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Hydrolysis of individual isomers of fluorogenic pyrethroid analogs by mutant carboxylesterases from *Lucilia cuprina*

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Abstract

We previously showed that wild-type E3 carboxylesterase of *Lucilia cuprina* has high activity against Type 1 pyrethroids but much less for the bulkier, α-cyano containing Type 2 pyrethroids. Both Types have at least two optical centres and, at least for the Type 1 compounds, we found that wild-type E3 strongly prefers the less insecticidal configurations of the acyl group. However, substitutions to smaller residues at two sites in the acyl pocket of the enzyme substantially increased overall activity, particularly for the more insecticidal isomers. Here we extend these analyses to Type 2 pyrethroids by using fluorogenic analogs of all the diastereomers of cypermethrin and fenvalerate. Wild-type E3 hydrolysed some of these appreciably, but, again, not those corresponding to the most insecticidal isomers. Mutations in the leaving group pocket or oxyanion hole were again generally neutral or deleterious. However, the two sets of mutants in the acyl pocket again improved activity for the more insecticidal acyl group arrangements as well as for the more insecticidal configuration of the cyano moiety on the leaving group. The activities of the best mutant enzyme against the analogs of the most insecticidal isomers of cypermethrin and fenvalerate were more than ten and a hundred fold higher, respectively, than those of wild-type. The implications for resistance development are discussed.

Keywords: Pyrethroid hydrolase; *In vitro* mutagenesis; Isomers; Esterases; Insecticide; Resistance

1. Introduction

Carboxylesterases play a major role in the degradation of ester insecticides across a broad range of animal and plant species, and increases in the amount or catalytic efficiency of such enzymes often contribute to the development of insecticide resistance in target insects. Previous studies have shown that the blowfly *Lucilia cuprina* develops resistance by mutations in the gene encoding carboxylesterase E3 that dramatically enhance the ability of the enzyme to hydrolyse various organophosphorus (OP) insecticides (Newcomb et al., 1997; Campbell et al., 1998). These mutations are located close to the serine of the catalytic triad, common to all catalytically competent members of this large α/β hydrolase fold family (Ollis et al., 1992), that is responsible for nucleophilic attack on the phosphorus or carbonyl carbon of a wide range of esters.

One of these resistance mutations, a glycine to aspartate (G137D) mutation in the so-called oxyanion hole, enhances hydrolysis of phosphate esters but diminishes carboxylester hydrolysis. This appears to be achieved through altering the location of a bound water molecule to favour nucleophilic attack on the tetrahedral phosphorus over a corresponding planar carboxyl group bound to the catalytic serine (Campbell et al., 1998; Devonshire et al., 2003). The other common resistance mutation, (W251L) in the acyl pocket (the p2 subsite of Järv, 1984), substitutes a smaller and more flexible residue that enables both phosphate ester hydrolysis and cleavage of the ethyl succinate groups in the leaving group of malathion. It has

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been proposed that the greater space generated by this mutation in the acyl pocket enables the enzyme to accommodate the larger acyl group and also to facilitate the inversion about the phosphorus that must occur during hydrolysis (Campbell et al., 1998; Devonshire et al., 2003). Mutation of this residue to serine has also been found in resistant blowflies (Claudianos et al., 2001; Oakeshott et al., 2005). Remarkably, analysis of DNA amplified from pinned specimens collected up to 70 years ago shows that W251L, W251T (which studies of *in vitro* expressed enzymes indicate also has OP hydrolase activity), and probably also W251S, all existed at polymorphic frequencies in *L. cuprina* prior to the first use of OP insecticides, which both explains the early outbreak of resistance in this species and suggests that these variants may have other functional significance (Hartley et al., 2006).

The G137D and W251L/S changes have been found in E3 orthologs in association with OP resistance in two other Diptera, the housefly *Musca domestica* (Claudianos, 1999; Claudianos et al., 1999; Taskin et al., 2004; Oakeshott et al., 2005) and screw worm fly *Cochliomyia hominivorax* (de Carvalho et al., 2006). Another small residue, glycine, has also been seen at the position corresponding to 251 in a homologous esterase from a malathion resistant parasitoid wasp, *Anisopteromalus calandrae* (Zhu et al., 1999).

Although not associated with resistance to synthetic pyrethroid (SP) insecticides, which are not registered for use against *L. cuprina*, the E3 enzyme also shows significant hydrolytic activity against some of these compounds (Heidari et al., 2005). In particular wild type E3 and the W251L/S/T mutants all show activity against the older Type 1 SPs, which lack an α-cyano in their alcohol (leaving) group. The structures of Type 1 SPs generally contain two asymmetric carbons in their acyl groups so that these compounds have four isomeric forms. Most commercial formulations of SPs are racemic mixtures, although the isomers can differ by up to two orders of magnitude in their insecticidal potencies (Chamberlain et al., 1998 and references therein); indeed it is likely that the low potencies measured for the least potent isomers can often be attributed to traces of the most potent isomers. Interestingly, the chiral preference of the W251L/S/T mutants differs from that of the wild-type enzyme. Whereas wild-type E3 prefers the *trans* over *cis* and 1*S* over 1*R* configurations around the cyclopropane ring common to the acyl group of Type 1 SPs, W251L in particular has greatly reduced *trans*/*cis* preference, and slightly elevated 1*S*/1*R* preference. Our analysis of several synthetic mutations of E3 also showed that a second mutation to a smaller residue in its acyl pocket, F309L, could reverse the 1*S*/1*R* preference of wild-type E3 (Heidari et al., 2005).

Whilst having acyl groups similar to the Type 1 compounds, the newer Type 2 SPs have an α-cyano moiety on their alcohol leaving group, which confers an *S* or *R* conformation on the α-carbon. The analyses in Heidari et al. (2005) found much reduced levels of activity against one of the Type 2 compounds, deltamethrin, for which a radiometric assay was available. Wild-type E3 had no detectable activity while the W251L/S/T and F309L variants, with their presumptively larger acyl pockets, had much reduced (relative to Type 1 compounds) but still measurable activity. Activity levels were too low for any meaningful analysis of chiral preferences in Heidari et al. (2005) but in the present work we have utilised a series of fluorogenic SP analogs designed to resemble all eight cypermethrin and all four fenvalerate diastereomers (Huang et al., 2005a) to redress this. Previous studies with these analogs have demonstrated marked chiral preferences of vertebrate esterases between SP isomers (Stok et al., 2004; Huang et al., 2005a, b; Nishi et al., 2006). We also analyse the activities of the various E3 enzymes for the corresponding acetates and butyrates of the analogs, in order to understand how the α-cyano geometries of the leaving groups affect activities against substrates with achiral and much less bulky acyl groups.

2. Materials and methods

2.1. Chemicals

The synthesis and structures of the fluorogenic pyrethroid analogs and the corresponding simpler acetates and butyrates used as substrates have been described previously (Huang et al., 2005a). As with commercial SPs, the insecticide analogs can be identified simply according to the chirality in the acid at the 2-position (for the four analogs of fenvalerate) or 1-position together with the geometric isomerism across the cyclopropane ring (for the eight analogs of cypermethrin), in both cases together with the chirality at the α-carbon carrying the cyano group in the alcohol (Fig. 1). Substrate solutions of all 12 insecticide isomers and four acetates/butyrates were prepared and stored at 4 °C as 2 mM stocks in dimethyl sulfoxide (DMSO).

2.2. Enzyme constructs and expression

Esterase constructs in a baculovirus vector were expressed in Sf9 cells and the molar concentration of esterase in the extracts determined by titration with a fluorogenic organophosphorus inhibitor as described previously (Devonshire et al., 2003). All extracts were prepared at the same concentration of 10^8 cells/ml, regardless of esterase titre, and stored at −80 °C; those used here were originally expressed during that earlier study.

2.3. Enzyme assays

The assays for hydrolysis of the SP analogs and their corresponding acetates and butyrates are based on the spontaneous formation of fluorescent 6-methoxynaphthaldehyde from the primary cyanohydrin hydrolysis product (Shan and Hammock, 2001). All assays were done in

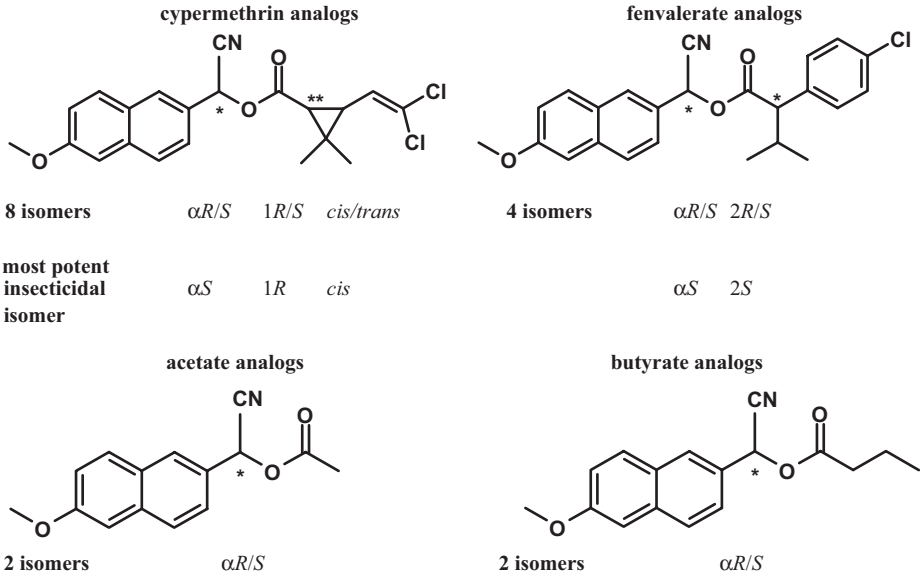

Fig. 1. Structures of fluorogenic pyrethroid analogs and the corresponding acetates and butyrates. * denotes a chiral centre.

25 mM Tris Cl^- pH 8.0 buffer at 30 °C. Substrates (2 mM in DMSO) were diluted 100-fold in buffer and 100 µl placed in the wells of NUNC white Polysorp fluoroplates, with all the wells of each vertical column having one substrate. The assays were started by adding 100 µl of the appropriately diluted enzymes, with one enzyme across each row, to give a final concentration of 10 µM substrate and 0.5% DMSO. Enzyme extracts were diluted the same regardless of esterase titre (final concentration corresponding to 5×10^5 cells/ml for the insecticide analogs or 5×10^3 cells/ml for the acetates and butyrates), so that control rates measured in host cells without insert were common to all assays. Control rates were negligible at the dilutions used, which is in line with the findings of Shan and Hammock (2001). (Lower rates of chemical hydrolysis of the α-cyano esters compared with naphthol or nitrophenol substrates are in fact expected because the cyano esters are aliphatic and therefore poorer leaving groups). All dispensing into plates was done with electronic multichannel pipettes (Biohit Plc., Finland).

Fluorescence of the 6-methoxynaphthaldehyde was monitored in a BMG FluoroStar (excitation 340 nm, emission 460 nm) for 20 min at 1 min intervals and the initial rate measured over the first four readings. Under the assay conditions used, less than 10% of any substrate was hydrolysed in this time, and initial rates were expressed as pmol hydrolysed/min/pmol esterase, based on a 6-methoxynaphthaldehyde calibration curve together with the measured esterase concentration. All assays were done in duplicate on separate occasions.

3. Results

Hydrolysis of the fluorogenic analogs of cypermethrin and fenvalerate and the corresponding acetates and butyrates was assessed using the panel of E3 variants expressed in baculovirus that have been characterised previously by their activity with radiolabelled pyrethroids (Heidari et al., 2005). All assays were done at 10 µM substrate as kinetic determinations were impractical for the large number of permutations of enzymes and substrates. Indeed, the limited solubility of pyrethroids, including these analogs, would compromise attempts to discriminate between substrate binding (K_m) and turnover (k_{cat}); any indications of activity reaching an asymptote at 'high' substrate concentrations are likely to result from its unavailability due to limited solubility rather than saturation of the enzyme's catalytic centre. Results for all substrates are shown in Fig. 2.

3.1. SP analog activities; general observations and wild type enzyme activities

The E3 WT enzyme has negligible activity for any of the fenvalerate analogs and very little activity for most of the cypermethrin analogs. The two exceptions are the analogs of 1*S trans* cypermethrin, for which the enzyme has quite high activities (~10 pmol/min/pmol enzyme), regardless of the chirality on the alcohol α-carbon. These exceptions aside, the generally low activities of the wild type enzyme accord well with the findings with radiolabelled deltamethrin in Heidari et al. (2005). All these data support the contention (Elliott and Janes, 1978) that esters of secondary alcohols found in Type 2 SPs are harder to hydrolyse, at least by this insect esterase (Heidari et al., 2005; Oakeshott et al., 2005).

Heidari et al. (2005) did not analyse 1*S trans* deltamethrin but the preference we see for this acyl group configuration in cypermethrin analogs mirrors the preferences shown among Type 1 SPs by wild-type E3 (Heidari et al., 2005), and also by the E4 esterase of the aphid *Myzus persicae* (Devonshire and Moores, 1982). SPs of this

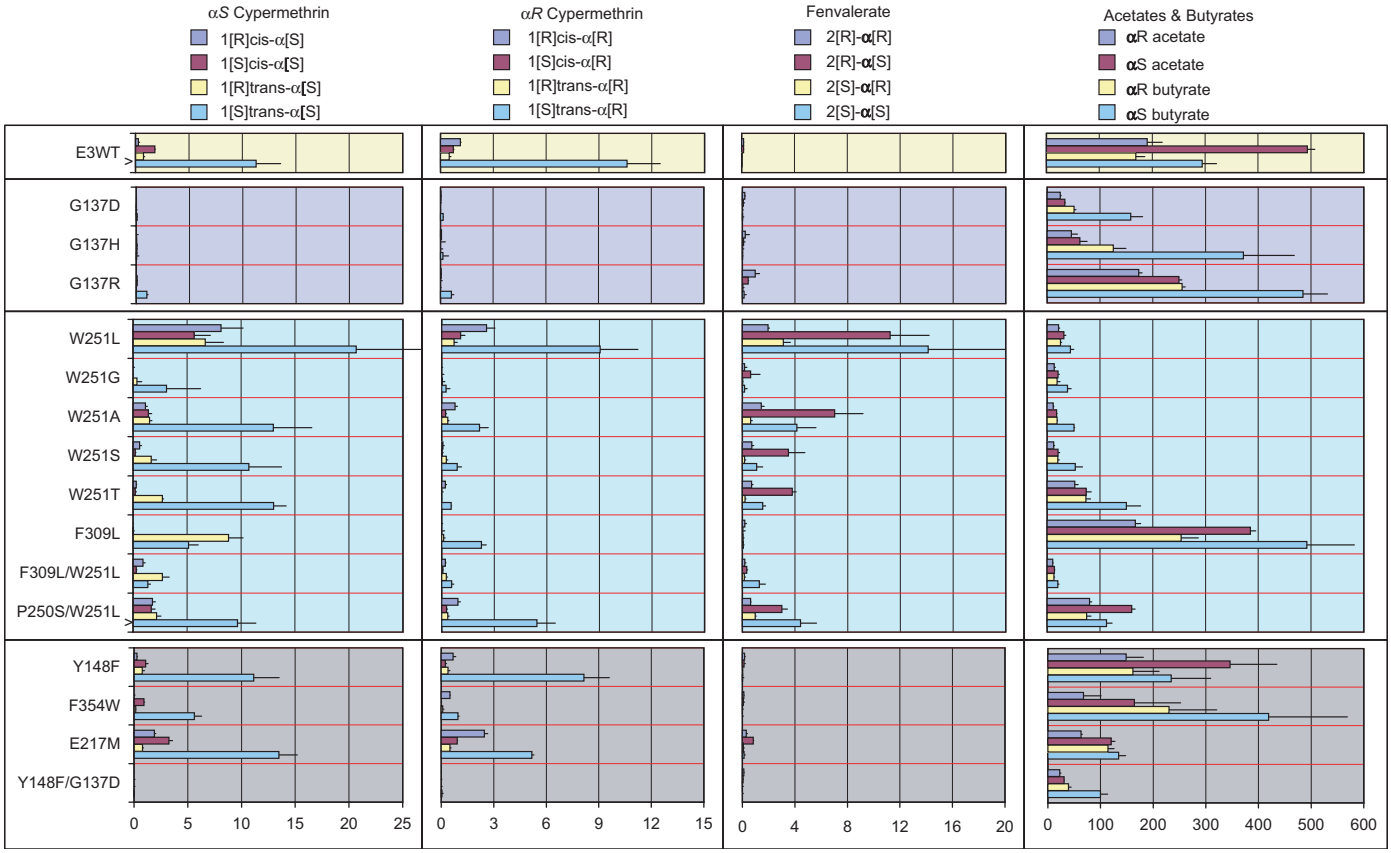

Fig. 2. Activities (pmol/min/pmol enzyme; mean of duplicates with s.d. bars) of E3 variants from *L.cuprina* with fluorescent analogs (10 μM) of fenvalerate, α*R* cypermethrin and α*S* cypermethrin, plus the corresponding acetates and butyrates. Results for E3 WT and mutants in the oxyanion hole, acyl pocket and leaving group pocket are given in separate panels. Note different scales on the activity axes. All enzyme/substrate combinations were assayed; the absence of a bar indicates that activity was too low to be recorded.

configuration have generally been found to be less potent insecticides than other configurations (Chamberlain et al., 1998 and references therein). The data here are thus consistent with their relatively low insecticidal potency being partly due to their susceptibility to esteratic degradation (Elliott and Janes, 1978; Chamberlain et al., 1998).

Wild-type E3 shows no obvious preference for α*R*/*S* chirality on the carbon bearing the cyano in the leaving group. Conformation of this carbon has a large effect on the insecticidal activity of the Type 2 SPs, with α*S* being much more potent (Chamberlain et al., 1998) at the sodium channel active site.

The panel of 15 mutant E3 variants analysed by Heidari et al. (2004, 2005) and herein cover all known natural mutants in the active site of E3 together with a range of synthetic ones designed to elucidate key sequence–structure–function relationships in the active site. The mutations are distributed across the three pockets in the carboxyl/cholinesterase active site, namely the leaving group pocket, where the alcohol group of the substrate is accommodated before it is hydrolysed away in the first step of the reaction, the oxyanion hole which accommodates the carbonyl or phosphate oxygen of the ester, and the acyl pocket, where the acyl group of the substrate sits through both the first and second steps of the catalytic cleavage. Although there is no empirical tertiary structure for E3 these assignments of residues to active site pockets have been made here and in previous papers (Campbell et al., 1998; Devonshire et al., 2003) using a model based on the known structures of related cholinesterases (Harel et al., 2000). Fig. 3 shows diagrammatically how the various mutations are distributed across the three pockets.

3.2. SP analog activities; oxyanion hole mutants

Heidari et al. (2005) examined four mutations at G137, the naturally occurring G137D and three synthetic variants, G137E, G137H and G137R, covering a range of charge states for this site. The G137D mutation corresponds to the 'mutant aliesterase' implicated in OP insecticide resistance, which has long been known to involve the simultaneous acquisition of phosphatase and drastic reduction in carboxylesterase activity (van Asperen and Oppenoorth, 1959; Newcomb et al., 1997; Devonshire et al., 2003). Heidari et al. (2005) found that all four of these mutations were deleterious to Type 1 SP esterase activity, as they were for the hydrolysis of other

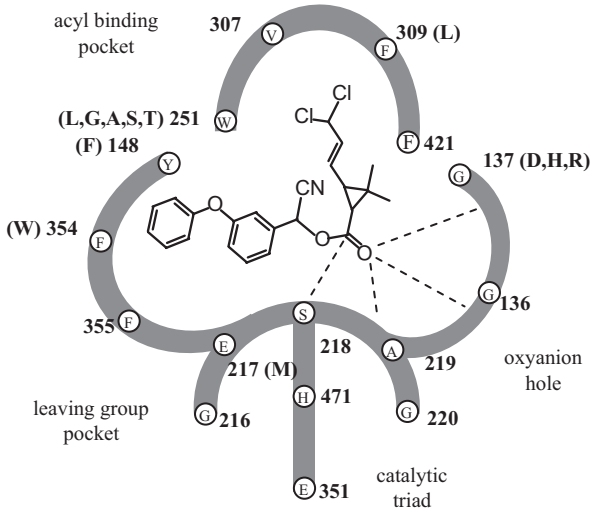

Fig. 3. Illustration, based on a generic model of esterase catalytic centres (Oakeshott et al., 2005), showing the major features of the E3 active site and the proposed orientation of a Type 2 pyrethroid (cypermethrin) within it. Dotted lines show the nucleophilic interaction of the catalytic serine with the carbonyl and the hydrogen bonds to the amides of the three residues in the oxyanion hole. All but one of the mutations analysed in this study are also shown (in brackets); one putative acyl pocket mutation, P250S, is not shown because its location is less clear—it had been included in the study on the basis of earlier evidence linking it to OP resistance (see text).

carboxylester substrates, but they had little effect on chiral preferences in respect of either the acyl or leaving groups. These effects could be readily understood in terms of a detrimental effect of substitutions to large charged or polar residues on the hydrogen bonding that occurs in the oxyanion hole during the enzyme reaction, regardless of substrate chirality.

We find here that G137D, G137H and G137R all show little activity with the Type 2 analogs, even with the most amenable 1*S trans* cypermethrin analogs. The activities for the cypermethrin analogs are all clearly lower than the corresponding wild-type values, as would be predicted from the results of Heidari et al. (2005). The values for both the wild-type E3 and G137 mutants with the fenvalerate compounds are all so low that no meaningful quantitative comparisons can be made.

3.3. SP analog activities; acyl pocket mutants

Heidari et al. (2005) analysed five mutations at W251 and one each at F309 and P250 in the acyl pocket. All five changes to W251 involve small amino acids and three of them, W251L/S/T, have been found as natural polymorphisms associated with malathion resistance in E3. The F309L change does not occur naturally in E3; it was selected because modelling suggests it sits on a side of the acyl pocket well away from W251 and it was reasoned that substitution to a small residue there might have quite different effects on stereo-preferences among acyl group configurations. P250S does not occur naturally in E3 either but does differ in an E3 ortholog associated with OP resistance in houseflies, where it occurs together with W251L (Claudianos, 1999; Claudianos et al., 1999).

Heidari et al. (2005) found no effect of the P250S on Type 1 SP activities but the mutations of W251 and F309 both substantially enhanced Type 1 SP hydrolytic activity and drastically altered the 1*S trans* chiral preference around the two optical centres in the acyl group of these compounds. Of the five changes to W251, W251L showed the greatest enhancement of activity and W251G and W251T the least. However all five greatly reduced the *trans* over *cis* preference, whilst somewhat enhancing the 1*S* over 1*R* preference. F309L, like W251L, greatly improved activity overall but it left the *trans*/*cis* preference unaltered while dramatically reversing the 1*S*/1*R* preference. These effects were rationalised in terms of the provision of extra room at parts of the pocket that enable better accommodation of particular acyl group geometries in the substrate.

In line with the pattern in Type 1 activities, we find that four of the five W251 mutations enhance hydrolysis of most of the Type 2 analogs, with W251L having the biggest effect, but with W251G not higher overall than wild-type. The enhancement is most marked for the fenvalerate series, where all four isomers are hydrolysed at least a hundred fold faster than the very low activities of wild-type enzyme. Substantial enhancements are also seen with several of the αS cypermethrin analogs but only rarely with those of αR cypermethrin. It is notable that the W251L change was again clearly superior to the other W251 mutants, which all involve even smaller amino acids, with two of them polar (Ser and Thr) and two of them neutral (Gly and Ala). This suggests that maintenance of hydrophobicity in an aliphatic side chain, but high flexibility compared to the aromatic tryptophan in the wild-type enzyme, could be optimal for hydrolysis of a wide range of SP structures.

With respect to cypermethrin, W251L also has the effect of reducing the *trans* over *cis* preference in the acyl group of the analogs, as it does for the Type 1 insecticides, albeit this effect is not consistent across the other four changes to W251. All the W251 mutants also reduce the 1*S* over 1*R* preference, whereas among Type 1 SPs they slightly exaggerate it (Heidari et al., 2005). Whilst still significant, the W251 changes therefore show somewhat less effect overall on acyl group isomer specificity among Type 2 analogs than they do among Type 1 SPs.

Importantly, the W251 changes, particularly W251L, also show consistent preferences for αS over αR cyano in the alcohol group of the cypermethrin analogs. This concurs with the limited data that Heidari et al. (2005) were able to obtain with ^{14}C labelled isomers of the other Type 2 SP, deltamethrin. Heidari et al. (2005) were unable to compare the deltamethrin isomer specificities of the W251 changes with those of wild-type E3 because the deltamethrin activities of the latter were too low for their radiometric assay. Our data now show that the αS

preference of the W251 mutants is indeed greater than wild-type. Notably this preference implies an effect of changes to the presumptive acyl pocket on the accommodation of the substrate leaving (alcohol) group. It is difficult to interpret this relayed effect in precise structural terms in the absence of an empirical structure for E3 but it is likely to reflect the tight constraints on the accommodation of SP substrates in the active site because of their bulk and the very limited rotational flexibility around the carbonyl group (Mullaley and Taylor, 1994), especially once anchored by the catalytic serine and oxyanion hole.

Overall, the enhancements among the cypermethrin analogs are greater with the more insecticidal conformations at each chiral centre. The turnover for the analog of the most insecticidal 1*R cis* α*S* isomer of cypermethrin by W251L is 8 pmol/min/pmol, 30 times that of the corresponding wild-type enzyme.

Although the acyl group of fenvalerate lacks the cyclopropane structure that generates the two acyl group optical centres of other SPs, it does have an acyl group optical centre and there is spatial correspondence between the isomers it generates and two of the configurations from the isomerism across the cyclopropane ring of the other SPs. In particular, for the most insecticidal isomers, the *gem*-dimethyl groups on the 1*R* cyclopropane lie in a similar spatial location to the dimethyl group in the substituted valeric acid of 2*S* fenvalerate, as do the terminal halogen groups; thus the 2*R* and 2*S* fenvalerate configurations respectively resemble the 1*S* and 1*R* arrangements of the cyclopropanecarboxylate SPs (Fig. 4 and see also Aketa et al., 1978; Chamberlain et al., 1998; Huang et al., 2005a).

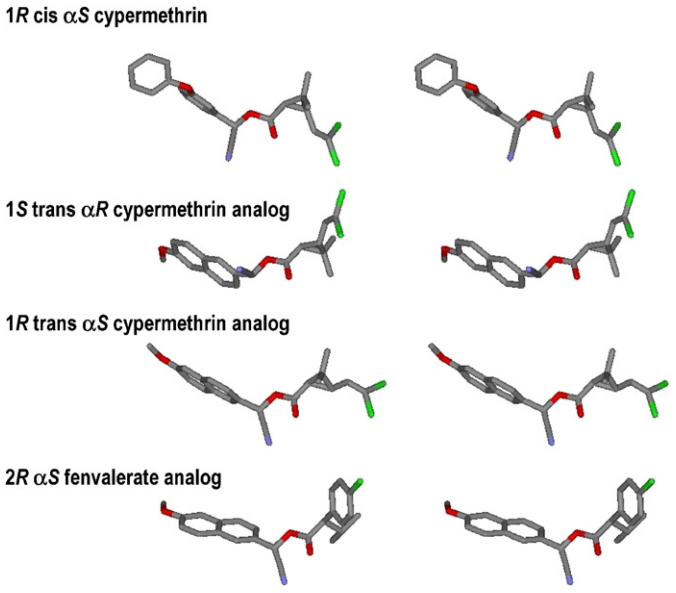

Fig. 4. Stereoview (crossed eyes) of 1*R cis* α*S* cypermethrin (Baert and Guelzim, 1991; Cambridge database ref. SISYUO) corresponding to the single active isomer in deltamethrin, and those analogs of cypermethrin and fenvalerate for which crystal structures have been determined. The structures were aligned manually so that the ester bonds were all in the same plane with the carbonyl oxygen orientated forwards.

Interestingly, there is little discrimination between the activities of the W251 mutants for the two acyl group isomers of fenvalerate, whereas these mutants had shown reduced but still significant preference for 1*S* over 1*R* arrangements around the cyclopropane rings of the other SPs. The difference presumably reflects the structural distinctiveness of the isovalerate and cyclopropanecarboxylate in the two classes of compounds.

The W251 mutants show the same clear preference for the α*S* over α*R* cyano conformation in the fenvalerate analogs as they do among those of cypermethrin. We cannot determine whether the difference is more marked than with wild-type in this case because wild-type levels with the fenvalerate analogs are simply too low for accurate comparisons.

The highest activity recorded for any mutant with any fenvalerate analog is the activity of 14 pmol/min/pmol esterase achieved by W251L for the most insecticidal 2*S* α*S* fenvalerate isomer, marketed as esfenvalerate (Chamberlain et al., 1998). Thus W251L is at least one (cypermethrin) or two (fenvalerate) orders of magnitude more active than wild-type E3 against the most insecticidal isomers of both the Type 2 SPs investigated here.

As noted earlier, Heidari et al. (2005) found that the F309L mutation enhanced E3's activities against all Type 1 SP isomers, but particularly the 1*R* isomers, leading to a reversal of the otherwise strong 1*S* over 1*R* preference. We find that F309 activity with all the fenvalerate analogs remains very low, as with E3 WT, and in the case of cypermethrin there is generally a marked decrease in activity, to negligible levels in the case of all the *cis* isomers. The only exception is the analog of 1*R trans* α*S* cypermethrin where there is a 12-fold increase in activity. Thus, in the *trans* α*S* context at least, the reversal of 1*S* over 1*R* preference seen in the Type 1 SPs carries over to the Type 2 analogs as well. A double F309L/W251L mutant also shows the reversal of 1*S* over 1*R* preference in this *trans* α*S* cypermethrin context. The overall conclusion from the F309L data is that the presence of the α-cyano group in the Type 2 substrates so constrains their accommodation in the active site that the benefits the mutation provides in accommodating 1*R* isomers of Type 1 compounds only translate across to certain cypermethrin analogs among the Type 2 isomers tested.

The P250S change has only been examined as a double mutant in combination with W251L (Claudianos, 1999). Heidari et al. (2005) found no major effect of P250S on Type 1 activities. We find that this double mutant generally gives activities for the Type 2 analogs intermediate between the corresponding values for E3 WT and W251L alone and we conclude that P250S has no major effect on Type 2 activities either.

3.4. SP analog activities; leaving group pocket mutations

Heidari et al. (2005) analysed the effects on Type 1 SP activities of mutations at three presumptive leaving group

pocket residues of E3 which modelling and empirical mutational analysis in the related acetylcholinesterase (AChE) enzyme suggest could affect interactions with the substrate leaving group via non-polar and π electron interactions. One mutation, E217M, had no detectable effect but the other two, Y148F and F354W, generally enhanced overall SP hydrolase activity. None of the mutations had major effects on acyl group chiral preferences.

We generally see a slight preference for the αS cyano leaving group arrangement in these mutants wherever there was sufficient activity for meaningful comparisons. In contrast to their behaviours with the Type 1 SPs, activities with F354W were if anything less than those of E3 WT while E217M generally gave a small but significant increase in activity. Like Heidari et al. (2005) however, we see no major change in acyl group chiral preferences.

We conclude that the leaving group pocket mutations have little effect on the small preference for the αS conformation of the leaving group cyano moiety seen in most of the variants to date. E217M accommodates this moiety more readily than do the other leaving group pocket mutations, or E3 WT, but this has no major impact on its preferences among acyl group conformations.

The results for the E217M substitution are also noteworthy because methionine is found naturally at the corresponding residue in the E4 esterase of the aphid *Myzus persicae*; overexpression of E4 confers OP resistance by a sequestration mechanism (Field et al., 1993). E217 is otherwise very strongly conserved throughout the α/β hydrolase fold superfamily and it is believed to function in the coordination of water molecules (Radic et al., 1992; Schrag and Cygler, 1993; Koellner et al., 2000). Our data support the evidence of Heidari et al. (2004, 2005) that E217M in E3 at least is permissive of activity against a wide range of carboxylester substrates.

3.5. SP analog activities; diastereomers versus enantiomers

As another perspective on the relationship between the location of mutations and the stereochemistry of the SP analogs, the activity of each enzyme towards its most readily hydrolysed isomer was compared with that against the corresponding enantiomer (i.e., differing in both its $\alpha R/S$ leaving group and $1R/S$ acyl group chirality for a given *cis*/*trans* acyl group geometry) and the diastereomer of each (i.e., one retaining the acyl and the other the alcohol conformation of the favoured isomer). This was done for each of the fenvalerate, *trans* cypermethrin and *cis* cypermethrin series (Table 1).

With E3 WT (Table 1, top panel), the diastereomer having the same acyl group arrangement as the favoured isomer in each series was also hydrolysed at a significant rate but, with one notable exception, there was little or no activity towards the other two isomers. The exception was for the *cis* cypermethrin analogs where the enantiomer of the preferred form was hydrolysed more readily than either diastereomer.

Mutations at G137 in the leaving group pocket (Table 1, second panel) virtually abolished activity for all the *cis* cypermethrin analogs, so between-isomer comparisons were not possible. For those of fenvalerate and *trans* cypermethrin, the diastereomer with the same acid as the preferred conformation was again hydrolysed at a significant rate, with little activity surviving for either other isomer.

By contrast, for the acyl pocket mutations (Table 1, third panel) it was often the diastereomer with the same alcohol as the preferred isomer that yielded the next best activities. This was true for most of the W251 replacements in the fenvalerate and *cis* cypermethrin series, although the two diastereomers of the preferred *trans* cypermethrin form gave more equivalent activities. It was also true in all cases for F309L, whether alone or in combination with W251L, and for two of the series with P250S/W251L, the exception being for *trans* cypermethrin analogs where the same acid diastereomer of the preferred isomer was next most readily hydrolysed.

The Y148F, F354W and E217M mutations in the leaving group pocket (Table 1, fourth panel) all showed remarkably similar patterns to E3 WT. Thus the fenvalerate and *trans* cypermethrin results showed the same strong preference for the diastereomer with the same acid conformation as the most preferred form while the *cis* cypermethrin data were again exceptional in showing the enantiomer was hydrolysed more readily than either diastereomer of the preferred analog.

These observations indicate another aspect to the difference between the acyl pocket mutations and most of the other enzymes tested in their SP activities. We have already seen that some of the acyl pocket changes can substantially enhance these activities and drastically alter chiral preferences. We now see that whereas most of the other enzymes show a greater tolerance for altered alcohol than altered acid chirality, the acyl pocket mutants show more tolerance for altered acid than altered alcohol chirality. The behaviour of these mutations, which all involve substitutions to smaller residues in the acyl pocket, suggest that the greater space generated in the acyl pocket enables the accommodation of the large cyclopropanecarboxylate regardless of its conformation. The chirality of the alcohol group then becomes limiting to their activity.

3.6. Activities for the acetate and butyrate analogs

To complement the analysis of the Type 2 SP analogs we also assayed the various enzymes against the corresponding acetates and butyrates of the resolved alcohols. The goal of this was to understand the effect of the α-cyano conformation in the alcohol group on enzyme activity when not influenced by the bulkiness and chirality of the acids (Fig. 2).

In contrast to the behaviour of many of the enzymes with the insecticide analogs, all the enzymes tested had readily detectable levels of activity with the acetates and

Table 1
Activities of each E3 variant for its preferred isomer (in pmol/min/pmol) within each of the *cis* cypermethrin, *trans* cypermethrin and fenvalerate series, together with its relative activity (% of activity for preferred isomer) for the corresponding two diastereomers (same (i.e. =) acid, different (i.e. Δ) alcohol and different acid, same alcohol conformers respectively) and enantiomer (different acid and alcohol conformer)

	Cis cypermethrin				*Trans* cypermethrin				Fenvalerate			
	Isomer	= acidΔalc	Δacid = alc	ΔacidΔalc	Isomer	= acidΔalc	Δacid = alc	ΔacidΔalc	Isomer	= acidΔalc	Δacid = alc	ΔacidΔalc
E3WT	1*S*α*S*				1*S*α*S*				2*R*α*S*			
	1.81	40	15	62	11.29	94	7	4	0.15	97	14	10
G137D					1*S*α*R*				2*R*α*R*			
	–	–	–	–	0.15	56	0	0	0.18	45	5	8
G137H					1*S*α*R*				2*R*α*R*			
	–	–	–	–	0.13	38	0	0	0.22	39	5	6
G137R					1*S*α*S*				2*R*α*R*			
	–	–	–	–	1.01	61	0	0	0.97	45	5	14
W251L	1*R*α*S*				1*S*α*S*				2*S*α*S*			
	9.14	31	70	13	20.67	44	32	3	14.11	22	79	14
W251G					1*S*α*S*				2*R*α*S*			
	–	–	–	–	3.11	8	12	1	0.63	27	26	3
W251A	1*S*α*S*				1*S*α*S*				2*R*α*S*			
	1.41	16	81	54	13.00	17	12	3	7.00	9	59	21
W251S	1*R*α*S*				1*S*α*S*				2*R*α*S*			
	0.61	11	34	1	10.72	8	16	2	3.49	20	31	5
W251T	1*R*α*S*				1*S*α*S*				2*R*α*S*			
	0.28	73	72	0	13.02	4	21	0	3.79	19	41	5
F309L					1*R*α*S*				2*R*α*R*			
	–	–	–	–	8.84	1	58	26	0.18	17	31	43
F309L/W251L	1*R*α*S*				1*R*α*S*				2*S*α*S*			
	0.92	22	32	7	2.72	10	60	21	1.29	12	26	13
P250S/W251L	1*R*α*S*				1*S*α*S*				2*S*α*S*			
	1.80	52	94	16	9.68	56	23	4	4.42	22	68	14
Y148F	1*S*α*S*				1*S*α*S*				2*R*α*R*			
	1.07	22	24	63	11.13	73	7	3	0.16	78	4	14
F354W	1*S*α*S*				1*S*α*S*				2*R*α*R*			
	0.93	0	0	53	5.62	17	3	2	0.11	69	0	0
E217M	1*S*α*S*				1*S*α*S*				2*R*α*S*			
	3.24	28	58	76	13.49	38	6	4	0.82	37	16	7
Y148F/G137D									2*R*α*R*			
	–	–	–	–	–	–	–	–	0.11	48	1	0

– indicates that preferred isomer activities were less than 0.05 pmol/min/pmol.

butyrates. Moreover activity towards these smaller substrates was always much greater (up to 2–3 orders of magnitude) than for the respective insecticide analogs, confirming that spatial constraints to substrate access were limiting for the SP analog substrates.

The E3 WT enzyme was unique in showing a preference for the α*S* cyano (but not the α*R*) acetate esters over both butyrates. By contrast, the mutants typically had a 3-fold preference for each of the larger butyrates over its corresponding acetate. Why this should change given the wide range of substitutions assayed is not clear, but the relative activities for the mutants are more in line with the general preference of carboxylesterases for the longer, more lipophilic substrates (Aldridge and Reiner, 1972).

All enzymes showed a preference for α*S* over α*R* cyano conformation in the acetate and butyrate series, as they had done in the insecticide analog series. The differences were generally smaller with the acetates and butyrates (always less than 2-fold) than with the analogs (typically greater than 2-fold). Qualitatively, however, we conclude that this preference for α*S* in the alcohol group survives in combinations with a wide range of acyl groups.

Enzymes with any of the three mutations of G137 in the oxyanion hole retained substantial activity against all four compounds in the acetate and butyrate series. This was not unexpected in the case of G137H and G137R, which we have previously shown retained activities broadly comparable to E3 WT with conventional carboxylester substrates like naphthyl acetate (Heidari et al., 2004, 2005; plus the data for the SP analogs earlier herein). However G137D is the naturally occurring 'mutant aliesterase' mutation (van Asperen and Oppenoorth, 1959) which confers OP hydrolase activity on the enzyme and OP resistance on the insect bearing it. This is achieved at the expense of much of its

carboxylesterase activity as measured with substrates like naphthyl acetate (Campbell et al., 1998; Devonshire et al., 2003) and, as we have seen earlier herein, with the fluorogenic SP analogs as well. This disparity may be due to our assaying the enzymes with a single, relatively low, fluorogenic substrate concentration; Devonshire et al. (2003) found that the effects of G137D on carboxylesterase activities with other substrates were highly dependent on substrate concentration.

Activities of the five W251 series mutants in the acyl pocket were all low compared to E3 WT with the simple fluorogenic substrates, which compares well with their behaviour against naphthyl acetate (Campbell et al., 1998; Devonshire et al., 2003), and contrasts with their elevated activities with SPs (Heidari et al., 2005) and fluorogenic SP analogs (herein). It appears that, while the additional space in the acyl pockets of the W251 series mutants is advantageous to the docking of substrates with relatively very large acyl groups like the SPs and their analogs, it provides too much room for productive docking of substrates with much smaller acyl groups like acetate or butyrate.

Changes in the putative leaving group pocket generally had little systematic effect, except for E217M, which reduced activity with all isomers.

Overall, analysis of these smaller esters showed that the much more marked activity differences seen with the insecticidal analogs are a consequence of their size and more complex geometry conferred by multiple sites of chirality in the diastereomers.

4. Discussion

Fig. 5 is a semi-quantitative summary of the activity and isomer preference data obtained by Heidari et al. (2005) and this study for E3WT and the mutations of major effect against the Type 1 and Type SPs. As was evident among the Type 1 SPs (Heidari et al., 2005), E3 shows strong chiral preferences among acyl group arrangements in the analog of the Type 2 SP cypermethrin. Again E3 and most mutants (other than the W251 series and F309L) show a marked preference for *trans* over *cis* and 1*S* over 1*R* conformations within the cyclopropane moiety. Our data also now show that this strong acyl group preference is

	WT	W251L	F309L
Heidari et al. (2005)			
Permethrin (Type 1, 1RS dichloro)			
activity	moderate - high	high - very high	moderate - very high
cis vs *trans* preference	strong *trans*	clear *trans*	strong *trans*
NRDC157 (Type 1, cis dibromo)			
activity	moderate	moderate - high	high
1R vs 1S preference	clear 1S	strong 1S	weak 1R
Deltamethrin (Type 2, cis dibromo)			
activity	ND	ND - low	low
1R vs 1S preference	-	{weak 1S and αR}	no 1RS
αR vs αS preference	-		weak αR
This study			
Cypermethrin analogs (Type 2, dichloro)			
activity	very low - moderate	low - moderate	ND - moderate
1R vs 1S preference	{clear - strong 1S *trans*}	{weak - clear 1S *trans*}	variable 1R
cis vs *trans* preference			weak - strong *trans*
αR vs αS preference	no αRS	variable αRS	weak - strong αS
Fenvalerate analogs (Type 2, chloro)			
activity	ND - very low	low - moderate	ND - very low
2R vs 2S preference	-	weak 2S	-
αR vs αS preference	-	clear αS	-

Fig. 5. Summary of activities and chiral preferences of wild-type E3 enzyme and two acyl pocket mutant E3 enzymes for various radiolabelled pyrethroids from Heidari et al. (2005) and for the fluorogenic pyrethroid analogs in this study. For the Heidari et al. (2005) study: very high, high, moderate and low activities mean $\sim 10^5$, 10^4, 10^3 and 10^2 mol substrate hydrolysed/s/$[S]$/$[E_0]$, respectively. For this study: moderate, low and very low mean ~10, 1 and 0.1 pmol substrate hydrolysed/min/pmol enzyme, respectively. ND = not detectable in both studies. For both studies: strong, clear and weak chiral preferences mean >10-fold, 2– ⩽10-fold and significant but <2-fold differences in activities, respectively. Stated chiral preferences need to be interpreted with caution where both activities are low. The analogs differ from the SPs in having 6-methoxy-2-naphthyl instead of 3-phenoxyphenyl structures in their leaving groups (see Fig. 1). Fenvalerate differs from the other substrates in having isovalerate instead of cyclopropane carboxylate structures in their acyl groups (see Fig. 1).

little affected by the chirality of the α-cyano group in the Type 2 leaving group, for which the enzyme shows only a small but consistent preference for the α*S* arrangement.

Thus, even though addition of the α-cyano to generate the Type 2 leaving groups drastically reduces activity across all mutants tested (Heidari et al., 2005), it remains true for E3 WT and most mutants that conformational variation around the cyclopropane moiety found in the acyl groups of most SPs is the primary determinant of their relative rates of hydrolysis. Interestingly, this latter limitation imposed by the acyl group conformation was evident between the two isomers in analogs of the alternate substituted valeric acid found in the other Type 2 analog we tested, fenvalerate; in this case hydrolytic rates with E3 and mutants except those in the acyl pocket were very low for both arrangements.

The fact that most enzymes other than the acyl pocket mutants give lower activities with the fenvalerate than the cypermethrin analogs is also informative in mechanistic terms. One might expect that the chrysanthemic acid esters such as cypermethrin (and deltamethrin and permethrin) would in fact be more stable to chemical and enzymatic hydrolysis than the fenvalerate derivatives because the aromatic character of the cyclopropane ring places the olefin, cyclopropane and carbonyl of the chrysanthemic acid esters into conjugation. The formation of a tetrahedral transient intermediate or transition state during base hydrolysis of these esters would have to force the carbonyl out of conjugation. Since the cypermethrin analogs are actually better hydrolysed than the fenvalerate analogs by most enzymes outside the acyl pocket series, one concludes that steric properties are more important than electronic ones in determining the rates of these hydrolyses.

Given the limitations on hydrolysis due to acyl group arrangement, it is not surprising that the mutations that we and Heidari et al. (2005) find have greatest effects on hydrolysis are in the acyl pocket. These mutations comprise all five changes made to W251 and the only one tested for F309. All the changes are to smaller residues, which we consider to make the acyl pocket larger and therefore more accommodating for the bulky acyl groups of the SPs and their analogs. However, the W251 and F309 are predicted to sit on different sides of the pocket, so it is also not surprising to find that some of the effects of the W251 series and F309L on chiral preferences are quite distinct.

The W251 mutants had generally enhanced hydrolysis of all isomers among the Type 1 SPs but the degree of enhancement was very different across isomers, so that the *trans* over *cis* preference was drastically reduced and the 1*S* over 1*R* preference slightly exaggerated (Heidari et al., 2005). We also see here clear enhancements of activity for the fluorogenic analogs of fenvalerate and cypermethrin isomers with the favoured α*S* leaving group conformation, albeit with little consistent difference for the α*R* isomers. There is also little chiral preference among the 2*S*/2*R* acyl group isomers of fenvalerate and, although still significant, less preference among acyl group isomers of cypermethrin analogs than there was among the Type 1 SPs. Specifically the reduction in *trans*/*cis* preference is only evident for W251L, and the 1*R* over 1*S* preference is retained in all five mutants but reduced in magnitude rather than increased as was the case for the Type 1 SPs. Thus some of the effects are less marked than for the Type 1s but the general view of Heidari et al. (2005), that the greater acyl pocket space created by the W251 series mutants permits more productive accommodation of most SP conformers, remains valid for the Type 2 analogs tested here. For the Type 2 compounds this general effect of the W251 mutants to lessen the limitation on hydrolysis due to acyl group conformation leaves the α*R*/α*S* difference in the leaving group as a larger determinant of activity.

F309L had enhanced activity for all the Type 1 SPs Heidari et al. (2005) tested, but particularly the 1*R* conformers, which led to a reversal of the 1*S* over 1*R* preference seen with all other variants. However F309L is generally detrimental to the analogs of both fenvalerate and cypermethrin, the only exception being 1*R* *trans* α*S* cypermethrin where there is a clear enhancing effect. In this case at least there is sufficient activity to see the same preference for 1*R* over 1*S* as was evident in the Type 1s. Otherwise however it appears that the depressing effect on activity by the cyano group on Type 2 leaving groups is not overcome by greater space in the acyl pocket due to F309L, even though this mutation had enhanced activities in the Type 1 compounds lacking the cyano group.

If we exclude all the W251 series and F309L mutants, some of our major findings bear striking similarities with those from an analysis of the chiral preferences of five wild-type mammalian liver carboxylesterases among the same Type 2 analogs (Huang et al., 2005b). They also found a small preference for α*S* rather than α*R* leaving group arrangements, with the chiral centres in the acyl groups having much larger effects on susceptibility to hydrolysis and a strong preference for *trans* geometry in the cyclopropane group of the cypermethrin analogs. There were variable effects of the 1*R*/1*S* difference, depending on the other chiral centres, but, as in the present study, they found the 1*S* *trans* α*S* isomer to be the most easily hydrolysed cypermethrin analog. Like us, they found generally lower levels of hydrolysis of the fenvalerate analogs. Perhaps the only substantive difference between the two studies is that the mammalian enzymes showed a general preference for 2*R* over 2*S* acyl groups among the fenvalerate analogs, whereas we found no consistent difference between these forms.

Although homogenates from *Heliothis* species were assayed during the development of these fluorescent SP analogs (Shan and Hammock, 2001), no other insect esterases have been analysed using the panel of substrates we and Huang et al. (2005b) have used, but Devonshire and Moores (1982) have shown that esterase E4 of *Myzus persicae* also has the 1*S* *trans* preference across the cyclopropane ring of Type 1 SPs. Although the patterns

are thus equivocal or inconsistent for the leaving group cyano configuration of Type 2 SPs and the acyl group arrangements of fenvalerate, the available data do suggest some generality across wild-type carboxylesterases in their preferences for acyl group conformation in cyclopropanecarboxylate SPs.

The question then is whether such differences relate to the relative potencies of the various isomers as insecticides. Although their potencies must depend primarily on their interactions with their sodium channel target sites, there has also been speculation that they are likely to be affected to some degree by rates of hydrolytic and oxidative metabolism (Elliott and Janes, 1978; Chamberlain et al., 1998 and references therein). In respect of acyl group configurations, the most potent isomers of cypermethrin and most other cyclopropanecarboxylate SPs (be they Type 1 or Type 2) have 1*R* and *cis* arrangements (or the conformationally equivalent 2*S* in the case of fenvalerate). Indeed Huang et al. (2005b), Heidari et al. (2005) and the current study all find these to be the acyl group arrangements least readily hydrolysed by wild-type carboxylesterases. Thus it seems that sensitivity to esteratic cleavage may contribute to relative potencies among acyl group isomers of Type 1 and Type 2 cyclopropanecarboxylate SPs.

The altered activity spectra and chiral preferences of the W251 and F309 mutants deserve further consideration in this context. Heidari et al. (2005) had shown that these mutants generally enhance activity against Type1 SPs but particularly enhance the activities for *cis* and 1*R* conformers of Type 1 SPs respectively. We find that these mutants do not enhance activities for the analogs of Type 2 SPs universally, although the same chiral preferences are often evident and W251L, in particular, enhances activities for all acyl group isomers esterified with the more potent α*S* cyano conformation. Moreover this (and the other W251 mutants tested) dramatically enhances activities for all isomers of the fenvalerate analogs. Whilst it must be acknowledged that our data are based on surrogate substrates rather than the commercial SPs, it is nevertheless tempting to suggest that the naturally occurring W251L mutant in particular could contribute to metabolic resistance to a wide range of insecticides.

About 30 cases of esterase-mediated metabolic resistance to SPs have been described across a range of insect species although none has yet been elucidated at a molecular level (Oakeshott et al., 2005 and references therein). Some cases may be due to regulatory or copy number mutations increasing enzyme amount but there is also evidence that some are due to amino acid changes affecting enzyme structure. SP resistance has not been investigated in *L. cuprina* because SPs are not registered for use against this species but substitutions of W251 to L or other small amino acids are not uncommon in E3 in pesticide-naïve *L. cuprina*; residue 251 was polymorphic for Leu, Thr and probably Ser in E3 in *L. cuprina* populations in the 1930s and 1940s, even before the first use of OP or SP insecticides (Hartley et al., 2006). Similarly, small amino acids (Leu, Ser, Ala, Gly, Ile or Val) occupy this site in 11 out of 104 presumptively catalytically active insect carboxyl/cholinesterases in the comparative genomic studies of Claudianos et al. (2006). Another 17 of these 104 sequences have small amino acids (Leu, Ser or Thr) at residue 309. Given that the insect genomes sequenced to date each have about 4–20 carboxylesterases with potential dietary or detoxification functions (Claudianos et al., 2006), it will be interesting to see whether any of these changes are found at the corresponding sites in carboxylesterases implicated in SP tolerance or resistance in other species.

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