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Isomer-specific comparisons of the hydrolysis of synthetic pyrethroids and their fluorogenic analogues by esterases from the cotton bollworm *Helicoverpa armigera*



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ABSTRACT

The low aqueous solubility and chiral complexity of synthetic pyrethroids, together with large differences between isomers in their insecticidal potency, have hindered the development of meaningful assays of their metabolism and metabolic resistance to them. To overcome these problems, Shan and Hammock (2001) [7] therefore developed fluorogenic and more water-soluble analogues of all the individual isomers of the commonly used Type 2 pyrethroids, cypermethrin and fenvalerate. The analogues have now been used in several studies of esterase-based metabolism and metabolic resistance. Here we test the validity of these analogues by quantitatively comparing their hydrolysis by a battery of 22 heterologously expressed insect esterases with the hydrolysis of the corresponding pyrethroid isomers by these esterases in an HPLC assay recently developed by Teese et al. (2013) [14]. We find a strong, albeit not complete, correlation ($r = 0.7$) between rates for the two sets of substrates. The three most potent isomers tested were all relatively slowly degraded in both sets of data but three esterases previously associated with pyrethroid resistance in *Helicoverpa armigera* did not show higher activities for these isomers than did allelic enzymes derived from susceptible *H. armigera*. Given their amenability to continuous assays at low substrate concentrations in microplate format, and ready detection of product, we endorse the ongoing utility of the analogues in many metabolic studies of pyrethroids.

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1. Introduction

Notwithstanding their ongoing importance in pest control, many aspects of the biochemistry underlying insects' metabolism of synthetic pyrethroids (SPs) and its relationship to SP resistance remain poorly understood. Much of this is because of the technical difficulty in working with these molecules, which have very low aqueous solubility (nM) and high chiral complexity (up to three optical centres in many major products). Isomer-specific degradation assays remain technically challenging and even now there are relatively few data on the relative potencies of different isomers and their susceptibility to degradation, despite the evidence from such data as are

available suggesting qualitatively different potencies and degradation rates among isomers [1–6].

In an attempt to redress the problems, Shan and Hammock [7] and Huang et al. [8] prepared a full set of optically pure isomers of fluorogenic analogues of the Type 2 SPs (i.e. those containing an α -cyano moiety on their phenoxybenzyl alcohol group [9]), cypermethrin and fenvalerate. The α -cyano-phenoxybenzyl alcohol group of these SPs is replaced in the analogues with an α -cyano-methoxynaphthalen-2-yl group (Fig. 1). Hydrolysis of the analogues releases a cyanohydrin that spontaneously converts to a fluorescent aldehyde. The fluorogenic and water-soluble (high μ M) nature of the analogues mean that assays with useful dynamic ranges can be performed on extracts and isolated enzymes to address important issues relating to metabolism, toxicity and resistance, particularly as they relate to esteratic degradation. Assays with isolated mammalian liver esterases [8,10,11] and an insect esterase [12,13] showed biologically relevant levels of turnover of all of the analogues, with

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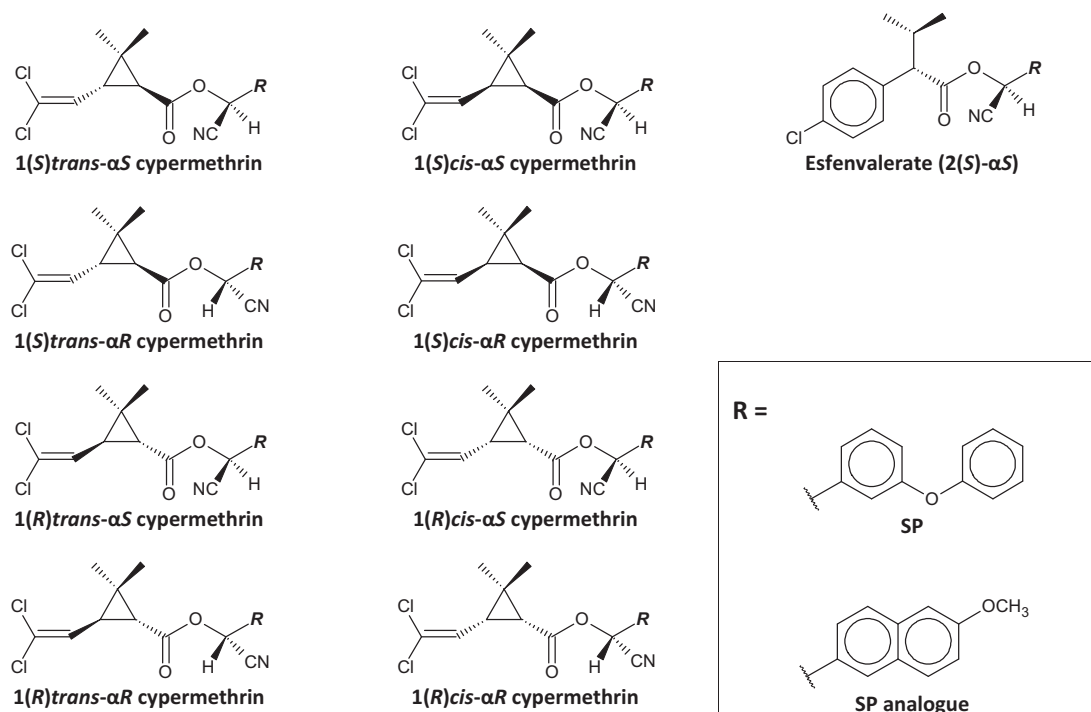


Fig. 1. Structures of the SP and SP analogue substrates analysed in this study.

an interesting but not invariant trend for lower turnover rates for analogues of the particular isomers which the available evidence suggests are most potent as insecticides. These data suggest that the analogues may indeed be useful surrogates for the real SP isomers in various metabolism and resistance studies.

Recently, Teese et al. [14] have also published isomer-specific assay procedures for the esteratic degradation of the SPs themselves. Although the dynamic range of their assay is still limiting and strict kinetic analyses are therefore not yet possible, it does now enable a direct comparison of the esteratic degradation of the SPs and their analogues, providing a more comprehensive test of the biological relevance of the analogues. This paper presents such a comparison for the eight isomers of cypermethrin and the widely used 2(S)-α(S) isomer of fenvalerate, all of which have been assayed against a panel of 22 heterologously expressed esterases from the cotton bollworm *Helicoverpa armigera*.

2. Materials and methods

Eight of the *H. armigera* esterases used here were derived from the Australian GR strain, which is largely susceptible to SPs [14]. All eight were from esterase Clade 1 and several of them have been shown to be overexpressed in SP-resistant strains [14,15]. Amino acid identities among the eight esterases range from 53 to 86%. A further five esterases were synthetic mutants of five of the GR esterases above into which a mutation had been introduced which is associated with increased activity for particular isomers of Type 1 SPs (which lack the α-cyano group) in another insect esterase (the Trp251Leu mutation of the E3 enzyme from the sheep blowfly *Lucilia cuprina* [16,17]). The last nine esterases were naturally occurring allelic variants of five of the GR esterases from Chinese populations, four of them from the largely SP-susceptible YG strain and five from the highly resistant YGF strain (~1700 fold resistant) selected from YG [15]. These nine esterase alleles were sequenced by Sanger sequencing at Micromon (Australia) and their Genbank ac-

cession numbers and an alignment of their amino acid differences are given in Suppl. Fig. S1.

All 22 of the esterases were expressed in Sf9 insect cells using the baculovirus expression system; the expression of the first 13 is described in Teese et al. [14] and Li et al. [17] and that of the last nine also follows their procedures.

The expressed esterases were titrated with diethyl 4-methylumbelliferyl phosphate (dEUP) following the methods of Coppin et al. [13] using the burst calculation described in Li et al. [17]. They were then assayed for the hydrolysis of the eight isomers of cypermethrin and 2(S)-α(S) fenvalerate following the High Pressure Liquid Chromatography (HPLC) methods of Teese et al. [14] and Li et al. [17] – the data for the first 13 esterases have already been tabled in those papers. Whilst we note that the SP concentration in these assays (100 μM) is orders of magnitude above the published aqueous solubility limits for the SPs, we find that enzyme activity is not limited by substrate solubility under these conditions – increases and decreases in substrate concentration result in corresponding Michaelis–Menten-like changes in enzyme activity (C.W. Coppin, A.L. Devonshire and J.G. Oakeshott, unpublished results). Thus it appears that the rate of SP solubilisation during the reaction does not limit the rate of the reaction. The fluorometric methods of Coppin et al. [13] were then used to assay the 22 esterases with the fluorogenic analogues of the eight cypermethrin isomers and the 2(S)-α(S) isomer of fenvalerate.

3. Results and discussion

Activities were found to be higher for the real SPs than for the analogues (Table 1, Suppl. Fig. S2) – for example, mean activities across all enzymes for the two least-readily degraded cypermethrin isomers (1(S)cis-α(S) and 1(R)trans-α(S)) and their analogues were in the ranges 2–4 and 0.2–0.5 min⁻¹, respectively whilst for the most readily degraded pair (1(S)trans-α(R) and -α(S)) they were in the ranges 30–33 and 10–11 min⁻¹, respectively. However, bearing in

Table 1
Means and standard errors of the activities of the 22 *H. armigera* esterases towards SPs and their analogues.

	SP ^a		SP analogue ^b	
Cypermethrin isomers				
1(S) <i>trans</i> - α (S)	33.03	(5.55)	11.36	(3.64)
1(S) <i>trans</i> - α (R)	30.26	(5.22)	9.68	(3.08)
1(R) <i>trans</i> - α (S)	3.95	(0.99)	0.50	(0.16)
1(R) <i>trans</i> - α (R)	5.27	(0.81)	0.36	(0.12)
1(S) <i>cis</i> - α (S)	2.25	(0.60)	0.15	(0.07)
1(S) <i>cis</i> - α (R)	4.85	(0.81)	0.17	(0.08)
1(R) <i>cis</i> - α (S)	5.31	(0.89)	0.04	(0.01)
1(R) <i>cis</i> - α (R)	6.54	(1.22)	0.06	(0.02)
Esfenvalerate				
2(S)- α (S)	2.09	(0.86)	1.00	(0.32)

^a Specific activity and SE (brackets) (min^{-1}) at 100 μM substrate.

^b Specific activity and SE (brackets) (min^{-1}) at 10 μM substrate.

mind the 10-fold higher substrate concentrations used in the SP assays (100 μM compared to 10 μM for the analogues), and with no knowledge of the relative K_m values, any precise inferences are impossible. Nevertheless, there is a good correlation overall between the activities for the SPs and their analogues (e.g. $r_{196} = 0.70$ in the total data across all enzymes and isomers).

Like us, Huang et al. [10] found that the mammalian liver esterase they tested showed a preference for the (1(S)*trans*- α (R) and - α (S) isomer pair among the eight analogues of the cypermethrin isomers, and we have reported previously the same preference for the major variant of the only other insect esterase tested thus far (the E3 enzyme of the sheep blowfly *L. cuprina*), both among the cypermethrin isomers [17] and among their analogues [13]. However we did find one naturally occurring variant of E3 (bearing the Trp251Leu mutation mentioned above) with a preference for several cypermethrin and fenvalerate isomer(s)/analogue(s) that the major variant had not preferred [13,17]. A preference for the 1(S)*trans*- α (R) and - α (S) isomer pair may thus be a common but not invariant property of eukaryote esterases, or at least the great majority of them that lie in the carboxyl/cholinesterase gene family [13].

The three cypermethrin and fenvalerate isomers which show the highest insecticidal potencies against a variety of insects, including the closely related *Heliothis virescens* (1(R)*cis*- α (S) and 1(R)*trans*- α (S) cypermethrin and 2(S)- α (S) fenvalerate, the latter being the steric equivalent of the other two), are all relatively slowly degraded by the

H. armigera esterases, both as SPs and as their analogues (Table 1, Fig. 2). This is consistent with some earlier suggestions [6,18,19] that the most potent SP isomers might be relatively resistant to esteratic degradation. However, as Fig. 2 shows, there are also isomers, such as 1(S)*cis*- α (S) and - α (R) cypermethrin, which have relatively low potencies but are also degraded relatively slowly by the esterases we have tested. There is thus no simple relationship between the susceptibilities of different isomers to degradation by the 22 esterases tested and their relative potencies to various insects. It is, of course, also important that the conformation of a potent insecticidal isomer should be a good match to the binding site on the primary target sodium channel protein in the nervous system [20]. Modelling studies such as this applied to the docking of pyrethroid isomers to the expressed E3 esterase, as done for the putative natural substrates of the E3 esterase [21], could provide a better insight into the basis for the spectrum of activity observed in the present study.

It is worth noting that the r^2 value for the correlation between the activities with the SP isomers and their analogues noted above is around 0.5. Thus, there is still significant activity variation among the SPs that is not represented in the analogue data. Nevertheless, the correlation is sufficiently strong to justify ongoing use of the analogues as SP surrogates for much biochemical work, given their advantages in respect of amenability to continuous assays at low substrate concentrations in microplate format with ready fluorescent detection of product, compared to the limitations with the end point SP assays of low throughput, limited dynamic range, the need for much greater concentrations that generate emulsions, and requirement for extraction and HPLC separation to monitor substrate depletion. Also bearing out the value of the analogues, Coppin et al. [13] found that laboratory selection on the E3 enzyme above for increased activity against the analogues of the insecticidal isomers of cypermethrin (and fenvalerate) led to the identification of a mutant E3 that had over a hundred-fold higher activity for the highly insecticidal 1(R)*cis*- α (S) isomer of the closely related deltamethrin.

Interestingly, one of the three amino acid substitutions in this mutant E3, Trp251Leu (which confers resistance to organophosphorus insecticides), also occurs at low frequencies in natural populations of *L. cuprina* (and PCR on museum specimens has shown that it did so even before the first use of chemical insecticides [22]) and is also found in a significant minority of the insect esterase sequences recovered from various genome sequencing projects [13]. This includes two of the Clade 1 *H. armigera* esterases studied here

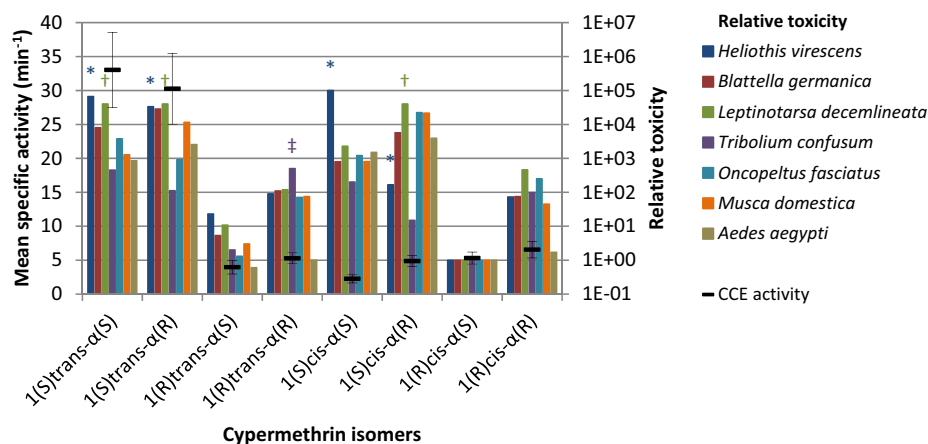


Fig. 2. Comparison of the mean specific activity of the 22 *H. armigera* esterase variants herein towards the isomers of cypermethrin, and the published toxicity of these isomers against other insect species (relative to the 1(R)*cis*- α (S) isomer). The LD₅₀ of the reference isomer, 1(R)*cis*- α (S), in each insect species is as follows: *Heliothis virescens* – 0.3 ng/insect; *B. germanica* – 1.4 ng/insect; *L. decemlineata* – 1.8 ng/insect; *T. confusum* – 0.02 mg/disc; *O. fasciatus* – 0.9 ng/insect; *M. domestica* – 0.9 ng/insect; and *A. aegypti* – 1.0 $\mu\text{g/L}$. *Heliothis virescens* data are from Ackermann et al. [2], whilst all the data for all the other species are from Pap et al. [6]. *LD₅₀ values are estimated from mortality/dose data because LD₅₀ values were not determined in original reference. †LD₅₀ quoted as >40,000 (value used). ‡LD₅₀ quoted as >500 (value used).

Table 2

Comparison of activities of wildtype Clade 1 *H. armigera* esterases and their '251L' mutant forms towards insecticidal SPs and their analogues. Esterase nomenclature follows Teese et al. [23].

Enzyme		Cypermethrin				Esfenvalerate							
		1(R)trans- α (S)		1(R)cis- α (S)		2(S)- α (S)							
		SP ^a	SP analogue ^b	SP ^a	SP analogue ^b	SP ^a	SP analogue ^b						
001b	GR	4.42	(1.72)	0.97	(0.18)	3.91	(3.91)	0.01	(0.01)	1.53	(1.53)	1.38	(0.09)
	GR F236L	6.34	(1.23)	3.37	(0.26)	5.72	(4.47)	0.11	(0.04)	9.13	(3.26)	6.36	(0.14)
001d	GR	6.20	(3.75)	0.48	(0.07)	4.10	(2.69)	0.02	(0.02)	0.81	(0.53)	1.20	(0.19)
	GR F235L	1.66	(1.66)	0.41	(0.22)	18.32	(3.33)	0.00	(0.00)	5.46	(3.78)	0.08	(0.04)
001f	GR	0.00	(0.00)	0.00	(0.00)	1.30	(1.30)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
	GR F238L	1.85	(1.16)	0.00	(0.00)	5.65	(0.85)	0.00	(0.00)	0.39	(0.39)	0.00	(0.00)
001g	GR	0.00	(0.00)	0.00	(0.00)	9.27	(9.27)	0.00	(0.00)	16.96	(11.37)	0.00	(0.00)
	GR F238L	1.62	(1.62)	0.00		4.90	(0.87)	0.00		1.80	(1.80)	0.00	
001j	GR	0.52	(0.52)	0.06	(0.02)	11.61	(3.03)	0.00	(0.00)	1.80	(1.80)	1.52	(0.13)
	GR F236L	0.38	(0.38)	0.61	(0.05)	9.08	(5.99)	0.09	(0.02)	0.00	(0.00)	0.43	(0.04)

^a Specific activity and SE (brackets) (min^{-1}) at 100 μM substrate.

^b Specific activity and SE (brackets) (min^{-1}) at 10 μM substrate.

Table 3

Comparison of activities of Australian (GR) and Chinese (YG is SP susceptible, YGF SP resistant) Clade 1 *H. armigera* esterases towards insecticidal SPs and their analogues.

Enzyme		Cypermethrin				Esfenvalerate							
		1(R)trans- α (S)		1(R)cis- α (S)		2(S)- α (S)							
		SP ^a	SP analogue ^b	SP ^a	SP analogue ^b	SP ^a	SP analogue ^b						
001a	YG	5.66	(0.70)	0.13	(0.10)	1.56	(0.54)	0.00	(0.00)	0.20	(0.20)	1.47	(0.21)
	YGF	5.95	(0.64)	0.15	(0.11)	3.31	(1.17)	0.03	(0.03)	0.01	(0.01)	1.59	(0.24)
001c	GR	12.51	(3.51)	0.23	(0.04)	1.86	(0.85)	0.01	(0.01)	1.16	(1.16)	0.38	(0.07)
	YG	14.47	(4.29)	1.45	(0.15)	2.57	(1.46)	0.08	(0.05)	0.01	(0.01)	0.09	(0.07)
001d	YGF	13.82	(1.05)	1.07	(0.12)	4.81	(1.93)	0.31	(0.18)	0.88	(0.52)	0.44	(0.09)
	GR	6.20	(3.75)	0.48	(0.07)	4.10	(2.69)	0.02	(0.02)	0.81	(0.53)	1.20	(0.19)
001i	YG	0.76	(0.71)	1.07	(0.20)	2.60	(0.69)	0.05	(0.03)	0.00	(0.00)	3.68	(2.86)
	YGF	1.44	(0.11)	0.37	(0.04)	0.95	(0.40)	0.06	(0.01)	0.04	(0.04)	0.90	(0.13)
001j	GR	7.92	(3.00)	0.00	(0.00)	4.51	(2.06)	0.00	(0.00)	5.37	(3.61)	0.00	(0.00)
	YGF	0.80	(0.80)	0.00	(0.00)	4.48	(1.21)	0.08	(0.08)	0.54	(0.34)	0.01	(0.01)
001j	GR	0.52	(0.52)	0.06	(0.02)	11.61	(3.03)	0.00	(0.00)	1.80	(1.80)	1.52	(0.13)
	YG	0.00	(0.00)	0.03	(0.02)	3.83	(1.81)	0.03	(0.02)	0.00	(0.00)	1.33	(0.11)
	YGF	0.60	(0.36)	0.15	(0.09)	1.91	(0.97)	0.00	(0.00)	0.00	(0.00)	1.27	(0.22)

^a Specific activity and SE (brackets) (min^{-1}) at 100 μM substrate.

^b Specific activity and SE (brackets) (min^{-1}) at 10 μM substrate.

(HaCCE001c and -001i). Li et al. [17] made and expressed the Leu251 forms for five of the other Clade 1 esterases studied here (i.e. HaCCE001b, -001d, -001f, -001g and -001j), finding that they did not consistently have higher activities for the insecticidal isomers of cypermethrin than the corresponding wild-type enzymes. Our data (Table 2, Suppl. Fig. S2) show the same to be true for the analogues. It appears that the ability of the Leu251 residue to enhance activity for the isomers generally associated with greater potency depends on the sequence/structure context in which it sits.

Comparisons between the naturally occurring Australian and Chinese variants and between the Chinese variants associated with SP susceptibility and resistance (Table 3) failed to find any significant differences in activity between allelic variants for any of the three most potent SP isomers tested (for the equivalent analogues, three significant differences were found, all affecting the hydrolysis of the 1(R)trans- α (S) cypermethrin isomer, but these activities were low and in the case involving a resistance allele, activity actually decreased; Suppl. Fig. S2). A few differences were found among the activities for the other six SP isomers (four between the variants from the Australian and Chinese susceptible strain and five between the variants from the Chinese susceptible and resistant strains; Suppl. Fig. S2) but, given the large number of contrasts performed, their biological significance is uncertain. No consistent pattern was evident among the significant contrasts between the Australian and Chinese material although, intriguingly, the five significant contrasts between the susceptible and resistant Chinese

strains actually showed the enzymes from the resistant strain to have lower activity than those from the susceptible strain (Suppl. Fig. S2). Thus we find no evidence that the highly successful selection for SP resistance in the YGF resistant strain has led to the selection of variants of the Clade 1 esterases studied which have higher specific activities. This is consistent with a range of studies ([24,25] for reviews) suggesting that the greater esterase activity often associated with SP resistance in this species is due to differences in the expression levels rather than amino acid sequences of relevant esterases.

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Appendix: Supplementary material

Supplementary data to this article can be found online at [doi:10.1016/j.pestbp.2014.12.010](https://doi.org/10.1016/j.pestbp.2014.12.010).

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