. Analysis of discriminating dose bioassay data

Generalized Linear Model (GLM) was fitted to the data calculated as proportions (number of dead aphids out of the total) assuming a Binomial distribution for the probability of death and using a logit link function.

The general model was:

\[
\log\left(\frac{P_{ijk}}{1 - P_{ijk}}\right) = \mu + Day_i + (Control vs Treatment)_j + \\
[(Control vs Treatment).Treatment]_{jk} + [Day.(Control vs Treatment)]_{ij} + \\
(Day.Treatment)_{ik} + [Day.(Control vs Treatment).Treatment]_{ijk}
\]

where \( P_{ijk} \) is the probability of death, \( i = 1, 2, 3 \) days (this refers to temporal replicates), \( j = 1, 2 \) (control or treated) and \( k = 1, 2, 3 \ldots \) (for the treatments: e.g. imidacloprid, imidacloprid + PBO and imidacloprid + EN 16/5-1).

The model accounted for:
the effects of day of assay – \( Day; \)
the control compared with the treated situation as a whole – \( (Control vs Treatment); \)
the different effects of the treatments having accounted for the control – \( [(Control vs Treatment).Treatment]; \)
and the interactions of control and treated situations with time – \( [Day.(Control vs Treatment)] \)
and \( (Day.Treatment)_{ik} + [Day.(Control vs Treatment).Treatment]. \)

On examination of residuals, the model was found to fit well, with no problem of overdispersion (variability over and above that which would be expected for the binomial distribution assumed). Following the fit of the model, the predicted probabilities of death were output with standard errors. The least significant difference (LSD) at the 5% level of significance between any pair of predictions was used to compare the treatments.
Appendix 6. Analysis of data for the esterase interference assay

The IC$_{50}$ value found using Grafit was converted into a percentage and given a new terminology: I (for “Index value”). The IC$_{50}$ for ‘E4’ was taken as 100% activity and for ‘no E4’ taken as 0% activity.

The IC$_{50}$ of the synergist sample, (I), was converted into a percentage by the following formula:

\[
I = \left( \frac{I_{\text{synergist}} - I_{\text{no E4}}}{I_{\text{E4}} - I_{\text{no E4}}} \right) \times 100 = \frac{y - x_1}{x_2 - x_1} \times 100
\]

Variance of the index (I) was approximated using the following expression:

\[
\text{var}(I) = \frac{100^2}{(x_2 - x_1)^2} \left[ \text{var}(y) + \text{var}(x_1) \right]
\]

It is important to note that this calculation often gives an underestimated value for the variance because the denominator is taken as a fixed quantity in the formula.

Here, \( \text{var}(y) = (\text{se}(y))^2 \) where se(y) is the standard error for the IC$_{50}$ (for the synergist) as given by the fit provided using Grafit; and similarly for \( x_1 \) (‘no E4’).

Following this,

\[
\text{se}(I) = \sqrt{\text{var}(I)}
\]

\[
= \sqrt{\frac{100^2}{(x_2 - x_1)^2} \left[ \text{var}(y) + \text{var}(x_1) \right]}
\]

The 95% Confidence Interval, denoted CI (95%), is given by

\[
I \pm t_{0.05, 21} \times \text{se}(I)
\]

where \( t_{0.05, 21} \) is the t-value at the \( p = 0.05 \) level of significance on 21 degrees of freedom (df). These are 21 because there are 33 data points (11 data points for each curve and 3 curves (no E4, E4, E4+synergist), less 12 parameters for fitting the 3 logistic curves.
Appendix 7. Formula for the variance of the ratio

Given the means and standard errors for all the compounds, the ratios of analogues to the control were calculated. The formula for the variance of this ratio is:

\[
\text{var}(a/b) = \left(\frac{a^2}{b^2}\right) \left[\frac{\text{var}(a)}{a^2} + \frac{\text{var}(b)}{b^2}\right]
\]

where \(a\) is the mean and \(\text{var}(a)\) is the square of the standard error of the mean for an analogue and where \(b\) is the mean and \(\text{var}(b)\) is the square of the standard error of the mean for the control.
Appendices

Appendix 8. Publications arising from this thesis

An analogue of piperonyl butoxide facilitates the characterisation of metabolic resistance

Graham D Moores, a, ∗ Despina Philippou, a Valerio Borzatta, b Paolo Trincia, b Philip Jewess, a Robin Gunning c and Georgina Bingham a

Abstract

BACKGROUND: Previous work has demonstrated that piperonyl butoxide (PBO) not only inhibits microsomal oxidases but also resistance-associated esterases. The ability to inhibit both major metabolic resistance enzymes makes it an ideal synergist to enhance xenobiotics but negates the ability to differentiate which enzyme group is responsible for conferring resistance.

RESULTS: This study examines an analogue that retains the ability to inhibit esterases but is restricted in its ability to act on microsomal oxidases, thus allowing an informed decision on resistance enzymes to be made when used in conjunction with the parent molecule.

CONCLUSION: Using examples of resistant insects with well-characterised resistance mechanisms, a combination of PBO and analogue allows identification of the metabolic mechanism responsible for conferring resistance. The relative potency of PBO as both an esterase inhibitor and an oxidase inhibitor is also discussed.

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Keywords: PBO; PBO analogues; esterase; synergist; resistance; cytochrome P450

1 INTRODUCTION

Insecticide resistance is often regarded as a major threat to increased agricultural production and the control of vector-borne diseases. Such resistance can be the result of mutations occurring at the insecticide target-site protein, e.g. modified acetylcholinesterase in the case of organophosphates and carbamates 1 or kdr in the case of pyrethroids, 2 or as a result of enhanced metabolic degradation of the parent insecticide molecule by specific enzymes. 3 Metabolic resistance can be diminished by the use of insecticide synergists: non-toxic chemicals that can be added to insecticides to increase their efficacy against insecticide-resistant insect pests. 4 The best known synergist is probably the methylendioxyphenyl (MDP) compound piperonyl butoxide (PBO), which is reported to act through a metabolic intermediate and form a complex with the haem moiety of the microsomal oxidase. 5 MDP compounds are thus thought to inhibit the biotransformation of the insecticide at phase 1 of its detoxification, and as such have been widely cited as specific inhibitors of microsomal oxidases. 6,7 However, recent reports have highlighted their effect on non-specific esterases. 6,9 This finding means that using a synergist assay incorporating PBO as a means of identifying the resistance mechanism may no longer be viable. It must also cast doubt on the previous wealth of literature where this procedure has been used.

The process by which MDP chemicals exert their effect on oxidases is well known and documented, 10 but no such system has yet been proposed for the action of similar compounds on resistance-associated esterases. Consequently, an analogue of PBO was synthesised with the purpose of defining a synergist that would act as effectively as inhibiting an important resistance-associated enzyme, but with greater specificity, and could therefore be used to characterise the resistance mechanism conferring insensitivity in an insect towards a particular xenobiotic.

2 MATERIALS AND METHODS

2.1 Analogue preparation

The analogue [EN 16/5-1; 6-[2-butoxyethoxy]ethoxymethyl]-5-propyl-2,3-dihydrobenzofuran] (Fig. 1) was synthesised through a four-step synthesis from 2,3-dihydrobenzofuran.

(c) 5-Propoxy-2,3-dihydrobenzofuran (acylation)

To a mixture of 2,3-dihydrobenzofuran (0.1 mol) and zinc chloride (0.005 mol), propionic anhydride (0.05 mol) was added under nitrogen while stirring, and the mixture was heated to 90 °C. The mixture was kept at 90 °C and stirred for another 4 h. It was then cooled to room temperature, and water (30 mL) and dichloromethane (20 mL) were added. The organic solution was separated and the aqueous phase was extracted with dichloromethane (20 mL). The organic phases were collected and washed with 2% hydrochloric acid (20 mL), aqueous sodium


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Appendices

PBO and analogue characterise metabolic resistance

Figure 1. 6-(2,2-Butoxyethoxyethoxymethyl)-3-propyl-2,3-dihydrobenzofuran, EN 16/5-1.

carbonate (50 g L\(^{-1}\)) and water (2 x 50 mL). The organic phase was then dried on sodium sulfate, filtered and evaporated under vacuum (40°C/23 mbar). The resulting oil was distilled under vacuum (100°C/0.3 mbar) to give (i) as a colourless oil (12.1 g); the structure was confirmed by NMR and GC/MS.

(ii) 5-Propyl-2,3-dihydrobenzofuran (reduction)

A mixture of 5-propanoyl-2,3-dihydrobenzofuran (0.068 mol), isopropanol (25 mL) and Pd/C 5% (50 wt%; 1.2 g) was put into an autoclave, purged with nitrogen and heated to 70°C. Hydrogen was added to a pressure of 5 bar, and this pressure was kept for 4 h at 70°C while stirring. The mixture was then cooled to room temperature, purged with nitrogen and filtered. The solvent was evaporated under vacuum (50°C/23 mbar). The resulting oil was distilled under vacuum (70-72°C/0.5 mbar) to give (ii) as a colourless oil (10.72 g); the structure was confirmed by NMR and GC/MS.

(iii) 6-Chloromethyl-5-propyl-2,3-dihydrobenzofuran

(Chloromethylation)

A mixture of 5-propyl-2,3-dihydrobenzofuran (0.057 mol), paraformaldehyde (0.091 mol) and 37% hydrochloric acid (0.23 mol) was heated to 50°C under nitrogen. The reaction was kept at 50°C for 6 h and then cooled to room temperature, and the organic phase was separated and used for the following reaction.

(iv) 6-(2,2-Butoxyethoxyethoxymethyl)-2,3-dihydrobenzofuran

(Alkylation)

A mixture of diethylene glycol butyl ether (0.53 mol) and sodium hydroxide (20-40 mesh; 0.06 mol) was heated to 40°C for 2 h. 6-Chloromethyl-5-propyl-2,3-dihydrobenzofuran (0.046 mol) was added at 40°C. After the addition, the mixture was heated to 100°C for 2 h and cooled to room temperature, and the organic phase was separated, washed with water (2 x 10 mL) and distilled under vacuum (180-185°C/0.1 mbar). EN 16/5-1 (iv) was obtained as a colourless oil (0.79 g), and the structure was confirmed by NMR and GC/MS.

2.2 Chemicals

Piperonyl butoxide (PBO ‘Ultra’ technical, 94%, and PBO 30 EW (30% PBO microemulsified in water) were both from Endura SpA, Italy. Imidacloprid 192 g L\(^{-1}\) SC (Provado) was obtained from Bayer CropScience. Technical l- cypermethrin (analytical standard) was obtained from Sigma (UK), as were all other chemicals, e.g. 7-ethoxy coumarin, l-naphthyl butyrate, NADPH and Fast Blue RR.

2.3 Insects

Tobacco whitefly, Bemisia tabaci Genn., were maintained on cotton plants (Gossypium hirsutum L. cv. Deltapine 16) at 26 ± 2°C with a 16:8 h light:dark photoperiod. SUDS is a standard susceptible strain collected from the Sudan in 1978; ‘GUJ A6’ is a B-type resistant strain collected from Guatemala in 2003.

Peach-potato aphid, Myzus persicae Sulzer, were reared on three-week-old Chinese cabbage plants (Brassica rapa (L.) Thell. Var. Pekenissi Wong Bok) maintained at 18 ± 2°C with a 16:8 h light:dark photoperiod. Clone 794A is a highly resistant (R3) variant obtained from a glasshouse at Evesham in 1982.

2.4 Insect Homogenisation

Approximately 100 adult B-type whitefly were homogenised in 700 μL of 0.2x phosphate buffer (pH 7.0) and centrifuged at 10,000 × g for 10 min, and the supernatant was taken as the enzyme source for the assay.

Approximately 1000 adult B-type whitefly were homogenised on ice in 250 μL homogenisation buffer (0.1x phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1.46 μM sucrose) and then diluted with the same buffer to give a final volume of 1 mL. This was centrifuged at 10,000 × g for 10 min, and the supernatant was taken as the enzyme source for oxidase activity.

2.5 Enzyme assays

2.5.1 Determination of esterase activity

Total esterase activity was measured in 96-well microplates using a colourimetric assay modified from Grant et al.\(^\text{11}\) Briefly, the rate of hydrolysis of 1-naphthyl butyrate (0.5 mM or 31.25 μM, final concentration) was assayed at 450 nm by measuring complex formation of 1-naphthol with the diazonium salt Fast Blue RR. Kinetic assays were performed using a Thermomax microplate reader (Molecular Devices, Mentlo Park, CA) reading for 20 min at 10 s intervals. The integrated ‘Softmax’ software was used to fit a linear regression to the kinetic plots.

2.5.2 Determination of esterase inhibition (in vitro)

Stock solutions of PBO and EN 16/5-1 (10 mM) were prepared in acetone. Aliquots of whitefly supernatant were incubated for 10 or 120 min with serial dilutions of PBO ‘Ultra’ or EN 16/5-1 to give final concentrations of between 1 mM and 10 mM. Esterase activity was measured as in Section 2.5.1. IC\(_50\) values were calculated using Graph 3.0 (GraphPad, Entrichis software).

2.5.3 Determination of microsomal oxidase activity

O-deethylation of 7-ethoxycoumarin was measured according to Ullrich and Weber\(^\text{12}\) and adapted to the microplate format as described by De Sousa et al.\(^\text{13}\). Briefly, ethoxycoumarin was dissolved in ethanol to make either a 20 mM or a 1.25 mM stock solution and diluted by the addition of 0.1x sodium phosphate buffer (pH 7.8) to give a concentration of 0.5 mM or 31.25 μM. A quantity of 50 μL of enzyme was added to separate wells of a microplate, followed by the addition of 80 μL ethoxycoumarin. The microplate was incubated for 5 min at 30°C, and the reaction was initiated by the addition of 10 μL of 9.6 mM NADPH. Final substrate concentrations were either 205 μM or 18 μM. Enzyme activity was read in a Victor® 1420 multilabel counter (Wallac, Milton Keynes, UK).
2.5.4 Determination of O-deethylatation inhibition (in vitro)

Stock solutions of PBO ‘Ultra’ and EN 16/5-1 (10 mg and 1 mg) were prepared in acetone. Dilute enzyme (50 μL enzyme + 50 μL 0.1 m phosphate buffer, pH 7.5) was incubated with 3 μL PBO/analogue to give final concentrations of 300 μM and 30 μM. After 10 or 120 min, 50 μL incubated enzyme was mixed with 80 μL of 0.5 m or 31.25 μL ethoxymercurodinitrile, and oxidase activity was monitored as in Section 2.5.3.

2.6 Insect bioassays

2.6.1 Bemisia tabaci

Leaf-disc bioassays were modified from those described by Cahill et al.10 Leaf discs (3 cm diameter) were dipped into serial dilutions (1000 to 0.001 mg AI L⁻¹) of imidacloprid SC and left to dry. The discs were then placed onto agar (1%) in petri dishes (37 mm diameter, 15 mm high), and the pest insects were placed onto the discs. Each unit was sealed with a close-fitting ventilated lid. After 48 h, at standard rearing conditions, mortalities were assessed. For the PBO 30 EW or EN 16/5-1 pretreatment, the insects were placed on leaves treated with 500 mg L⁻¹ inhibitor and then, after 5 h, transferred to discs treated with imidacloprid. Controls were treated with distilled water only. All bioassays were completed in triplicate. The bioassay data were analysed using probit analysis15 revised for microconfiguration In the statistical program PC PolYPlus (LuOra Software, Berkeley, California). Control mortality was corrected using Abbott’s formula.16 The results of the probit analyses were used to generate resistance factors (RFs), calculated as the LD₅₀ of the resistant strain divided by the LD₅₀ of the susceptible strain under the same conditions, and effective synergist ratios (ESR). Calculated as the LD₅₀ for the synergised strain divided by the LD₅₀ for the unsynergised susceptible strain.

2.6.2 Myzus persicae

Young aphid were transferred to the abaxial surface of Chinese cabbage leaf discs (ten adults per leaf disc, three replicates per experiment) held on 1% agar in plastic containers (4 cm diameter). The upper edge of each container was coated with Fluro (Whittford Plastics, UK) to prevent subsequent aphid escape from the leaf surface. After allowing 2 h for the aphids to settle, each was dosed with 0.25 μL of either 1 μL L⁻¹ PBO ‘Ultra’ or 1 μL L⁻¹ EN 16/5-1 in acetone (96% AR) using a Burkard microapplicator (Burkard, Rickmanworth, UK). After a further 4 h, aphids were dosed with 0.25 μL of 0.1 g L⁻¹ 1-benzylimidazole in acetone. Controls were treated with acetone only. Treated aphids were stored at 18 ± 2 °C with a 16:8 h light:dark photoperiod. All bioassays were scored at the endpoint of 72 h after treatment. Adults incapable of coordinated movement (after gentle touching with a paintbrush if necessary) were scored as dead. All bioassays were completed in triplicate. The bioassay data were analysed by means of a generalised linear model for a binomial distribution using a logit link.

3 RESULTS AND DISCUSSION

3.1 Analogue structure

The PBO analogue EN 16/5-1 (Fig. 1) was characterised by NMR and MS to confirm the structure.

Table 1. IC₅₀ values of B-type Bemisia tabaci esterase against PBO and analogue EN 16/5-1. All assays performed in triplicate

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate concentration (μM)</th>
<th>Incubation time (min)</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO</td>
<td>500</td>
<td>10</td>
<td>4.7 (±0.6)</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>500</td>
<td>10</td>
<td>6.5 (±1.7)</td>
</tr>
<tr>
<td>PBO</td>
<td>500</td>
<td>120</td>
<td>5.1 (±2.4)</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>500</td>
<td>120</td>
<td>5.4 (±3.3)</td>
</tr>
<tr>
<td>PBO</td>
<td>33.25</td>
<td>10</td>
<td>5.4 (±3.8)</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>33.25</td>
<td>10</td>
<td>4.8 (±1.9)</td>
</tr>
</tbody>
</table>

2.50 [L, 2H, J] 7.5 Hz, CH₃]; 3.45 [L, 2H, J] 6.5 Hz, CH₃]; 3.65 [m, 8H, 4CH₃]; 4.55 (m, 4H, 2CH₂); 6.88 [s, 2H, CH (4, 7)];

1H NMR (400 MHz; deuterochloroform), δ ppm: 0.95 [m, 6H, 2CH₃ (10, 21)]; 1.35 [m, 2H, CH₂ (20)]; 1.55 [m, 4H, 2CH₂ (19, 9)]; 1.95 [m, 2H, CH₂ (19, 9)].

3.2 Esterase inhibition

Table 1 shows the calculated IC₅₀ values from incubations of whitefly homogenate with PBO and EN 16/5-1. It can be seen that there is no significant difference between the two chemicals with regard to esterase inhibition potency. IC₅₀ values were not changed significantly with a 16-fold decrease in substrate concentration, suggesting a non-competitive inhibitory action. Furthermore, IC₅₀ values were not significantly changed when the incubation time was increased from 10 min to 2 h, suggesting that both MDP compounds act as reversible inhibitors.

3.3 O-deethylatation inhibition

The analogue (EN 16/5-1) was tested together with PBO as an oxidase inhibitor. At the lower inhibitor concentration (30 μM), no inhibition of oxidase activity was found with either inhibitor (results not shown). At the higher concentration (300 μM), PBO was found to inhibit 76% of the O-deethylatation activity from B. tabaci, while the analogue, having a modified MDP ring, was less effective, inhibiting only 22% (Table 2). Modification of the MDP ring, therefore, does seem to decrease the potency of the compound as a microosomal oxidase inhibitor, as expected, but perhaps surprisingly does not remove this action altogether. These inhibition percentages were not significantly different when the incubation time was increased to 2 h, but inhibition did increase when the final substrate concentration was reduced to 18 μM.

3.4 Insect bioassays

Analysing the effects of synergists in bioassays is usually done by calculating the resistance factor (RF) or synergist factor (SF). Although this can give useful information, it can be misleading when considering control in the field. For this reason, the authors have chosen to give additionally the effective synergist ratio (ESR). A value of 1.0 is defined as a standard susceptible population, with values higher than 1.0 indicative of the ‘resistance’ beyond an unsynergised susceptible, and values less than 1.0 indicative of hypersensitivity. With resistant B. tabaci, pretreatment with PBO gives excellent synergism against imidacloprid, bringing
Appendices

Table 2. *Bemisia tabaci* (GU1 M8O) oxidase inhibition by PBO and analogue EN 16/5-1 (100 μM). All assays performed in triplicate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate concentration (μM)</th>
<th>Incubation time (min)</th>
<th>β-O-deethylhydrol Activity remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>285</td>
<td>10</td>
<td>1.4 (±0.6) 100</td>
</tr>
<tr>
<td>PBO</td>
<td>285</td>
<td>10</td>
<td>10.1 (±1.5) 24</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>285</td>
<td>10</td>
<td>32.2 (±2.2) 79</td>
</tr>
<tr>
<td>Acetone</td>
<td>265</td>
<td>120</td>
<td>42.7 (±5.3) 100</td>
</tr>
<tr>
<td>PBO</td>
<td>265</td>
<td>120</td>
<td>11.4 (±2.0) 27</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>265</td>
<td>120</td>
<td>35.3 (±6.3) 82</td>
</tr>
<tr>
<td>Acetone</td>
<td>28</td>
<td>10</td>
<td>28.3 (±0.5) 100</td>
</tr>
<tr>
<td>PBO</td>
<td>28</td>
<td>10</td>
<td>4.4 (±3.4) 16</td>
</tr>
<tr>
<td>EN 16/5-1</td>
<td>28</td>
<td>10</td>
<td>11.8 (±4.3) 42</td>
</tr>
</tbody>
</table>

4 CONCLUDING REMARKS

Although synergists, and PBO in particular, have long been used as an indicator of resistance mechanisms, such interpretations have often been misleading. Synergists may have multiple effects in biological systems, and inhibition of enzyme systems in one species cannot necessarily be reflected in other species. Each example should be thoroughly investigated in its own right.

The analogue of PBO, EN 16/5-1, retained the ability to inhibit esterase activity, but alteration of the MDP ring severely disrupted its ability to inhibit microsomal oxidase activity. This is perhaps expected, as Wen et al. reported that the removal of oxygen from MDP-like compounds to form benzofuran derivatives without an accompanying pyran ring resulted in a loss of oxidase inhibition. For inhibition of esterases, PBO and EN 16/5-1 acted as reversible and non-competitive inhibitors, with no effects seen by alteration of substrate concentration or the length of preincubation time (Table 1). For inhibition of oxidases, PBO and EN 16/5-1 acted as reversible and competitive inhibitors, with no influence by pretreatment time but higher inhibition observed with lowering of substrate concentration (Table 2). It is perhaps surprising that PBO and EN 16/5-1 acted similarly, suggesting that, although the MDP ring is important for conferring potency to oxidase inhibition, other parts of the molecule can be responsible for binding to substrates. It also suggests that, if a metabolic intermediate is formed with the whitefly oxidases, this complex is reversible.

While *in vitro* experiments with model substrates do not necessarily reflect *in vivo* situations of insects metabolising insecticide molecules, there is now the exciting prospect that these two molecules, PBO and EN 16/5-1, could be used in parallel for better characterisation of metabolic resistance factors. If equal synergism were found with both, it would be hypothesised that esteratic factors are the major metabolic resistance mechanism. If better synergism were found with PBO, it would seem that microsomal oxidases could be the primary metabolic enzyme conferring resistance. This premise is reinforced by the results with *M. persicae* (in which esterase E4 is known to be the major metabolic resistance mechanism against insecticides), where little difference was found between PBO and EN 16/5-1 in synergism studies. Conversely, bioassays of resistant *B. tabaci* against imidacloprid revealed PBO to be a significantly better synergist than EN 16/5-1, suggesting oxidase involvement and supporting the findings of Nauen et al.

Penetration and metabolism of EN 16/5-1 should be very similar to those of the parent molecule, and indeed the similar synergism observed in *M. persicae* would reinforce this supposition. However, it is possible that the analogue does not fully penetrate *B. tabaci* or is more readily metabolised in this insect.

Perhaps most surprising, given the long period of time that PBO was considered a specific inhibitor of microsomal oxidases, is the observation that it is no more potent an inhibitor of these enzymes than of esterases. At a concentration of 30 μM, a 10 min incubation of PBO against β-O-deethylhydrol activity revealed no significant inhibition. The same concentration would result in near-complete inhibition of esterase activity. Similarly, PBO did not inhibit CYP6B1 activity from the Black Swallowtail butterfly, and De Sousa et al. found only 32% inhibition of ECOQ activity in housefly abdomens after exposure to 100 μM PBO. As PBO acts as a reversible inhibitor of both esterases and oxidases, but is a competitive inhibitor in the latter, it may be that substrate protection gives the impression that it is a more potent esterase inhibitor. Most importantly, it is clear that esterase inhibition potency is at least comparable with oxidase inhibition potency.
Appendices

Table 3. Bioassay of *Bemisia tabaci* against imidacloprid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Strain</th>
<th>N</th>
<th>LC50 (mg L⁻¹)</th>
<th>95% FL</th>
<th>Slope</th>
<th>df</th>
<th>χ²</th>
<th>RF</th>
<th>ESR³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>SUD-S</td>
<td>867</td>
<td>1.49</td>
<td>1.29–1.73</td>
<td>3.70</td>
<td>24</td>
<td>9.99</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>GUAMIX</td>
<td>658</td>
<td>&gt;1000</td>
<td>n/c</td>
<td>n/c</td>
<td>24</td>
<td>8.11</td>
<td>&gt;670</td>
<td>&gt;670</td>
</tr>
<tr>
<td>PBO + imidacloprid</td>
<td>SUD-S</td>
<td>577</td>
<td>0.49</td>
<td>0.34–0.70</td>
<td>4.18</td>
<td>24</td>
<td>98</td>
<td>1</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>GUAMIX</td>
<td>836</td>
<td>32.1</td>
<td>22.5–45.8</td>
<td>1.13</td>
<td>27</td>
<td>47.8</td>
<td>655</td>
<td>21.5</td>
</tr>
<tr>
<td>EN 16/51 + imidacloprid</td>
<td>SUD-S</td>
<td>591</td>
<td>0.94</td>
<td>0.79–1.11</td>
<td>2.87</td>
<td>24</td>
<td>7.0</td>
<td>1</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>GUAMIX</td>
<td>760</td>
<td>847</td>
<td>955–1340</td>
<td>1.11</td>
<td>27</td>
<td>8.67</td>
<td>900</td>
<td>508</td>
</tr>
</tbody>
</table>

³ ESR = effective synergism ratio (LC50, synergized strain/LC50, unsynergized susceptible strain).

and the combination of PBO and the analogue EN 16/51 could be useful tools to clarify metabolic resistance mechanisms in resistant pests. It is envisaged that a further analogue with specific oxidase activity will also be forthcoming, further enhancing metabolic characterisation and enabling the use of a specific synergist in field situations when beneficial. Such specific synergists would also allow ‘rotation’ in a similar manner to rotation of actives.

ACKNOWLEDGEMENTS

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REFERENCES

Metabolic enzyme(s) confer imidacloprid resistance in a clone of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) from Greece

Despina Philippou, Linda Field and Graham Moores*

Abstract

BACKGROUND: Previous studies have reported varying levels of resistance against imidacloprid in several insect species, including populations of the peach-potato aphid, *Myzus persicae* (Sulzer). These cases of resistance have been attributed to either target-site resistance or enhanced detoxification. In this study, a clone of *M. persicae* originating from Greece revealed a 60-fold resistance factor to imidacloprid.

RESULTS: The Greek clone is compared in terms of metabolic enzyme activity and synergism profiles with other *M. persicae* clones showing lower imidacloprid resistance.

CONCLUSION: A combination of *in vitro* biochemical assays and *in vivo* differential synergism studies using PBO and a close analogue EN 165-1 suggests that the mechanism conferring increased resistance in this clone is primarily due to enhanced oxidase activity.

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Keywords: PBO; imidacloprid; *Myzus persicae*; synergist; resistance

1 INTRODUCTION

The peach-potato aphid, *Myzus persicae* (Sulzer), is an extremely important worldwide pest that causes direct feeding injury to a variety of vegetable and ornamental species and indirect damage as a major vector of many plant viruses. As a result, many classes of insecticides have been used against this cosmopolitan pest, leading to the development of insecticide resistance.

Previous studies have identified three main mechanisms of resistance in this pest: enhanced levels of carboxylesterase E4 (or the closely allied FE4), modified acetylcholinesterase (MACE) and knockdown resistance (kdr). Enhanced E4/FE4 gives a broad spectrum of resistance against organophosphates, carbamates and pyrethroids. MACE, when present in *M. persicae*, gives very specific insensitivity against dimethyl carbamates such as pirimicarb. Knockdown resistance confers target-site insensitivity against pyrethroids. These neonicotinoids, however, have remained effective in the field against this pest, although reports have revealed both low and relatively high resistance levels.

The first commercially available neonicotinoid was imidacloprid, which is used extensively as a systemic, seed, soil or foliar treatment against many sucking pests. These neonicotinoids have enabled effective control against those species that have exhibited high resistance against other classes of insecticides. However, resistance to imidacloprid has now been identified in some insect species, although often at levels below that which would cause concern at field application rates. In common with other insecticides, resistance mechanisms to neonicotinoids have been identified as being either target site, for example in the brown planthopper, *Nilaparvata lugens* Stål; metabolic, for example as enhanced P450 activity in the tobacco whitefly, *Bemisia tabaci*.
in terms of resistance mechanisms. Clone 5191A was collected from tobacco in Greece in 2007. All clones have been established from single pathogenetic M. persiciana females.

2.2 Chemicals

Technical pirimethrin butoxide (PBO ‘Ultra’), 94%, and EN 16/5-1, 98%, were kind gifts of Endura SpA, Italy. Technical imidacloprid (analytical standard 99.7%) was obtained from Promochem Ltd, Welwyn Garden City, UK; technical α-cypermethrin (analytical standard 98%) was obtained from Sigma UK, as were other chemicals, e.g. 7-ethoxyphenyl, 1-naphthyl acetate, NADPH and Fast Blue RR.

2.3 Insect bioassays

2.3.1 Full dose response bioassays

Young aptera were transferred to the abaxial surface of Chinese cabbage leaf discs (ten adults per leaf disc; at least three replicates per concentration) held on 1% agar in plastic containers (4 cm diameter). The upper edge of each container was coated with Fluon (Whitford Plastics, UK) to prevent subsequent aphid escape from the leaf surface. After allowing 2 h for the aphids to settle, each was dosed with 0.25 μL of either 1 μg L⁻¹ PBO or 1 μg L⁻¹ EN 16/5-1 in acetone (99.6% AR) or acetone only in the absence of synergist pretreatment. After 4 h, aphids were further treated with 0.25 μL imidacloprid or α-cypermethrin in acetone using a Burkaid microapplicator (Burkaid, Rickmansworth, UK). To investigate response (mortality) to PBO alone, 0.25 μL aliquots of PBO from stock solutions of 300–100 000 mg mL⁻¹ in acetone were typically applied. Controls were treated with acetone only. Treated aphids were stored at 18 ± 2°C with a 16:8 h light-dark photoperiod. All bioassays were scored at the endpoint of 72 h after treatment. Adults incapable of coordinated movement (after gentle touching with a paint brush if necessary) were scored as dead. All bioassays were completed in triplicate.

2.3.2 Discriminating dose bioassays

Bioassays incorporating the two synergists were carried out as above, but using a sub-lethal concentration of imidacloprid (1 μg mL⁻¹) derived from the full dose response bioassays. This concentration was chosen to give low (5–10%) mortality with the 5191A clone in the absence of a synergist.

2.4 Polyacrylamide gel electrophoresis (PAGE)

Native gel electrophoresis was carried out as per Devonshire and Moores. Briefly, individual apterous aphids of each M. persicae clone were homogenised in 25 μL of 16 μL L⁻¹ Triton X-100 and 100 g L⁻¹ sucrose containing a few grains of bromocresol purpel as indicator. Following homogenisation, 10 μL samples were analysed by electrophoresis on a 7.5% polyacrylamide gel using barbitone buffer, based on the method described by Williams and Reisfeld.

After 1.5 h electrophoresis at 250 V, gels were rinsed in distilled water, then stained in 0.2 μ phosphate buffer, pH 6.0 (100 mL), containing 2 g L⁻¹ Fast Blue RR, to which 1 mL of 30 μL 1-naphthyl acetate in acetone was added. After 20 min, the esterase banding patterns were clearly visible on the gels. The stained gels were then washed with distilled water and 7% acetic acid was added to stop the reaction.

2.5 Enzyme assays

2.5.1 Esterase purification

The resistance-associated esterase from clone 5191A was purified as per Devonshire, with some modifications. Briefly, 5 g of aphids (stored at −20°C) was homogenised in 0.02 mL phosphate buffer, pH 7.0 and centrifuged at 20 000 × g for 10 min. The supernatant was passed through a G-25 column using 0.02 mL Tris/HCl, pH 8.5, as buffer. Unrelated fractions containing esterase activity were further chromatographed through DEAE Sepharose Fast Flow (Amersham Biosciences) and eluted with a linear 0–0.35 M NaCl gradient in 500 mL of Tris/HCl as above. Fractions containing carboxylesterase activity in the form of FE4 were concentrated, desalted and buffer exchanged to 0.02 mL phosphate, pH 7.0, for storage.

2.5.2 Indirect determination of esterase inhibition

Interactions of the purified resistance-associated esterase (FE4) with PBO, EN 16/5-1, α-cypermethrin and imidacloprid were performed using the ‘esterase interference assay’, as described by Khot et al, with some modifications. Briefly, stock solutions of synergists and insecticides (100 μL in acetone) were pre-incubated with pure FE4, to give a final concentration of 1 μμ, for 16 h at 4°C. Aliquots (15 μL) of FE4, FE4 + PBO, FE4 + EN 16/5-1, FE4 + α-cypermethrin and FE4 + imidacloprid were incubated for 1 h with 60 μL of serial dilutions of azamethiphos (in 0.02 mL phosphate buffer, pH 7.0), in separate wells of a NUNC microplate. Aliquots (25 μL) of housefly head homogenate (source of AChE) were added to each well and incubated for 15 min at room temperature. FE4 in acetone and buffer only served as the positive and the negative controls respectively. AChE activity was measured at 405 nm using a Tmmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). Readings were taken automatically for 10 min at 10 s intervals. The rate (mOD min⁻¹) was calculated by the integrated software Softmax Pro 4.6. Concentrations to inhibit 50% of the enzyme activity (IC50) were calculated using Graph 3.0 (Leatherbarrrow, Ehrlich Software). All treatments were performed in triplicate.

2.5.3 Determination of microsomal oxidase activity

Microsomal preparations. Fresh rabbit liver (1 g) was diced and homogenised on ice in 1 mL homogenisation buffer (0.1 μ phosphate buffer, pH 7.6, containing 1 mm EDTA, 1 mm DTT, 1 mm PTU, 1 mm PMSF and 1.46 μM sucrose), then diluted with the same buffer to give a final 100 μL L⁻¹ homogenate. This was centrifuged at 20 000 × g for 5 min. Supernatants were further centrifuged at 105 000 × g for 1 h at 4°C. The resulting pellet was resuspended in 300 μL homogenisation buffer and aliquots were used directly for the determination of microsomal oxidase activity.

Determination of O-deethylation activity. O-Deethylation of 7-ethoxyphenyl (7-EC) was measured according to Ulrich and Weber and adapted to the microplate format as described by de Sousa et al. Briefly, 7-EC was dissolved in ethanol (absolute high, 100% AR) to give a 20 μm stock solution and diluted by

the addition of 0.1 M sodium phosphate, pH 7.8, buffer to give a concentration of 0.5 μw. Aliquots of diluted enzyme (50 μl liver or aphid microsomal preparations, or a mixture of both) was added to separate wells of a microplate (OptiPlate™; Perkin Elmer), followed by the addition of 60 μl of 0.5 μw 7-EC. The microplate was incubated for 2 min at 30 °C and the reaction was initiated by the addition of 10 μl of 9.6 μM NADPH in 0.1 M sodium phosphate, pH 7.8. Enzyme activity was measured in a Victor® 1420 multilabel counter (Wallac, Milton Keynes, UK) for 60 min, with readings taken every 5 min, using an excitation wavelength of 370 nm and an emission wavelength of 460 nm. All assays were performed in triplicate.

2.6 Statistical analysis

For full dose bioassays, concentrations required to kill 50% of the population (LC50) were calculated by probit analysis using the statistical program PC Polo Plus (LeOra, Software, Berkeley, CA) after correcting for control mortality.12-20 Statistical comparisons using LC50 values were based on non-overlap of 95% confidence intervals.

For discriminating dose bioassays, a generalised linear model (GLM) was fitted to the data calculated as proportions (number of dead aphids divided by the total), assuming a binomial distribution for the probability of death and using a logit link function.

The model was:

$$\log(P_D - \mu) = \mu + \text{Day} + (\text{Control vs Treatment})_i + (\text{Control vs Treatment})_j + (\text{Day vs Control vs Treatment})_k + (\text{Day vs Control vs Treatment})_l$$

where $$P_D$$ is the probability of death, $$i = 1, 2, 3$$ (days this refers to temporal replicates), $$j = 1, 2$$ (control or treated) and $$k = 1, 2, 3$$ (for the treatments: imidacloprid, imidacloprid + PBO and imidacloprid + EN 16/5-1).

The model accounted for the effects of day of assay – Day; the control compared with the treated situation as a whole – (Control vs Treatment); the different effects of the treatments having accounted for the control – (Control vs Treatment)); and the interactions of control and treated situations with time – (Day vs Control vs Treatment) and (Day vs Control vs Treatment). On examination of residuals, the model was found to fit well, with no problem of overdispersion (variability observed was within that which would be expected for the binomial distribution assumed). Following the fit of the model, the predicted probabilities of death were output with standard errors. The least significant difference (LSD) at the 5% level of significance between any pair of predictions was used to compare the treatments.

The LC50 values obtained from the indirect determination of esterase inhibition were tested for significance using one-way ANOVA analysis (GenStat, 11th edition, VSN International). Following the ANOVA, the significance of the difference between selected pairs of means was assessed using the least significant difference (LSD) at the 5% level.

3 RESULTS

3.1 Insecticide bioassays

3.1.1 Full dose response

The results of the full dose response bioassays of clone 5191A, alongside three other M. persicae clones in which the resistance status had previously been characterised, are presented in Table 1. Considering the imidacloprid bioassay data, the Greek clone 5191A has a considerably higher resistance (RF 56.5) than clone 9268 (RF 4), also from Greece. The mechanism increasing the resistance in 5191A to imidacloprid does not confer cross-resistance to α-cypermethrin, i.e. 5191A and 9268 both have similar resistance factors (RF 33 and RF 4) to the pyrethroid. When PBO is used as a pretreatment (pretreatment on 5191A, the LC50 is reduced substantially, indicating that the resistance is at least partially due to a metabolic mechanism. When EN 16/5-1 is used as the pretreatment synergist on 5191A, the LC50 is again reduced, although to a lesser extent than with PBO. As expected, 794IZ showed a very high resistance against α-cypermethrin owing to the presence of target-site resistance.4

3.1.2 Discriminating dose

The effect of a 4 h pretreatment with either PBO or EN 16/5-1 on clone 5191A prior to exposure to a discriminating dose of imidacloprid (1 mg L\(^{-1}\)) is presented in Fig. 1. The insecticide alone (with a pretreatment of acetone) resulted in a mortality of 4.4%, pretreatment with EN 16/5-1 gave 13.5% mortality, while pretreatment with PBO resulted in 36.2% mortality. Acetone alone gave 1.9% mortality, EN 16/5-1 alone gave 0.9% mortality and

<p>| Table 1. Results of probit analysis on full dose response bioassay data |
|--------------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aphid clone</th>
<th>Esterase variant</th>
<th>LC50 (mg L(^{-1}))</th>
<th>95% CL</th>
<th>Slope (± SE)</th>
<th>df</th>
<th>$$\chi^2$$</th>
<th>RF</th>
<th>SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>4106A</td>
<td>S</td>
<td>1.2</td>
<td>1.2–1.9</td>
<td>1.0 (±0.10)</td>
<td>60</td>
<td>83</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>9268</td>
<td>F4 (R3)</td>
<td>5.5</td>
<td>5.3–6.0</td>
<td>1.6 (±0.15)</td>
<td>82</td>
<td>78</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5191A</td>
<td>F4 (R3)</td>
<td>51.8</td>
<td>51.1–52.5</td>
<td>0.9 (±0.07)</td>
<td>54</td>
<td>193</td>
<td>56.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+1 g L(^{-1}) PBO</td>
<td></td>
<td>4.0</td>
<td>4.0–4.7</td>
<td>1.5 (±0.14)</td>
<td>86</td>
<td>113</td>
<td>11.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+1 g L(^{-1}) EN 16/5-1</td>
<td></td>
<td>12.2</td>
<td>8.8–16.4</td>
<td>1.2 (±0.09)</td>
<td>89</td>
<td>105</td>
<td>14.5</td>
<td>–</td>
</tr>
<tr>
<td>α-Cypermethrin</td>
<td>4106A</td>
<td>S</td>
<td>0.17</td>
<td>0.14–0.2</td>
<td>0.3 (±0.03)</td>
<td>60</td>
<td>92</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>9268</td>
<td>F4 (R3)</td>
<td>7.0</td>
<td>5.6–8.7</td>
<td>2.9 (±0.03)</td>
<td>65</td>
<td>70</td>
<td>41.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5191A</td>
<td>F4 (R3)</td>
<td>5.6</td>
<td>5–6.0</td>
<td>3.3 (±0.02)</td>
<td>102</td>
<td>88</td>
<td>32.9</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>794IZ</td>
<td>F4 (R3)</td>
<td>1500</td>
<td>2060–1630</td>
<td>1.7 (±0.2)</td>
<td>63</td>
<td>56</td>
<td>7660</td>
<td>–</td>
</tr>
</tbody>
</table>

a RF = resistance factor (LC50 resistant strain/LC50 susceptible strain).
b SF = synergist factor (LC50 un synergised/LC50 synergised).
Appendices

Figure 1. Effect of synergist pretreatment on mortality of Aphis pancerceae clone 5191A exposed to imidacloprid.

PBO alone gave 1.7% mortality. Correcting for control mortality using Abbott's formula\(^2\) resulted in final mortalities of 2.9% (imidacloprid only), 12.7% (EN 16/5-1 pretreatment) and 33.1% (PBO pretreatment). Statistical analysis revealed a significant difference between insecticides only and the pretreatments (P < 0.001). There was also a significant difference in the synergism conferred by the two synergists (P = 0.001).

3.1.3 Prenylbutylate biosynthetic (P450) response

To investigate the response mortality to PBO only, high concentrations of the synergist had to be applied. For this study, three standard M. persicae clones were tested alongside clone 5191A. The LC50 values obtained for 4106A (S), 926B (R3, FE4) and 79412 (R3, E4) were not significantly different. Clone 5191A (R3, FE4), however, required approximately twice the dose of PBO to achieve equal mortality (Table 2).

3.2 PAGE

The enhanced resistance-associated enzyme present in 5191A was that of the E4 variant, commonly found in A. pancerceae samples from Mediterranean countries (Fig. 2). The mobility and intensity of the enzyme bands was identical to that found in 926B. Homogenates of TIV and 79412 show the mobility of E4 and a reference enzyme and also reveal the lower intensity of an R2 variant (TIV) alongside the R3 standard (79412).

3.3 Enzyme assays

3.3.1 Indirect determination of esterase inhibition

The induced protection of ACHE towards acaricides provided by FE4 following pre-incubation with PBO, EN 16/5-1,

<table>
<thead>
<tr>
<th>Aphid clone</th>
<th>Concentration (mM)</th>
<th>95%-CL</th>
<th>Slope</th>
<th>df</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>4106A</td>
<td>S</td>
<td>17.490-0.04</td>
<td>1.9 (0.18)</td>
<td>41</td>
<td>42</td>
</tr>
<tr>
<td>926B</td>
<td>FE4</td>
<td>0.030-0.004</td>
<td>1.4 (0.18)</td>
<td>55</td>
<td>150</td>
</tr>
<tr>
<td>79412</td>
<td>FE4</td>
<td>0.030-0.004</td>
<td>1.4 (0.18)</td>
<td>55</td>
<td>150</td>
</tr>
<tr>
<td>5191A</td>
<td>FE4</td>
<td>0.030-0.004</td>
<td>1.4 (0.18)</td>
<td>55</td>
<td>150</td>
</tr>
</tbody>
</table>

Figure 2. Polycatenar gel electrophoresis (PAGE) showing the presence of E4 in clones 79412 and the presence of FE4 in 926B and 5191A.

Enzyme activity (nM)

4. DISCUSSION

Resistance to neonicotinoids is becoming more widespread, with various insects developing a degree of resistance, reportedly

Figure 3. Inhibition of Microsomal ACHE activity: a, no FE4; b, FE4; c, FE4 + PBO; c, FE4 + EN 16/5-1; d, FE4 + acaricide.

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Appendices

Table 3. \( K_{IC_{50}} \) (concentration of azamethiphos required for 50% inhibition of AChE) values corresponding to Fig. 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>( K_{IC_{50}} ) (nm)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE only (no FE4)</td>
<td>0.165</td>
<td>±0.006</td>
</tr>
<tr>
<td>FE4</td>
<td>3.886</td>
<td>±0.016</td>
</tr>
<tr>
<td>FE4 + PRO</td>
<td>0.215</td>
<td>±0.016</td>
</tr>
<tr>
<td>FE4 + EN 16/5-1</td>
<td>0.197</td>
<td>±0.007</td>
</tr>
<tr>
<td>FE4 + α-cypermethrin</td>
<td>0.437</td>
<td>±0.034</td>
</tr>
<tr>
<td>FE4 + imidacloprid</td>
<td>0.908</td>
<td>±0.087</td>
</tr>
</tbody>
</table>

Figure 4. O-deethylation activity by (c) microsomal preparation of liver, (a) clone S191A and (b) a liver + clone S191A mixture.

Owing to target site or metabolic factors,\(^{7-13} \) while earlier reports had indicated low levels of resistance in \( M. \) persicae,\(^{12} \) similar to that observed in clone 9268, clone S191A revealed an additional degree of resistance, similar to that found previously in Greece, on tobacco.\(^{15} \) This enhanced resistance could be due to either target site or metabolic mechanisms, or to both. Synergism by PB0, however, as revealed in the full dose response biosays, suggests that the additional levels of imidacloprid resistance exhibited by S191A are due, partly at least, to metabolic enzyme activity. As PB0 is able to inhibit both phase-1 metabolic enzyme systems that can confer resistance, namely esterases and oxidases,\(^{24} \) differential synergist biosays were performed to identify which enzyme contributed the major effect. In vitro studies were performed to confirm the specificity of synergist action.

Using the esterase interference assay, it was found that the FE4 variant responded equally to inhibition by PB0 and the analogue EN 16/5-1.\(^{15} \) This assay also confirmed that FE4 is capable of sequestering α-cypermethrin and revealed that the esterase can also sequester imidacloprid, although to a lesser extent. The ability of FE4 to sequester imidacloprid probably accounts for the fourfold resistance observed in clone 9268 (Table 1).

Although measurement of O-deethylation by \( M. \) persicae has been reported previously,\(^{25} \) there was no appreciable activity found in the S191A microsomal preparation, possibly owing to high levels of oxidative inhibitors present in \( M. \) persicae homogenates, as reported by Devonshire.\(^{26} \) These inhibitors are probably the same membrane-bound proteases that were found to degrade rapidly aphid proteins, unless subjected to heat denaturation prior to homogenisation.\(^{17} \) It was therefore impossible to assess the relative O-deethylation inhibitory potencies of PB0 and the close analogue EN 16/5-1. However, in another insect species (\( B. \) tabaci) it was found that removal of an oxygen atom from the methylenedioxyphenyl ring gave the expected decrease in P450-inhibitory potency.\(^{17} \)

In populations of \( M. \) persicae where enhanced esterase activity confers resistance to a broad spectrum of insecticides, equal synergism was obtained by the use of PB0 or the analogue EN 16/5-1 when combined with a discriminating dose of insecticide.\(^{17} \) This was found not to be the case with S519A against imidacloprid, with both the discriminating dose biosay and the full dose biosay revealing PB0 to be conferring significantly more synergism than EN 16/5-1. There was a 14.5-fold synergism using PB0 and a 7.4-fold synergism with EN 16/5-1 (Table 1). The discriminating dose biosay result is in good agreement; following correction for control mortality, there is a fivefold increase in mortality with EN 16/5-1 and a 14-fold increase using PB0. Thus, both in vivo assays strongly suggest the involvement of enhanced P450s, in line with similar results with imidacloprid-resistant \( B. \) tabaci.\(^{17} \) This cannot be absolutely confirmed, as the in vitro oxidase assay was unable to validate the effect of PB0 and EN 16/5-1. However, it is most likely that PB0 inhibits \( M. \) persicae P450 activity, while EN 16/5-1 does not. The difference in the structure is unlikely to induce drastic changes in other pharmacokinetic parameters such as penetration, etc.

That resistance levels to α-cypermethrin in both 9268 and S519A are the same further suggests that enhanced levels of FE4 are not responsible for the additional imidacloprid resistance in S519A. In addition, the esteratic patterns of S519A and 9268 were found to be identical in terms of both esterase mobilities and intensities, again suggesting that enhanced esterases were not responsible for the difference in imidacloprid response between the two clones.

A final observation is that of the response of the S519A clone to PB0 alone. It is found to have an ‘insensitivity’ of around twofold when compared with other \( M. \) persicae clones. This ‘resistance’ to PB0 is not found in relation to esterase content; hence, the R3 clones 7941Z and 9268 have the same response as the susceptible 4106A. Previous reports of PB0 resistance have intimated high levels of P450 activity, although full characterisation of the mechanism was never carried out.\(^{25,26} \) These same reports also noted a reduction in the ability of PB0 to synergise, which could explain why complete susceptibility was not restored when S519A was pretreated with PB0. Alternative explanations would be the presence of a target-site mechanism, albeit one that confers little resistance, or that the synergist:insecticide ratio needed to be increased.\(^{25} \)

In conclusion, the results indicate that the additional resistance to imidacloprid present in this clone (compared with the lower resistance reported previously in field populations of \( M. \) persicae) is metabolic and is due primarily to enhanced oxidative activity, although low levels of resistance due to a target-site mutation cannot be completely ruled out. This is in agreement with the metabolic mechanism identified in \( B. \) tabaci. As such, a synergist such as PB0 could be used judiciously to regain maximal efficacy against imidacloprid-resistant pests.

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# Patent of Invention

Filed as

European Patent Application

for

"SUBSTITUTED ALKYNYL PHENOXY COMPOUNDS AND THEIR USES"

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