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Biochemical evaluation of interactions between synergistic molecules and phase I enzymes involved in insecticide resistance in B- and Q-type *Bemisia tabaci* (Hemiptera: Aleyrodidae)

Michela Panini,[a,b] Francesco Tozzi,[c] Christoph T Zimmer,[d,e] Chris Bass,[d,e]

Linda Field,[e] Valerio Borzatta,[c] Emanuele Mazzoni[b] and Graham Moores[a*]

Abstract

BACKGROUND: Metabolic resistance is an important consideration in the whitefly *Bemisia tabaci,* where an esterase-based mechanism has been attributed to pyrethroid resistance and over-expression of the cytochrome P450, CYP6CM1, has been correlated to resistance to imidacloprid and other neonicotinoids.

RESULTS: *In vitro* interactions between putative synergists and CYP6CM1, B and Q-type esterases were investigated, and structure–activity relationship analyses allowed the identification of chemical structures capable of acting as inhibitors of esterase and oxidase activities. Specifically, methylenedioxyphenyl (MDP) moieties with a polyether chain were preferable for optimum inhibition of B-type esterase, whilst corresponding dihydrobenzofuran structures were potent for the Q-esterase variation. Potent inhibition of CYP6CM1 resulted from structures which contained an alkynyl chain with a terminal methyl group.

CONCLUSIONS: Synergist candidates could be considered for field control of *B. tabaci*, especially to abrogate neonicotinoid resistance.
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Supporting information may be found in the online version of this article.

Keywords: *Bemisia tabaci*; esterase; P450s; piperonyl butoxide; synergist; SAR

1 INTRODUCTION

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a globally important pest of agricultural and ornamental crops, with a host range of more than 900 plants, capable of causing damage to the leaves by direct feeding or honeydew contamination, and also by transmitting many plant virus species.[1] Among the wide range of biotypes reported, type 'B' (also known as 'Middle East–Asia') and type 'Q' (also known as 'Mediterranean species') represent the two major groups which have shown an ability to develop resistance to different insecticide classes used extensively for their control.[2]

Numerous reports from different parts of the world document the widespread occurrence of resistant whitefly populations, mainly correlated to enhanced levels of phase I enzymes: esterases and oxidases.[3−8]

Pyrethroid, organophosphate (OP) and carbamate resistance has been attributed to an esterase-based resistance mechanism. The over-produced esterases are responsible for the cleavage or sequestration of these insecticides, thus preventing modulation of the voltage gated sodium channel or the irreversible inhibition of the acetylcholinesterase enzyme.[3,5,6]

Neonicotinoid insecticides with a different mode of action (they are selective agonists of nicotinic acetylcholine receptors) were introduced against numerous pests, including *B. tabaci*, had the considerable advantage of being less-affected by the existing resistance mechanisms of the previously mentioned

* Correspondence to: G Moores, Biochemistry Department, ApresLabs Ltd, Research and Innovation Campus, Harpenden, Herts AL5 2JQ, UK. E-mail: graham.moores@apreslabs.co.uk

a ApresLabs Ltd, Harpenden, Herts, UK

b Dipartimento di Scienze delle Produzioni Vegetali Sostenibili, Università Cattolica del Sacro Cuore, Piacenza, Italy

c Endura SpA, Ravenna, Italy

d College of Life and Environmental Sciences, University of Exeter, Penryn, Cornwall, UK

e Department of Biological Chemistry and Crop Protection, Rothamsted Research, Harpenden, Herts, UK

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insecticides. Nevertheless, their extensive use to control resistant populations culminated in a decreased susceptibility of the whiteflies. The first report of neonicotinoid resistance was documented in a Spanish greenhouse population, showing a reduced efficacy of imidacloprid[4] and possible cross-resistance between a neonicotinoid insecticide (acetamiprid) and thiamethoxam was demonstrated.[9] Since then, cases of loss of efficacy were documented in different parts of Europe and correlated to enhanced levels of microsomal monooxygenases.[7,10] One single cytochrome P450 enzyme (CYP6CM1) was found to be highly over-expressed in imidacloprid resistant populations of both B- and Q-type *B. tabaci*.[8] Furthermore, laboratory studies performed with the recombinant form of the Q-biotype version of the enzyme (CYP6CM1vQ) showed the capability to cross-metabolise other neonicotinoid molecules like clothianidin and thiacloprid.[11]

Piperonyl butoxide (PBO) has been used for many years to aid diagnosis of oxidase-modulated resistance through inhibition of cytochrome P450 enzymes in household pests including *Cimex letularius*,[12] *Blatella germanica*,[13] disease vectors such as *Culex quinquefasciatus*,[14] and agriculturally important pests including *Plutella xylostella*[15] and *Pseudoplusia includens*,[16] reviewed by Glynne-Jones.[17] More recently, it has been shown that PBO also inhibits resistance-associated esterases,[18,19] and thus it should be considered as a diagnosis to metabolic resistance rather than specifically diagnosing enhanced P450 activity.

Many *in vitro* studies showed that there was a decreased P450 activity in microsomal fractions from insects treated with MDP compounds[20] and with PBO and NADPH.[21,22] On this basis it was suggested that MDP compounds form metabolic intermediates (MIs) preventing enzymatic activity.[23] The molecular mechanism by which PBO in the presence of NADPH interacts with cytochrome P450s is classically referred to as mechanistic (a suicide substrate), following the formation of an intermediate carbene moiety.

In order to detect the involvement of metabolic resistance mechanisms, PBO has previously been used in laboratory bioassays and field trials against resistant whitefly populations, confirming the importance of detoxifying enzymatic pathways in the analysed insects.[10,12–14]

This work aimed to better explore/abrogate metabolic resistance mediated by enhanced levels of the previously mentioned enzymes of the cotton whitefly *B. tabaci*, through the investigation of possible interactions with a wide range of putative PBO-like inhibitors.

2 MATERIALS AND METHODS

2.1 Whiteflies

The B-type resistant strain was a long term frozen (−80 °C) population originally collected from Cyprus in 2003. Adult Q-type resistant whiteflies were collected from ornamental plants kept in a greenhouse in Savona district (Liguria, Italy) in 2013 and maintained on tobacco plants, at a constant temperature of 26 °C and a 16:8 h light:dark photoperiod.

2.2 Chemicals

Technical PBO and analogues with modifications in the methylenedioxyphenyl (MDP) moiety, alkyl and polyether side chains were supplied by Endura SpA (Bologna, Italy) (Table 1).

Derivatives **7**, **11**, **12**, **25**, **40** were synthesised according to the chemical process reported in Bertok *et al.*[24] Derivatives **18**, **19**, **20**, **21**, **22**, **24**, and **43** were synthesised according to Borzatta *et al.*[25] Compound **10** was synthesised according to Moore and Hewlett.[26] Derivative **23** was synthesised according to Fellig and Rachlin.[27] Derivatives **41** and **42** were synthesised by enantioselective reduction of the corresponding acetyl dimethoxybenzene by using [$RuCl_2$(*p*-cymene)]complexed with (1*R*,2*R*)-(−)*N*-*p*-tosyl-1,2-diphenylethylenediamine) and (*S*,*S*)-(−)*N*-*p*-tosyl-1,2-diphenylethylenediamine) and further condensation with 2-butyn-1-ol. Derivatives **13**, **14**, **15**, **16**, **17**, **26**, **27**, **28**, **29**, **30**, **31**, **32**, **33**, **34**, **35**, **36**, **37**, **38** and **39** were synthesised following the synthesis procedures reported in Philippou *et al.*[28] Derivative **9** was synthesised according to Moores *et al.*[29] Derivatives **8** and **13** were synthesised according to Arvai *et al.*[30] Benzodioxole and benzodioxane derivatives, **2**, **3**, **4**, **5**, **6** and **44**, were synthesised following the synthesis procedure reported in Ugolini *et al.*[31] Benzodioxole derivative **45** was synthesised according to Tozzi.[32] Dimethoxybenzene derivative was synthesised following the procedure reported in Panini *et al.*[33]

All the derivatives were purified by using the suitable purification techniques such as distillation, crystallisation and column chromatography. The physico-chemical characteristics and NMR analyses of the novel compounds are reported in the supporting information.

2.3 Baculovirus-mediated CYP6CM1 expression

Full-length *B. tabaci* CYP6CM1 sequence (GenBank accession number GQ214539) and *Drosophila melanogaster* NADPH cytochrome P450 reductase (CPR) sequence (GenBank accession number Q27597) were obtained as described in Nauen *et al.*,[34] but using High 5 (Trichoplusa ni) cells in place of Sf9 for reduced background activity against model substrates. Expression of P450 was checked by measuring CO-difference spectra in reduced samples[35] and enzymatic activity checked with fluorescence substrates. Aliquots of recombinant CYP6CM1 were stored at −80 °C.

2.4 Inhibition of P450 activity

Inhibition of recombinant CYP6CM1 enzyme was measured as described by Moores *et al.*[36] Briefly, 7-ethoxycoumarin (7-EC) was dissolved in ethanol to make a 20 mM stock solution and diluted to 0.125 mM by the addition of 0.1 M sodium phosphate buffer, pH 7.8. Aliquots of the diluted recombinant enzyme (50 μL, 0.5 pmol) were dispensed into a 96-well microplate (Optiplate™, white; Perkin Elmer, Seer Green, Beaconsfield, UK).

In a preliminary experiment, 3 μL of three analogues (structures **1**, **14** and **18**; stock 10 mM in acetone) possessing the MDP moiety were incubated for 10 min (condition 1) or 30 min (condition 2) in the presence of 10 μL of 9.6 mM NADPH. After the incubation, 80 μL of 0.125 mM 7-EC were added, followed by 10 μL of 0.1 M sodium phosphate, pH 7.8.

In parallel, 3 μL of the same analogues were incubated for 10 min (condition 3) or 30 min (condition 4) in the absence of NADPH, replaced by 10 μL of 0.1 M sodium phosphate, pH 7.8. After the incubation, 80 μL of 0.125 mM 7-EC were added, followed by 10 μL 9.6 mM NADPH.

For all conditions, two negative controls containing cytochrome P450 reductase (CPR) only, or the absence of 7-EC, were included.

The reaction was monitored using a Spectramax Gemini EM (Molecular Devices, Menlo Park, CA, USA) for 60 min, with readings taken every min, using an excitation wavelength of 390 nm and an emission wavelength of 460 nm, with a 435 nm cut-off filter. *O*-Deethylation activity was calculated as the rate of production

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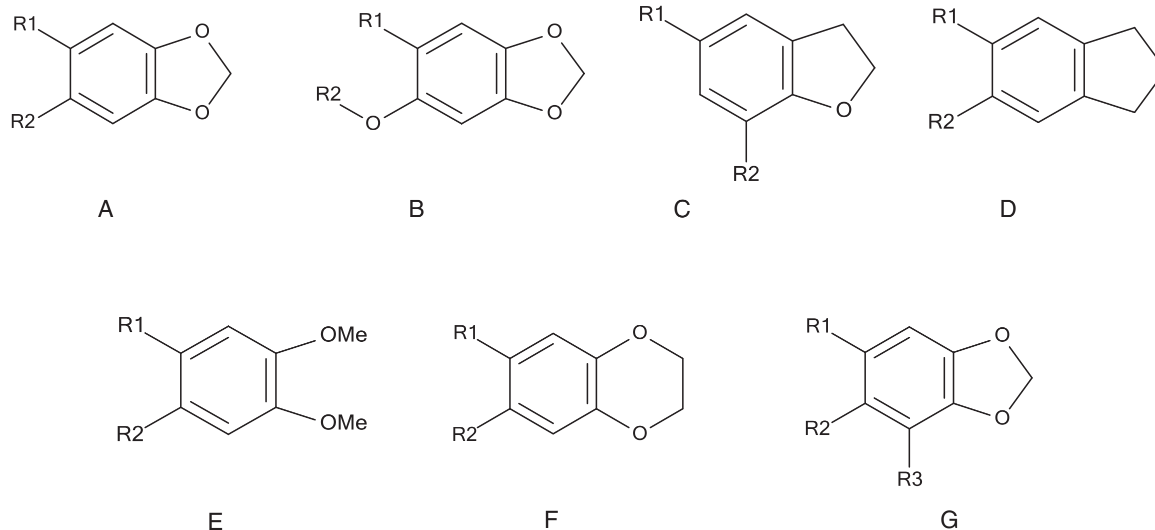
Table 1. Structures of PBO analogues



Structure number	Name	Aromatic ring	R_1	R_2
1	PBO	A	$CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$
2	EN 1-14	A	$CH_2CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$
3	EN 1-16	A	$CH_2CH_2CH_2CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$
4	EN 1-40	A	H	$(CH_2OCH_2)_3CH_2CH_2CH_3$
5	EN 1-42	A	$(CH_2OCH_2)_3CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$
6	EN 1-48	A	$CH_2CH_2CH_3$	$(CH_2OCH_2)_2C_6H_6$
7	EN 1-93	A	$CH_2CH_2CH_3$	COOH
8	EN 1-125	A	H	$CH_2OCH_2C\equiv CCH_3$
9	EN 1-126	A	$CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CCH_3$
10	EN 1-129	A	H	$(CH_2)_{11}CH_3$
11	EN 1-162	A	$CH_2CH_2CH_3$	$(CH_2OCH_2)_3COONa$
12	EN 1-182	A	H	$CH_2CH(CH_3)CH_2OCH_2C\equiv CCH_3$
13	EN 1-198	A	$CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CH$
14	EN 1-213	A	$CH_2CH_2CH_2CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CCH_3$
15	EN 1-215	A	$CH_2CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CCH_3$
16	EN 1-216	A	$CH_2CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CH$
17	EN 1-218	A	$CH_2CH_2CH_2CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CH$
18	EN 1- 163	B	H	$CH_2C\equiv CCH_3$
19	EN 1-164	B	$CH_2CH_2CH_3$	$CH_2C\equiv CCH_3$
20	EN 1-175	B	H	$(CH_2CH_2O)_2CH_2CH_2CH_2CH_3$
21	EN 1-179	B	$OCH_2C\equiv CCH_3$	$(CH_2CH_2O)_2CH_2CH_2CH_2CH_3$
22	EN 1-180	B	$CH_2CH_2CH_3$	$(CH_2CH_2O)_2CH_2CH_2CH_2CH_3$
23	EN 1-181	B	H	$CH_2C\equiv CH$
24	EN 1-183	B	$CH_2CH_2CH_3$	$CH_2C\equiv CH$
25	EN 1-186	B	$CH_2CH_2CH_3$	$CH_2CH_2OCH_3$
26	EN 16-06	C	$(CH_2OCH_2)_3CH_2CH_2CH_3$	H
27	EN 16-18	C	$OCH_2C\equiv CCH_3$	H
28	EN 16-55	C	$CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$
29	EN 16-56	C	$CH_2CH_2CH_3$	$OCH_2C\equiv CCH_3$
30	EN 16-28	C	$CH_2CH_2CH_2CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$
31	EN 16-39	C	$CH_2CH_2CH_2CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CH$
32	EN 16-40	C	$CH_2CH_2CH_2CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CCH_3$
33	EN 16-41	C	$CH_2CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CCH_3$
34	EN 16-42	C	$CH_2CH_2CH_2CH_2CH_2CH_3$	$OCH_2C\equiv CCH_3$
35	EN 16-43	C	$CH_2CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CH$
36	EN 16-44	C	$CH_2CH_2CH_2CH_2CH_2CH_3$	$OCH_2C\equiv CH$
37	EN 16-46	C	$CH_2CH_2CH_3$	$CH_2OCH_2C\equiv CCH_3$

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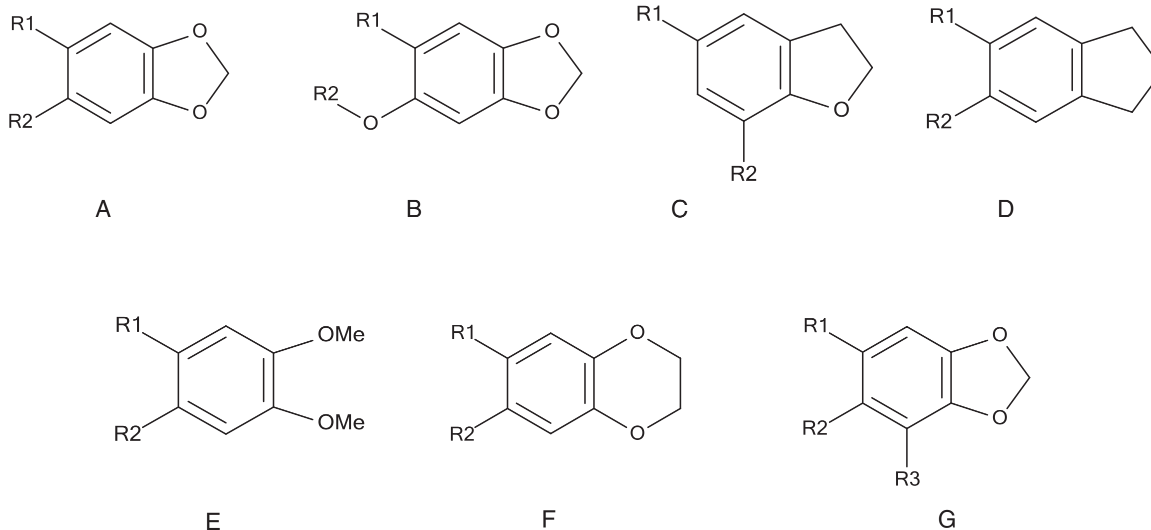
Table 1. continued



Structure number	Name	Aromatic ring	R_1	R_2
38	EN 16-47	C	$CH_2CH_2CH_3$	$CH_2OCH_2C{\equiv}CH$
39	EN 14-05	D	$CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$
40	EN 25-08	E	H	$CH(CH_3)OCH_2C{\equiv}CCH_3$ (*RS*)
41	EN 25-09	E	H	$CH(CH_3)OCH_2C{\equiv}CCH_3$ (*S*)
42	EN 25-10	E	H	$CH(CH_3)OCH_2C{\equiv}CCH_3$ (*R*)
43	EN 25-37	E	H	$OCH_2C{\equiv}CCH_3$
44	EN 18-05	F	$CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$
45	EN 1-44	G	$(CH_2OCH_2)_3CH_2CH_2CH_3$	$(CH_2OCH_2)_3CH_2CH_2CH_3$

In structure **45**, R3 is $(CH_2OCH_2)_3CH_2CH_2CH_3$.

of 7-hydroxycoumarin and expressed as fluorometric units per minute (FU min^{-1}), using the integrated software programme Softmax Pro version 5.4 to fit the linear regressions. All assays were repeated in triplicate.

Inhibition of CYP6CM1 by analogues was then performed as per the latter method above (condition 4).

2.5 Esterase purification

Since many resistance-associated esterases in insect pests are known to be glycosylated, the decision was made to purify the resistance-characterised esterases from *B. tabaci*, rather than make recombinant versions. Esterases (B and Q) were semi-purified from whitefly samples using size exclusion and ion exchange chromatography. Whiteflies were bulk homogenised on ice in 5 mL of 0.02 M phosphate buffer pH 7.0 and centrifuged at 10 000 × *g* for 5 min. The supernatant was passed through a Sephadex G-25 column (Fine) (Amersham Biosciences, Little Chalfont, Bucks, UK) (2 cm diameter × 10 cm height) to separate low molecular weight material. The proteins were eluted using 0.02 M Tris–HCl buffer pH 8.5. Unretarded fractions were collected, loaded onto an ion exchange column (DEAE Sepharose TM Fast Flow) (Amersham Biosciences) (2.5 cm diameter × 6 cm height) and eluted using a linear 0–0.35 M NaCl gradient in 250 mL of Tris–HCl pH 8.5.

Fractions (2 mL) were collected and tested for total esterase activity using a colorimetric assay with 1 mM (final) 4-nitrophenyl acetate as substrate. The assay was performed in a Tmax kinetic microplate reader (Molecular Devices, Menlo Park, CA, USA) taking readings every 20 s at 405 nm for 5 min. The integrated software programme Softmax Pro version 5.4 was used to fit linear regressions, the slopes of which were summarised as a rate of milli-optical density change per minute at 405 nm (mOD_{405} min^{-1}).

Fractions with the highest activities were pooled and passed through a Sephadex G-25 column (Fine; Amersham Biosciences) to desalt and buffer exchange to 0.02 M phosphate buffer pH 7.0 for storage. Aliquots were frozen at −20 °C.

2.6 Inhibition of esterase activity

Inhibition of esterase activity was measured using 4-nitrophenyl octanoate (pNO). Stocks (10 mM) of the substrate were prepared in acetone and diluted to 2 mM by the addition of 0.02 M phosphate buffer pH 7.0. Aliquots of the purified enzyme (50 µL) was dispensed into a 96-well microplate (maxisorb; NUNC, VWR International, Magna Park, Lutterworth, UK), in addition to 100 µL 0.02 M phosphate buffer pH 7.0, mixed with 2.5 µL of inhibitor (stock 10 mM in acetone) and incubated for 10 min at room temperature. Acetone only was used as uninhibited control. After the incubation, 100 µL of 2 mM pNO was added, and esterase activity monitored at 405 nm in a Spectramax Tmax kinetic microplate, taking readings every 5 s for 5 min. The integrated software programme Softmax Pro version 5.4 was used to fit linear regressions. The slopes were recorded as a rate of milli optical density

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change per minute at 405 nm (mOD_{405} min^{-1}). All assays were repeated in triplicate.

2.7 Homology modelling and molecular docking

The crystal structure of human liver P450, CYP3A4 (PDB entry 1TQN),[37] was used as a template for the protein model of CYP6CM1. Docking of PBO and selected analogues was carried out using Autodock Vina on the constructed enzyme model.[38] The protein hydrogen atoms, atom charges and solvation parameters were added to the model using Auto Dock Tools (version 1.5.4).[39] Grid boxes were generated to encompass the whole proteins. Docking simulations were performed with an ‘exhaustiveness’ of 200.

3 RESULTS

3.1 CYP6CM1 inhibition by PBO and analogues

Results of the preliminary assays (Table 2) showed that ‘pre-activation’ of the MDP moiety by NADPH (conditions 1 and 2) resulted in equal inhibition to ‘co-activation’ (conditions 3 and 4). It also confirmed that a longer incubation time (conditions 2 and 4) did not result in increased inhibition, as found previously with *B. tabaci* preparations.[36]

Results in Fig. 1A show the difference in the ability of the analogues to inhibit CYP6CM1 *O*-deethylation activity. The most effective compounds contained a triple bond located within the chain (**8**, **21**, **29**, **33**); removal of the terminal methyl group from the alkynyl moiety resulted in lower inhibition (**15** > **16**; **14** > **17**; **32** > **31**; **33** > **35**). Among the alkynyl structures, no consistent increase/decrease in potency was observed following removal of an oxygen from the MDP ring (**15** > **33**; **13** > **38**; **16** > **35** but **14** < **32**; **24** < **29**). The presence/absence of the methylene bridge seemed to be unimportant (**35** < **36**; **8** < **18** but **32** > **34**; **9** > **19**), whilst the presence of a butyl side chain was better than hexyl (**15** > **14**; **16** > **17**).

PBO-like structures (those with a polyether chain) were less effective than the corresponding alkynyl analogues (**2** < **15**; **20** < **21**; **30** < **32**). The absence of a methylene bridge to generate a sesamol structure improved the potency of the analogues (**22** > PBO), as did the removal of one oxygen from the MDP ring (**28** > PBO) and the absence of the alkyl chain (no chain: **4** > butyl: **2**/propyl: PBO/hexyl: **3**).

Structures that are electron-donating on both chains showed good inhibition (**21**, **5**); particularly, analogue **21** carrying both a polyether chain and an alkynyl chain which resulted in one of the most potent CYP6CM1 inhibitors.

In general, the most effective structures contained an alkynyl chain with a terminal methyl group, whilst PBO-like structures did not perform well, giving very low inhibition at this concentration.

Table 2. Comparison of CYP6CM1 activity remaining (%) after incubation with (1 and 2) or without (3 and 4) NADPH, followed by incubation with 7-EC and NADPH

Condition	Structure 18	Structure 14	Structure 1
1	31.0	44.3	93.3
2	36.2	47.3	87.2
3	34.9	46.7	89.4
4	37.7	49.6	91

Condition 1: 10 min pre-incubation of MDP and NADPH with CYP6CM1; Condition 2: 30 min pre-incubation of MDP and NADPH with CYP6CM1. Condition 3: preincubation without NADPH, 10 min incubation with CYP6CM1; Condition 4: preincubation without NADPH, 30 min incubation with CYP6CM1. Full details are given in the methods.

3.2 B-type esterase inhibition by PBO and analogues

Results in Fig. 1B show the different ability of the analogues to blockade esterases from the B-type strain. In MDP derivatives increasing the length of the alkyl chain was unimportant, as there were no differences in inhibition with structures having a propyl, butyl or hexyl (PBO = **2** = **3**; **13** = **16** = **17**). However, the loss of the alkyl chain resulted in a consistent decrease in inhibition, not only in MDP derivatives (PBO > **4**; **9** > **8**) but also in sesamol derivatives (**22** > **20**; **19** > **18**; **24** > **23**).

In structures with the polyether chain, the introduction of a second polyether chain partially restored the ability to inhibit esterase activity (**5** > **4**); the substitution of this second chain with an alkynyl ether chain fully restored the efficacy (**21** > **4**). The presence of a sodium carboxylate (—COONa) group at the end of the polyether chain (**11**), or a shorter polyether chain (**25**), or the replacement of the polyether chain with a dodecyl chain (**10**) or a carboxyl group (**7**) resulted in a significant reduction of inhibition, as did replacement with a butynyloxy chain when compared to its polyether counterpart (**8** < **4**; **9** < PBO).

Removal of one oxygen from the MDP moiety showed a small decrease of inhibitory potency of the structures if an alkyl chain was present (**29** < **19**; **28** < PBO), as did removal of both the oxygen atoms (**39** < PBO). If a single oxygen atom was retained in the absence of an alkyl chain, change in potency was dependent upon the presence of a polyether chain (**26** > **4**) or alkynyl moiety (**27** < **18**).

Considering the alkynyl structures, dihydrobenzofuran moieties were more effective in some cases (**32** > **14**; **31** > **17**), but not in others (**33** = **15**; **38** < **13**). Removal of the terminal methyl had negative effects for both MDP (**23** < **18**; **24** < **19**; **16** = **15**) and dihydrobenzofuran moieties (**35** < **33**; **38** < **37**; **31** = **32**).

Removal of the methylene bridge and changing from a MDP to a sesamol structure produced contrasting results, showing no differences in polyether derivatives (**4** = **20**; PBO = **22**) nor for MDP alkynyl structures (**31** = **36**; **32** = **34**), but increasing the efficacy in the case of dihydrobenzofuran alkynyl moieties (**8** < **18**; **9** < **19**).

In general, ‘PBO-like’ structures were more potent than alkynyl phenoxy structures, demonstrated by the presence of only a single alkynyl compound among the 10 most potent inhibitors. This is clearly confirmed by the comparison between PBO and **13**, where replacement of the polyether chain with the alkynyl chain resulted in lower inhibition.

3.3 Q-type esterase inhibition by PBO and analogues

Results in Fig. 1C show the difference in the ability of the analogues to blockade esterases from the Q-type strain. In MDP structures containing a polyether chain, a butyl alkyl chain (**2**) seemed to perform slightly better than the propyl or the hexyl (PBO or **3**), whilst there was a consistent decrease of inhibitory potency when the alkyl chain was removed (**4** < PBO; **20** < **22**). This was not always true when an alkynyl moiety was present, where the absence of the alkyl chain increased the efficacy (**18** > **19**) but decreased in others (**8** < **9**), perhaps due to the presence of the methylene bridge. Among dihydrobenzofuran moieties with alkynyl chain, again a butyl alkyl chain seemed to perform better than a propyl or hexyl chain (butyl: **33** > propyl: **37** > hexyl: **32**).

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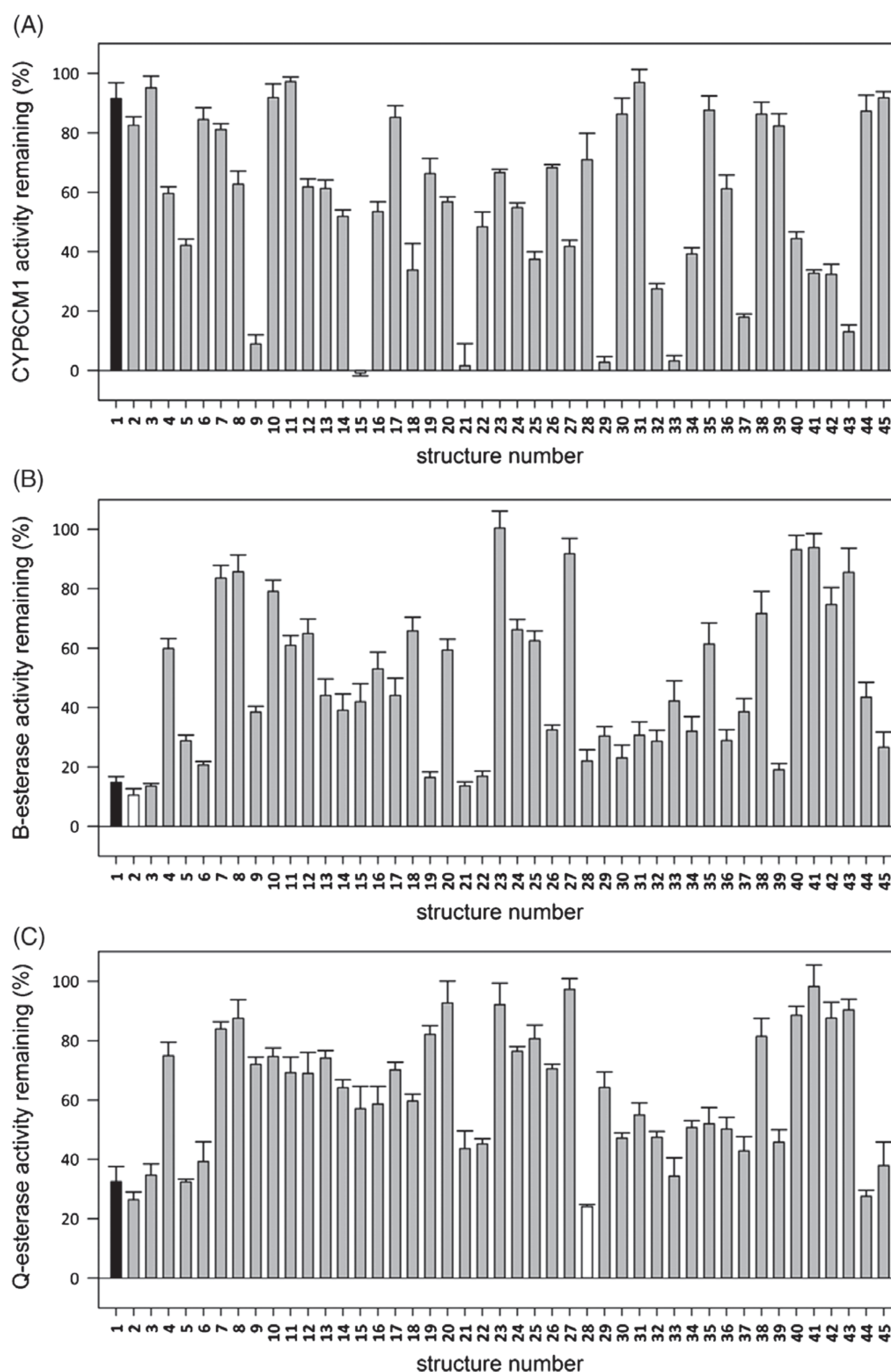

Figure 1. Activity remaining (%) calculated for different analogues, including PBO (black columns). (A) CYP6CM1; (B) B-esterase; (C) Q-esterase. Activity remaining was calculated as a percentage of uninhibited activity (not shown). The best performing analogue is reported in white.

The introduction of an alkynyl ether chain in structures with a polyether chain improved efficacy (**21** > **4**), and enhancement was also obtained with a second polyether chain (**5** > **4**), performing similar to PBO. The presence of a sodium carboxylate (—COONa) group at the end of the polyether chain (**11**) and replacement of the side chain with a carboxyl group (**7**) or with a dodecyl chain (**10**) showed low inhibitory potency, as did structure **25** with a shortened polyether chain and a propyl side chain. Also, replacement of the polyether chain with a butynyloxy chain reduced the inhibition potency when compared to its counterpart (**8** < **4**; **9** < PBO).

Removal of one oxygen from the MDP moiety resulted in higher inhibition potency of the structures (**28** > PBO; **29** > **19**), whilst structure **39**, with no oxygen in the ring, performed worse than

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PBO. The maintenance of only one oxygen atom in the absence of an alkyl chain and only a polyether chain in the *para* position negatively affected the inhibition rates for structures with alkynyl chain (**27** < **18** but **26** > **4**).

Removal of the methylene bridge and changing from a MDP to a sesamol structure produced lower affinity (**4** > **20**; PBO > **22**; **9** > **19**) unless superimposed on an alkynyl structure lacking an alkyl chain (**8** < **18**).

Alkynyl moieties showed higher potency with a dihydrobenzofuran moiety replacing the MDP ring, particularly if the terminal methyl group was present (**33** > **15**; **32** > **14**), but this was not always true if the terminal methyl group was absent (**35** > **16**; **31** > **17** but **38** < **13**). Modification of the alkynyl moiety by removal of the terminal methyl group showed similar (**24** > **19**) or decreased binding affinities (**23** < **18**); decreased inhibition potencies were detected for dihydrobenzofuran structures with shorter alkyl chains (hexyl: **31** < **32**; butyl: **35** < **33**; propyl: **38** < **37**).

In general, structures with a polyether chain, expecially with dihydrobenzofuran moieties, were more effective than alkynyl phenoxy equivalents, provided that the alkyl chain length was shorter than hexyl.

3.4 Bi-variate analysis

Results from all the inhibition assays are summarised in Fig. 2A and B. In general, no clear correlations between oxidase and esterase inhibition were observed, for either B- or Q-type whiteflies. Several analogues were found to be potent inhibitors of CYP6CM1 at the diagnostic concentration, unlike PBO, which gave only 10% inhibition. A bi-variate plot between CYP6CM1 and B-type esterase highlighted structure **21** (analogue EN 1–179) as the most potent inhibitor of the two enzymes (Fig. 2A). A bi-variate plot between CYP6CM1 and Q-type esterase highlighted both structures **21** and **33** (analogue EN 16–41) as the optimum analogues, with no significant difference between them (Fig. 2B).

3.5 Protein modelling of interactions

Representations of the predicted docking positions and conformations of PBO and analogues with CYP6CM1 are shown in Fig. 3 and Fig. 4.

Both PBO and analogue **21** (structure EN 1–179) are held in position by aromatic interactions with Phe-501, Phe-64 and possibly Pro-117; analogue **21** is further stabilised by hydrogen bonds which are predicted to occur with Arg-224 and Phe-501 (Fig. 3A and B). The former residue has already been shown to be involved in interactions between imidacloprid and the biotype Q variant of the CYP6CM1 enzyme (CYP6CM1vQ).[40] The docking energies for CYP6CM1 and PBO was calculated to be -7.4 Kcal mol^{-1} and -7.5 Kcal mol^{-1} respectively.

The docking energies for CYP6CM1 and analogue **15** (structure EN 1–215) was calculated to be -8.0 Kcal mol^{-1} and -7.9 Kcal mol^{-1} for PBO and analogue **33** (structure EN 16–41) respectively. The analogues are both held in position by aromatic interactions with Pro-117, Phe-501 and Phe-64, and further anchorage is provided by a hydrogen bond to Lys-412 (Fig. 4). The oxygens on the MDP moiety of EN 1–215 do not, therefore, enter into binding directly, and the dihydrobenzofuran equivalent is in the same docking position.

4 DISCUSSION

Insecticide resistance continues to be a major concern for the control of agricultural insect pests; a problem that is exacerbated by the phasing out of several active ingredients. One possible solution would be the optimisation of permitted compounds by the addition of a synergist that inhibits the action of hyper-expressed metabolic enzymes in resistant populations.

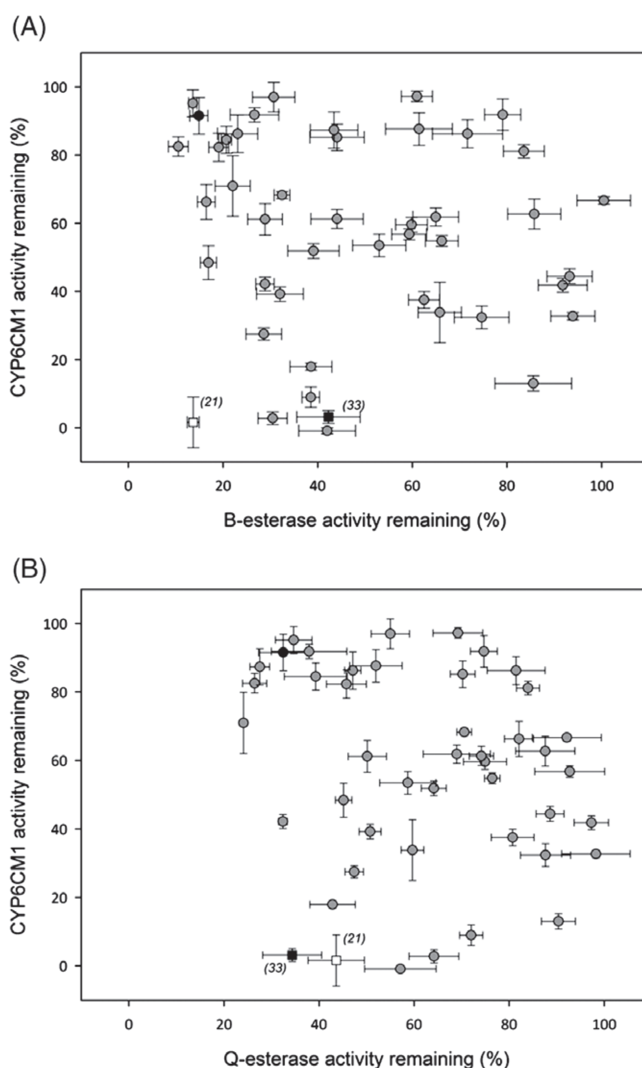

Figure 2. Bi-variate plots summarising the inhibition against CYP6CM1 and B-esterase (A) or Q-esterase (B). PBO (structure number 1, black circle) and analogues EN 1–179 (structure number 21, white square) and EN 16–41 (structure number 33, black square) are indicated.

The work described here explores the interactions between detoxifying enzymes of the whitefly *B. tabaci* and PBO analogues designed against the metabolic enzymes of another important agricultural pest, the green peach aphid *Myzus persicae*.[33]

Previous findings demonstrated that PBO-pyrethroid applications may enhance the insecticide efficacy in resistant populations of B-type *B. tabaci*, following the inhibition of pyrethroid resistance-associated esterases.[41] The effect is more evident if PBO is applied before the insecticide, allowing a reduction of the insecticidal dose to be applied, as demonstrated with small-scale field trials performed by the same authors. Starting from these observations, a selection of PBO analogues were explored, looking for more potent inhibitors on the basis of specific interactions with the enzymatic structures of both B- and Q-type esterases.

Inhibition of esterase activities were detected through the assay with pNO using the semi-purified enzymes, without requiring the

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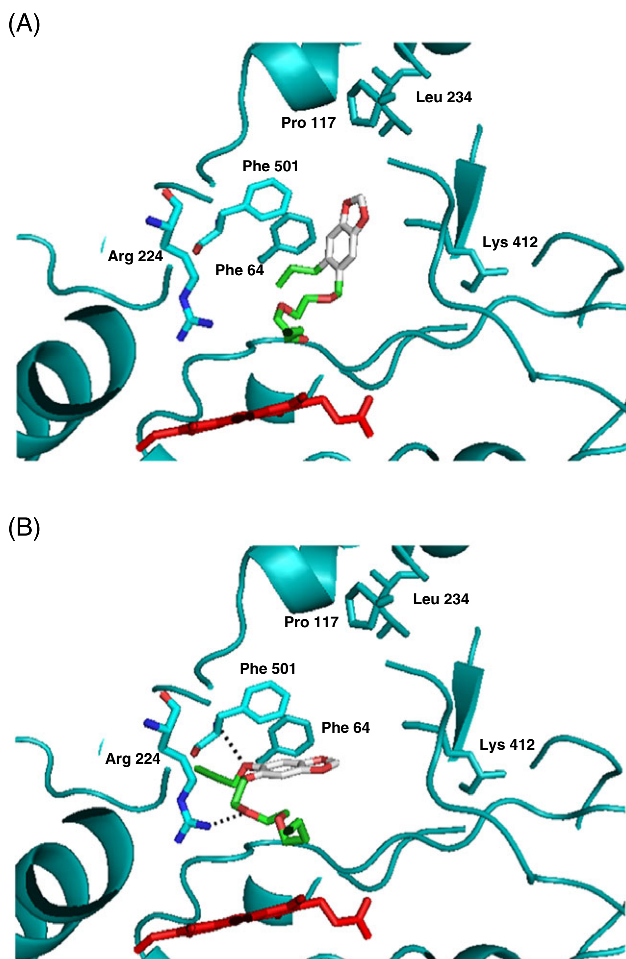

Figure 3. *In silico* model of PBO (A) and EN 1–179 (B) docked with CYP6CM1. Hydrogen bonds (dotted lines) between EN 1–179 and Arg-224 and Phe-501 are shown.

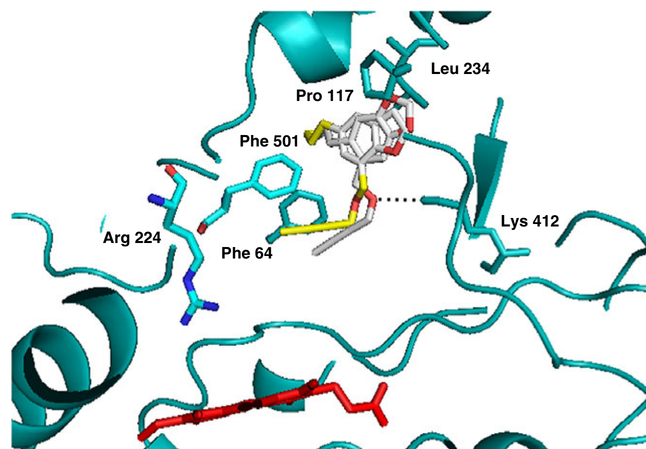

Figure 4. *In silico* model of EN 1–215 (white) and EN 16–41 (yellow) docked with CYP6CM1. Hydrogen bonds (dotted lines) between EN 1-215/ EN 16–41 and Lys-412 are shown.

production of recombinant proteins. The relatively large model substrate was used to overcome the difficulties previously found in visualising inhibition of esterases with PBO analogues.[19] In general, no great improvements were obtained in comparison with PBO, with just a few analogues performing slightly, but not significantly, better. Analogues with a polyether chain were more effective than the alkynyl phenoxy equivalents against both esterase types, with just one alkynyl structure (no. **19**, in B-type analyses) among the ten most potent (Fig. 1A and B).

Whilst for the B-esterase retention of both the MDP moiety and the polyether chain is preferable for optimum inhibition, with the Q-type a dihydrobenzofuran moiety is preferred, again with the polyether chain and an alkyl chain shorter than hexyl. This is in direct contrast to the *M. persicae* resistance-associated esterase (FE4) where it was found that a dihydrobenzofuran moiety with an alkynyl side chain conferred optimum inhibition.

The model substrate chosen for the inhibition studies of CYP6CM1 was 7-EC, due in part to the findings of Karunker *et al.*,[40] who examined the metabolic efficiency of CYP6CM1vQ against a number of model P450 fluorescent substrates and found that 7-EC gave the highest specific activity (20 pmol min^{-1} $pmol^{-1}$ P450).

Results of the preliminary P450 inhibition assays (Table 2) also found that CYP6CM1 inhibition was not increased considering a 10 min to 30 min incubation; indeed, equal inhibition was found from all four conditions.

Suicide substrates form a complex with the enzyme, whereupon the enzyme catalyses the reversible production of a complex with the activated intermediate, which could then be released as a product, releasing free enzyme or modify the enzyme. If the latter, inhibition would be expected to be time dependent. If the final modification of the enzyme is relatively slow, the enzyme: activated intermediate complex can be dissociated by other substrates. The degree of MDP activation by P450s has also been reported to be 'quantitatively unique' for each P450,[42] so it could be that relatively little activation occurs with CYP6CM1.

Since the research is to find a structure that would work well in the field, it was decided that the closest condition to spraying an insect would be to subject the analogue to both NADPH and substrate (albeit a model substrate) at the same time, i.e. not to pre-activate the MDP moieties with NADPH prior to incubation with substrate.

Similar to the results found both with the *M. persicae* P450 (CYP6CY3), and with house fly P450 (CYP6D1): removal of the alkyl chain from the PBO-like structures, resulted in a loss of inhibitory potency.[33,43]

In contrast to the *M. persicae* P450, CYP6CY3, incubation of CYP6CM1 with PBO did not result in high inhibition at the diagnostic concentration, and many of the alternative analogues actually resulted in superior inhibition. Indeed, the 'PBO-like' structures containing both an MDP ring and ethoxy chain did not reveal potent inhibition, which seemed the preserve of those structures containing an alkynyl chain. The exception was structure **21**, but this contained both ethoxy and alkynyl chains.

It was reported that the constitutive over-expression of *CYP6CM1* gene is correlated to imidacloprid resistance in both B and Q biotypes.[8] Metabolism of imidacloprid was confirmed by LC-MS and molecular docking and dynamic simulations suggested key residues responsible for imidacloprid binding and metabolism.[40]

5 CONCLUSIONS

In this study, although no clear correlations could be detected between CYP6CM1 and B- or Q-esterases, bi-variate plots highlighted structure no. **21** as one of the most potent inhibitors. Preliminary *in silico* modelling of this analogue suggested a shift in

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position when compared with PBO, resulting in hydrogen bonds being formed with Arg-224 and Phe-501. The former residue was identified by Karunker and colleagues[40] as a key residue in stabilising imidacloprid binding by aromatic interactions; the presence of PBO or analogue **21** would therefore occupy a space preventing interactions between CYP6CM1 and imidacloprid, with no. **21** the more difficult to displace.

The presently used set of analogues was synthesised to investigate interactions on specific detoxifying enzymes derived from resistant samples of *M. persicae*[33] and on whole homogenates from *M. persicae* and pollen beetle *Meligethes aeneus* (Fabricius).[44] In these studies some common structural features were identified as key elements for the interaction with the P450 specifically involved in insecticide detoxification, CYP6CY3 for *M. persicae* and total P450 activity from *M. aeneus*. Thus benzodioxole derivatives containing an alkynyl chain and a propyl or a butyl side chain were the most potent inhibitors. Since both CYP6CM1 and CYP6CY3 have been reported as P450s that have likely evolved to allow colonisation of cotton by these insect species,[45,46] it is perhaps surprising that they have different model substrate preferences and a different inhibition matrix to the PBO analogues. However, both insects are generalist feeders, and inevitably with a limited supply of P450s available the enzymes will therefore span a multitude of potentially toxic xenobiotics. It is possible that were they both specialist cotton feeders, the response of the nicotine-metabolising P450s would be closer.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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