ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Analytical Biochemistry 315 (2003) 208–222

ANALYTICAL BIOCHEMISTRY

www.elsevier.com/locate/yabio

Evaluation of α-cyanoesters as fluorescent substrates for examining interindividual variation in general and pyrethroid-selective esterases in human liver microsomes

Craig E. Wheelock,[a] Åsa M. Wheelock,[b] Rong Zhang,[a] Jeanette E. Stok,[a] Christophe Morisseau,[a] Susanna E. Le Valley,[c] Carol E. Green,[c] and Bruce D. Hammock[a,*]

[a] *Department of Entomology and Cancer Research Center, University of California, Davis, CA 95616, USA*
[b] *Department of Molecular Biosciences, University of California, Davis, CA 95616, USA*
[c] *Biopharmaceutical Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA*

Received 30 August 2002

Abstract

Carboxylesterases hydrolyze many pharmaceuticals and agrochemicals and have broad substrate selectivity, requiring a suite of substrates to measure hydrolytic profiles. To develop new esterase substrates, a series of α-cyanoesters that yield fluorescent products upon hydrolysis was evaluated for use in carboxylesterase assays. The use of these substrates as surrogates for Type II pyrethroid hydrolysis was tested. The results suggest that these novel analogs are appropriate for the development of high-throughput assays for pyrethroid hydrolase activity. A set of human liver microsomes was then used to determine the ability of these substrates to report esterase activity across a small population. Results were compared against standard esterase substrates. A number of the esterase substrates showed correlations, demonstrating the broad substrate selectivity of these enzymes. However, for several of the substrates, no correlations in hydrolysis rates were observed, suggesting that multiple carboxylesterase isozymes are responsible for the array of substrate hydrolytic activity. These new substrates were then compared against α-naphthyl acetate and 4-methylumbelliferyl acetate for their ability to detect hydrolytic activity in both one- and two-dimensional native electrophoresis gels. Cyano-2-naphthylmethyl butanoate was found to visualize more activity than either commercial substrate. These applications demonstrate the utility of these new substrates as both general and pyrethroid-selective reporters of esterase activity.

Keywords: Esterase; Pyrethroid; Fluorescent substrate; High-throughput assay; Human liver microsomes; Cytochrome P450; Native 2D gel electrophoresis

Carboxylesterases (EC 3.1.1.1) are a family of enzymes that are important in the hydrolysis of numerous xenobiotics and pharmaceuticals [1]. They are of clinical importance due to their high abundance, ability to hydrolyze many drugs, and ubiquitous distribution throughout the body [2]. Esterases have been used extensively in the design of prodrugs, including chemotherapeutic agents (CPT-11) [3,4], anti-HIV[1] drugs (glycovir) [5], and anti-Parkinsonian agents (L-DOPA esters) [6]. They are also vital in the metabolism and detoxification of insecticides including pyrethroids [7], organophosphates [8], and carbamates [9].

The use of pyrethroid insecticides is steadily increasing [10] and human exposure to these compounds is likely to increase commensurately. One of the main routes for pyrethroid metabolism is via esterase-mediated hydrolysis [11] as shown in Fig. 1. As most existing assays rely upon laborious analytical techniques

* Corresponding author. Fax: +530-752-1537.
E-mail address: bdhammock@ucdavis.edu (B.D. Hammock).

[1] *Abbreviations used*: HIV, human immunodeficiency virus; L-DOPA, L-dopamine; PNPA, *p*-nitrophenol acetate; EI, electron impact; TMS, trimethyl silane; BSA, bovine serum albumin; PBS, phosphate-buffered saline; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate; ALDH, aldehyde dehydrogenase; RFU, relative fluorescence unit.

0003-2697/03/$ - see front matter © 2003 Published by Elsevier Science (USA).
doi:10.1016/S0003-2697(03)00002-2

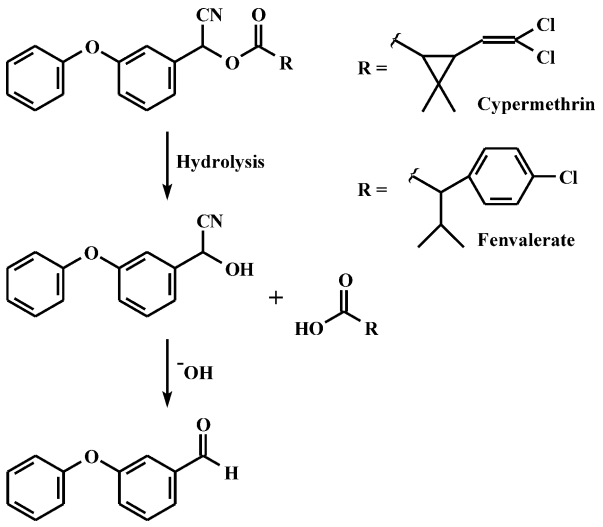


Fig. 1. The hydrolysis degradation pathway for Type II pyrethroids. After hydrolysis, the cyanohydrin and corresponding acid are formed. The cyanohydrin spontaneously forms the aldehyde at basic pH [15]. Hydrolysis of the α-cyanoester substrates is similar, except that the resulting aldehyde is strongly fluorescent.

[12], it is time-consuming and difficult to directly measure pyrethroid hydrolysis. Earlier attempts have been made to synthesize pyrethroid surrogate substrates for high-throughput screening, for example, using spectrophotometric assays. However, these new substrates often vary considerably in structure from the actual pyrethroids [13] or lack sensitivity [14]. With the aim of solving these problems, we have developed a series of esterase reporter assays based upon the hydrolysis of α-cyanoesters to produce fluorescent aldehydes [15]. These substrates are structurally very similar to Type II pyrethroids (e.g., cypermethrin and fenvalerate). Using human liver microsomes, the activity and assay performance of the α-cyanoester substrates were compared against standard substrates: *p*-nitrophenyl acetate (PNPA) and α-naphthyl acetate. The ranges of activity and interindividual variation in carboxylesterase activity in these human liver microsomes were then evaluated.

Another common use of esterase substrates is to visualize enzyme activity in native polyacrylamide gel electrophoresis (PAGE). These applications are of importance in proteomics for the study of the effects of xenobiotic exposure or physiological state upon esterase isozyme regulation and abundance. In-gel activity assays are commonly performed with the commercial substrates α-naphthyl acetate [16] or 4-methylumbelliferyl acetate [17]. However, given the large variability in esterase hydrolysis activity, there are undoubtedly esterase isozymes not visualized with these substrates. Therefore the α-cyanoesters were also evaluated as native PAGE stains.

This work examines the use of new esterase substrates as reporters of hydrolysis activity. Additionally, we evaluate the ability of these compounds to report on interindividual variations in human liver carboxylesterase activity, providing information on the range of human esterase activity. This variability is compared to that in other classic xenobiotic metabolizing enzymes (cytochrome P450 and microsomal epoxide hydrolase). The tools developed in this study will be useful for studying hydrolysis activity and determining levels of pyrethroid-hydrolyzing enzymes.

Materials and methods

Reagents

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) unless otherwise noted and were used without further purification. 4-Methylumbelliferyl acetate was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and all pyrethroid standards were purchased from either Chem Services, Inc. (West Chester, PA, USA) or Riedel de Haen (Seelze, Germany). Racemic 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid was generously supplied by E.I. duPont de Nemours & Co. (Wilmington, DE, USA). The two isomers (*S*)-(+)-4-chloro- and (*R*)-(−)-4-chloro-α-(1-methylethyl)benzene acetic acid were available from previous work by Shan et al. [18].

Porcine liver esterase (Catalog No. E-3019), bovine serum albumin (BSA), and ovalbumin were obtained from Sigma. Pooled human liver microsomes were purchased from Gentest Corp. (Woburn, MA, USA; Lot No. 18; Catalog No. 452161). Livers were from 16 individuals (10 males, 6 females) and were characterized for pathogenicity and cytochrome P450 activity. This information can be obtained directly from Gentest (http://www.gentest.com/index.html). Swiss Webster mouse liver microsomes were available from work by Wheelock et al. [19] and Fisher 344 rat liver microsomes were from Wheelock et al. [20].

Instruments for structural confirmation

Structural characterization and purity were provided by ^{1}H and ^{13}C NMR and electron impact (EI) GC/MS. NMR spectra were acquired on a Mercury 300 spectrometer (Varian, Palo Alto, CA, USA). Chemical shift values were recorded in *ppm* using TMS as the internal reference. For GC analysis, samples were analyzed on a HP 6890 GC (Agilent Technologies, Engelwood, CO, USA) equipped with a 0.25 mm i.d. × 30 m, 0.25-μm film DB-17MS column (J&W Scientific, Folsom, CA, USA) with a constant He flow rate of 0.8 ml/min. The injector temperature was 250 °C. The initial column temperature of 50 °C was held for 5.00 min and then ramped at 15 °C/min to 320 °C and held for 2.00 min. The GC was

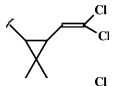
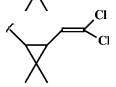
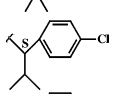
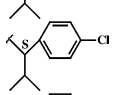
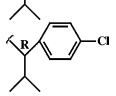
interfaced with a HP 5973 MS run in full scan mode from 50 to 550 m/z with a quadrupole temperature of 186 °C and a source temperature of 240 °C. The EI–GC/MS fragmentation patterns and NMR spectra supported all reported structures. Melting point data were collected with a Thomas–Hoover apparatus (A.H. Thomas Co., Philadelphia, PA, USA) and are uncorrected. Compound purity was >97% as determined by NMR, GC/MS, and melting point.

Synthesis

Compounds were synthesized under N_2 and reaction progress was monitored using thin-layer chromatography (TLC) 0.2-mm glass plates precoated with silica gel 60 F_{254} (E. Merck, Darmstadt, Germany) and are listed in Table 1. Chemical detection was based on the quenching of fluorescence from ultraviolet light at 254 nm. Flash chromatographic separations were carried out using 40-μm average particle size Baker silica gel. The compounds (*R*/*S*)-α-cyano(6-methoxy-2-naphthyl)methyl acetate (compound **1**) and (*R*/*S*)-α-cyano (6-methoxy-2-naphthyl)methyl (*R*/*S*)-*trans*/*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (compound **3**) were available from earlier work in this laboratory [15].

The racemic cyanohydrin (*R*/*S*)-2-hydroxy-2-(2-naphthyl)ethanenitrile was readily prepared from 2-naphthaldehyde via hydrolysis of the α-cyano trimethylsilyl ether according to methods of Gassman and Talley [21] as shown in Fig. 2. The product was isolated as a cream-colored solid in 99% yield (mp 108–109 °C). TLC R_f 0.44 (hexane:EtOAc 3.5:1, v/v). ^{1}H NMR

Table 1
Synthesized compounds as described in Fig. 2

No.	X	*R*[a]
1	OCH_3	CH_3
2	H	CH_3
3	OCH_3	
4	H	
5	OCH_3	
6	H	
7	OCH_3	
8	H	$CH_2CH_2CH_3$

[a] *R* or *S* indicates stereochemistry at the labeled chiral center and both stereocenters at positions 1 and 3 are unresolved.

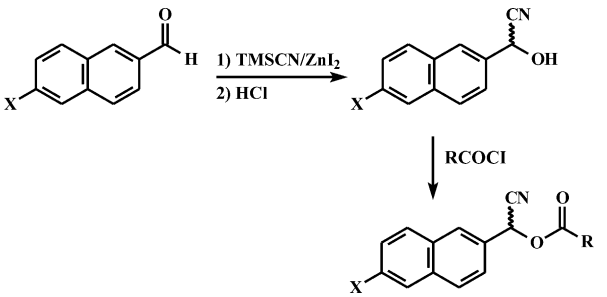


Fig. 2. Synthetic pathway for all α-cyanoester substrates in this study.

($CDCl_3$) δ 2.80 (s, 1 H, OH), 5.67 (s, 1 H, CH), 7.54–7.62 (m, 3 H, Ar), 7.86–8.03 (m, 4 H, Ar). The racemic cyanohydrin (*R*/*S*)-2-hydroxy-2-(6-methoxy-2-naphthyl) ethanenitrile was prepared as described by Shan and Hammock [15]. All cyanohydrins and ester compounds were synthesized as racemic mixtures, which showed only one UV-visible spot on TLC and no visible trace of the starting naphthaldehyde. Esterification was carried out by forming the acid chloride of the starting acid using thionyl chloride followed by reaction with the cyanohydrin according to the procedure described by Shan and Hammock [15].

(R/S)-α-Cyano-2-naphthylmethyl acetate (compound **2***)*. Acetyl chloride was combined with (*R*/*S*)-2-hydroxy-2-(2-naphthyl)ethanenitrile and purified with flash chromatography to give a white creamy solid in 71% yield (mp 37–38 °C). TLC R_f 0.64 (hexane:EtOAc 3.5:1, v/v). ^{1}H NMR ($CDCl_3$) δ 2.2 (s, 3 H, CH_3), 6.6 (s, 1 H, CH), 7.54–7.62 (m, 3 H, Ar), 7.85–8.04 (m, 4 H, Ar); ^{13}C NMR ($CDCl_3$) δ 20.8 (CH_3), 63.4 (*C*CN), 116.4 (CN), 124.4, 127.2, 127.6, 128.0, 128.2, 128.5, 129.0, 129.6, 132.9, 133.8, 169.0 (C=O); GC/MS EI m/z 225 (M^+, 36%), 183 (90%), 165 (100%), 155 (22%), 139 (28%), 127 (34%).

(R/S)-α-Cyano-2-naphthylmethyl (R/S)-trans/cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (compound **4***)*. This compound was synthesized from the acid chloride of (*R*/*S*)-*trans*/*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid and (*R*/*S*)-2-hydroxy-2-(2-naphthyl)ethanenitrile and purified by flash chromatography to give a pale orange liquid that solidified upon storage in 95% yield (mp 52–60 °C). TLC R_f 0.88 (hexane:EtOAc 3.5:1, v/v). ^{1}H NMR ($CDCl_3$) δ 1.18–1.40 (m, 6 H, $2CH_3$), 1.91 (d, $J = 8.72$ Hz, 1 H, CH), 2.12 (dd, $J = 8.72$, 4.41 Hz, 1 H, CH), 6.22 (d, $J = 4.41$ Hz, 1 H, CH=CCl_2), 6.53 (s, 1 H, CH), 7.52–7.60 (m, 3 H, Ar), 7.84–8.14 (m, 4 H, Ar); ^{13}C NMR ($CDCl_3$) δ 15.2, 28.4, 29.0, 31.4, 33.7, 63.2 (*C*CN), 119.8 (CN), 124.1, 124.2, 124.3, 124.5, 125.0, 127.3, 127.8, 128.1, 128.2, 128.7, 133.1, 169.0 (C=O); GC/MS EI m/z 373 (M^+, 3%), 166 (100%), 139 (15%).

(R/S)-α-Cyano-(6-methoxy-2-naphthyl)-methyl-(S)-(+)-2-(4-chlorophenyl)-3-methyl butanoate (compound **5***)*. This compound was synthesized from (*S*)-(+)-4-

chloro-α-(1-methylethyl)benzene acetic acid and was recrystallized from hexanes to give a white powder in 5% yield (mp 139–140 °C). TLC R_f 0.37 (hexane:EtOAc 3.5:1, v/v). ^{1}H NMR ($CDCl_3$) δ 0.72 (d, $J = 7.0$ Hz, 3 H, CH_3), 1.07 (d, $J = 6.5$ Hz, 3 H, CH_3), 2.34 (m, $J = 10.5$ Hz, 1 H, CH_3CH), 3.24 (d, $J = 10.6$ Hz, 1 H, CHC=O), 3.92 (s, 3 H, OCH_3), 6.48 (s, 1 H, CHCN), 7.11–7.34 (m, 7 H, Ar),7.66–7.73 (m, 3 H, Ar); ^{13}C NMR ($CDCl_3$) δ 20.0 (CH_3), 21.2 (CH_3), 32.2 (Me_2C), 55.3 (*C*C=O), 58.6 (OCH_3), 63.2 (*C*CN), 105.6, 116.0 (CN), 119.9, 124.6, 126.2, 127.4, 128.0, 128.1, 128.8, 129.8, 133.5, 135.2, 135.4 158.8, 171.7 (C=O); GC/MS EI m/z 407 (M^+, 9%), 196 (100%), 167 (27%), 125 (34%).

(R/S)-α-Cyano-1-(2-naphthyl)-methyl-(S)-(+)-2-(4-chlorophenyl)-3-methyl butanoate (compound **6***)*. This compound was prepared from (*S*)-(+)-4-chloro-α-(1-methylethyl)benzene acetic acid and was recrystallized from ethanol to give a white powder in 93% yield (mp 100–101 °C). TLC R_f 0.86 (hexane:EtOAc 3.5:1, v/v). ^{1}H NMR ($CDCl_3$): δ 0.73 (d, $J = 6.7$ Hz, 3 H, CH_3), 1.08 (d, $J = 6.5$ Hz, 3 H, CH_3), 2.35 (m, $J = 6.6$, 10.5 Hz, 1 H, CH_3CH), 3.24 (d, $J = 10.5$ Hz, 1 H, CHC=O), 6.52 (s, 1 H, CHCN), 7.16–7.98 (m, 11 H, Ar), ^{13}C NMR ($CDCl_3$): δ 20.4 (CH_3), 21.6 (CH_3), 32.5 (Me_2C), 58.9 (*C*C=O), 63.4 (*C*CN), 116.2 (CN), 124.1, 127.2, 127.7, 128.0, 128.5, 129.0, 129.5, 130.0, 132.9, 133.3, 133.8, 134.0, 135.6, 171.9 (C=O); GC/MS EI: m/z 377 (M^+, 22%), 183 (8%), 166 (100%), 139 (27%).

(R/S)-α-Cyano-(6-methoxy-2-naphthyl)-methyl-(R)-(−)-2-(4-chlorophenyl)-3-methyl butanoate (compound **7***)*. Synthesis was performed as described above from (*R*)-(−)-4-chloro-α-(1-methylethyl)benzene acetic acid. The product was recrystallized from hexanes, giving a white spongy solid in 21% yield (mp 137–139 °C). TLC R_f 0.36 (hexane:EtOAc 3.5:1, v/v). ^{1}H NMR ($CDCl_3$) δ 0.72 (d, $J = 7.0$ Hz, 3 H, CH_3), 1.07 (d, $J = 6.5$ Hz, 3 H, CH_3), 2.34 (m, $J = 10.5$ Hz, 1 H, CH_3CH), 3.24 (d, $J = 10.5$ Hz, 1 H, CHC=O), 3.91 (s, 3 H, OCH_3), 6.47 (s, 1 H, CHCN), 7.11–7.34 (m, 7 H, Ar), 7.65–7.76 (m, 3 H, Ar); ^{13}C NMR ($CDCl_3$) δ 20.0 (CH_3), 21.2 (CH_3), 32.2 (Me_2C), 55.3 (*C*C=O), 58.6 (OCH_3), 63.2 (*C*CN), 105.6, 116.0 (CN), 119.9, 124.6, 126.2, 127.4, 128.0, 128.1, 128.8, 129.8, 133.5, 135.2, 135.4 158.8, 171.7 (C=O); GC/MS EI m/z 407 (M^+, 9%), 196 (100%), 167 (24%), 125 (31%).

(R/S)-α-Cyano-2-naphthylmethyl butanoate (compound **8***)*. The acid chloride of butyric acid was prepared as described above and combined with (*R/S*)-2-hydroxy-2-(2-naphthyl)ethanenitrile. The mixture was purified by flash chromatography to give a pale yellow oil in 75% yield. TLC R_f 0.82 (hexane:EtOAc 3.5:1, v/v). ^{1}H NMR ($CDCl_3$) δ 0.96 (t, $J = 7.2$ Hz, 3 H, CH_3), 1.60–1.75 (m, 2 H, CH_2), 2.40 (t, $J = 7.2$ Hz, 2 H, CH_2), 6.61 (s, 1 H, CH), 7.46–7.60 (m, 3 H, Ar), 7.82–8.04 (m, 4 H, Ar); ^{13}C NMR ($CDCl_3$) δ 13.6, 18.4, 36.1, 63.3 (*C*CN), 116.2 (CN), 124.2, 127.0, 127.5, 127.7, 127.8, 128.3, 129.0, 129.3, 132.8, 133.8, 173.6 (C=O); GC/MS EI m/z 253 (M^+, 56%), 183 (95%), 166 (100%), 139 (41%).

Aldehyde stability assays

Aldehyde stability determination. The effects of temperature, ionic strength, pH, and protein concentration upon the rate of aldehyde degradation and quenching were examined. Aldehyde fluorescence was measured for 20 min as described under *α-Cyanoester hydrolysis activity*. Unless otherwise stated, all assays were performed in Tris/HCl buffer (pH 8.0, 20 mM) and each well contained 0.5 nmol of aldehyde. A typical measurement involved the addition of the aldehyde (1 μl in ethanol of appropriate stock solution) to 200 μl of buffer. The effects of three different proteins, BSA, ovalbumin, and rat liver microsomes, were examined. Temperature effects were examined at 30 and 37 °C. Ionic strength was studied at 20, 50, and 100 mM, while pH effects were measured from 7.0 to 9.0 at 0.5 pH unit intervals.

Aldehyde solubility determination. The solubility of 6-methoxynaphthaldehyde and 2-naphthaldehyde was determined according to methods of Nellaiah et al. [22] as described in Wheelock et al. [20]. Briefly, compounds were dissolved in dimethylformamide and 10 μl of solution was added to 1 ml sodium phosphate buffer (pH 7.4, 100 mM) in a cuvette at 25 °C. The turbidity of the solution was then measured by reading the absorbance at 700 nm on a Cary 100 Bio spectrophotometer (Varian). Compound solubility was determined by a significant increase in absorption (greater than fourfold) and reported as a range of concentrations.

Substrate pH stability determination. The stability of (*R/S*)-α-cyano-(6-methoxy-2-naphthyl)methyl acetate (compound **1**) versus that of PNPA was determined for a range of pH values (7.0–9.0). A series of buffers was prepared at varying pH using either sodium phosphate (100 mM) or Tris/HCl buffer (100 mM). The substrate was incubated in buffer at 37 °C as described in the following section. Overlapping pH values were used to verify that there were no buffer effects upon substrate hydrolysis.

Preparation of human liver microsomes

Human liver was stored at -135 ± 5 °C until use. For microsome preparation, livers were thawed and rinsed in 1.15% KCl. Samples were then homogenized with a motor-driven Teflon–glass homogenizer (five passes) in sodium potassium phosphate buffer (pH 7.4, 100 mM) at 4 °C. Homogenates were centrifuged at 10,000*g* at 4 °C for 30 min. The supernatant was removed and centrifuged at 105,000*g* at 4 °C for 60 min. The pellet was resuspended in phosphate buffer, centrifuged again at 105,000*g* at 4 °C for 60 min, and then resuspended in phosphate buffer. The microsomal protein of each microsome preparation was determined according to Bradford [23] and stored at -135 ± 5 °C.

Carboxylesterase assays

Standard substrates. Assays were performed in 96-well microtiter styrene flat-bottom plates (Dynex Technologies, Inc., Chantilly, VA, USA) and analyzed on a Spectramax 200 plate reader (Molecular Devices, Sunnyvale, CA, USA). All assays were designed such that no more than 10% of the substrate was hydrolyzed during the assay and solvent content never exceeded 1% of the total assay volume. Reported results are all corrected for background hydrolysis of the substrate.

p-Nitrophenyl acetate activity. Assays were performed using sodium phosphate buffer (pH 7.4, 100 mM) at 37 °C according to methods of Ljungquist and Augustinsson [24] as described in Wheelock et al. [20]. The amount of microsomal protein added per assay varied with each individual preparation, but ranged from 1.7 to 4.8 μg/well. Activity was monitored for 2.0 min at 405 nm.

α-Naphthyl acetate activity. All assays were performed by methods of Van Asperen [25] using sodium phosphate buffer (pH 7.4, 100 mM) at 37 °C. Protein added per well was the same as for PNPA assays. The plate was analyzed by monitoring absorbance at 450 nm for 2.0 min.

α-Cyanoester hydrolysis activity. All assays were performed under the optimized conditions as determined by the aldehyde stability studies. Fluorescence assays were conducted with a Spectrafluor Plus (Tecan, Research Triangle, NC, USA) running Magellan v. 2.50 software. Assays were conducted in black 96-well polystyrene clear flat-bottom microtiter plates (Corning, Inc., New York, NY, USA) at 37 °C. The total assay volume was 200 μl, consisting of 180 μl Tris/HCl buffer (pH 8.0, 20 mM) and 20 μl of enzyme preparation. Substrate solutions were prepared in ethanol (10 mM; except for compounds **5**, **6**, and **7**, which also contained 10% Me_2SO) and assays were initiated by the addition of 1 μl substrate solution (final concentration 50 μM) followed by shaking for 5 s. Production of 6-methoxynaphthaldehye was monitored with excitation at 330 nm (bp 35) and emission at 465 (bp 35). Assays with 2-naphthaldehyde as the product were performed with an excitation wavelength of 346 nm (bp 35) and an emission wavelength of 450 nm (bp 35). All assays were performed with the instrument gain set to 60. Assays were configured such that no more than 10% of the substrate was hydrolyzed during the assay and solvent added never exceeded 1% of the total assay volume. Reported activities were all corrected for background hydrolysis. Standard curves were generated by adding an equivalent amount of rat liver microsomal protein to each well to account for protein-induced aldehyde quenching. Assays for compounds **1**, **2**, and **8** were performed with 3 flashes and 10 cycles to give an ~3-min linear assay. Assays for compounds **3**, **4**, **5**, **6**, and **7** were performed with 10 flashes and 30 cycles for an ~20-min linear assay. The amount of protein added in each assay varied with the substrate and was adjusted such that the assay was linear over the reported time.

Substrate sensitivity determination

Substrates (PNPA, α-naphthyl acetate, compound **1**, and compound **8**) were incubated with a range of porcine esterase concentrations (1×10^{-5}–1×10^{-3} mg/ml) in Tris/HCl buffer (pH 8.0, 20 mM) at 37 °C. Each substrate assay was performed as described above.

Pyrethroid hydrolysis assays

All assays to determine pyrethroid hydrolysis rates were conducted with porcine esterase due to low activity with human liver microsomes. Assays were configured such that no more than 10% of the substrate was hydrolyzed over the life of the assay. Assays were initiated by adding 4 μl of substrate (25 mM in ethanol) to 2 ml of enzyme preparation for a final concentration of 50 μM and incubating for 5.0 min at 37 °C. After 5.0 min, 1 ml of ethyl acetate and 1 ml of brine were added to each sample, followed by vortexing for 30 s and centrifugation for 5.0 min. Next, 250 μl of ethyl acetate was removed from the sample and placed into a 1.5-ml autosampler vial containing a silanized 300-μl insert (National Scientific Co., Quakertown, PA, USA). Last, 10 μl of the 3-(4-methoxy)phenoxybenzaldehyde internal standard (2 mM in ethanol) were added (final concentration 77 μM). The assay was based upon detecting the esterase hydrolysis products (see Fig. 1): 3-phenoxybenzylalcohol for Type I pyrethroids (extraction efficiency $103 \pm 3\%$) and 3-phenoxybenzaldehyde for Type II pyrethroids (extraction efficiency $96 \pm 4\%$).

Samples were analyzed on a HP 6890 GC (Agilent Technologies) equipped with a 0.25 mm i.d. × 30 m, 0.25-μm DB-XLBms column (J&W Scientific, Folsom, CA, USA) with a constant He gas flow rate of 0.8 ml/min. The injector temperature was 250 °C. The initial column temperature of 100 °C was held for 5.00 min and then ramped at 20 °C/min to 320 °C and held for 2.00 min. The GC was interfaced with a HP 5973 MS run in selected-ion monitoring mode scanning for ions 228 and 213 (3-(4-methoxy)phenoxybenzaldehyde), 198 and 141 (3-phenoxybenzaldehyde), and 200 and 141 (3-phenoxybenzylalcohol). The quadrupole temperature and source temperature were 186 and 240 °C, respectively.

Cytochrome P450 assays

Microsome standards and antibodies. Microsomes were prepared by Gentest Corp. (Woburn, MA) from

cells expressing only one isoform of P450. The microsomal protein content and total cytochrome P450 activity were determined by Gentest Corp. Microsomes from P4501A1-, 2E1-, and 3A4-induced cell lines were run with the human microsomes as a reference. The various goat anti-rat polyclonal antibodies were donated by Daichi Pure Chemical Co. (Tokaimura, Japan): a polyclonal antibody against rat 1A1 that also recognizes human 1A1 and 1A2, a polyclonal antibody against rat 2E1 that recognizes 2E1 and 2A6 human P450 forms, and a polyclonal antibody against rat cytochrome 3A2 that recognizes human 3A4.

SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. SDS–PAGE was performed according to the method of Laemmli [26], using a 4% stacking gel and a 10% running gel. The proteins were electrophoresed for 1 h at 100 V and then electroblotted onto nitrocellulose membranes according to the method of Towbin et al. [27]. The nitrocellulose membranes were blocked for 1 h in 1% Blotto (nonfat powdered milk) in phosphate-buffered saline (PBS), rinsed in PBS with 0.1% Tween, and incubated with primary antibody for 1 h. Western blots were probed with the anti-rat cytochrome P450 1A1, 2E1, and 3A2 described above. The blots were then incubated with a rabbit anti-goat affinity-purified IgG Fab fragments coupled with alkaline phosphatase and visualized with NBT/BCIP (Pierce, Rockford, IL).

Total cytochrome P450. The content of microsomal cytochrome P450 was determined from the dithionite difference spectra of CO-treated samples, assuming an extinction coefficient of $91\,mM^{-1}\,cm^{-1}$ for P450 and $111\,mM^{-1}\,cm^{-1}$ for P420 by the method of Omura and Sato [28].

P450 activity assays. All activity assays were performed using standard procedures and are not further described. 7-Ethoxycoumarin *O*-deethylase activity was determined according to Greenlee and Poland [29]. Ethoxyresosufin activity was measured by methods of Pohl and Fouts [30]. Bufuralol 1′-hydroxylase activity was determined according to the method of Kronbach et al. [31]. Mephenytoin 4′-hydroxylase activity was measured as described by Goldstein et al. [32]. Testosterone 6β-hydroxylase activity was determined according to the method of Pearce et al. [33].

Microsomal epoxide hydrolase activity. Activity was determined using *cis*-[^{3}H]stilbene oxide as the substrate [34]. Samples were incubated for 5 min in Tris/HCl buffer (pH 9.0, 10 mM) containing 0.1 mg/ml BSA at 30 °C with a final substrate concentration of 50 μM. The activity was determined by measuring the quantity of radioactive diol formed using a liquid scintillation counter (Wallac Model 1409; Gaithersburg, MD, USA). Added protein varied from 0.8 to 1.6 μg and activity was reported as nmol of diol formed/min/mg protein.

In-gel hydrolytic activity assays

For two-dimensional electrophoresis, 150 μg microsomal protein was diluted to 125 μl with 5% glycerol and 2% IPG buffer (Amersham Biosciences, Uppsala, Sweden). Seven-centimeter IPG Drystrips (Amersham Biosciences), pH 4–7, were rehydrated in the protein solution at 4 °C overnight in a reswelling tray (Amersham Biosciences). The Drystrip was transferred to a precooled 2117 Multiphore II IEF unit (LKB, Bromma, Sweden), and the strips were focused for a total of 44 kVh at 4 °C using a EPS 3501 XL power supply (Amersham Biosciences) with the following program: 50 V for 1 h, 50–1000 V gradient for 3 h, 1000–3500 V gradient for 2.5 h, 3500 V for 10.5 h. The focused Drystrips were equilibrated in cooled native running buffer (26 mM Tris, 192 mM glycine, pH 8.1) for 15 min and were then loaded onto 1.5-mm 10% Novex Tris–glycine minigels (Invitrogen, Carlsbad, CA, USA). The 2D well was sealed with 1% isogel agarose (BioWhittaker Molecular Applications, Rockland, ME, USA), and electrophoresis was performed at an 80–125 V gradient the first hour, then at 125 V for 3 h. The gels were equilibrated in room temperature Tris/HCl buffer (pH 8.0, 20 mM) for 0.5–1 h.

Ester hydrolysis was measured in a Fluor-S Imager (Bio-Rad, Hercules, CA, USA). Esterase substrates were prepared immediately before hydrolysis from a 20 mM stock solution in Me_2SO for a final concentration of 100 μM (10 μM for 4-methylumbelliferyl acetate) in Tris/HCl buffer (pH 8.0, 20 mM). The gel was placed on the imaging plate and substrate was applied to just cover the gel. After 8 min incubation excess substrate was removed, the gel was transilluminated with UV light (290–365 nm), and fluorescence was measured at high sensitivity with a clear filter for 10–20 s depending on substrate. Afterward gels were either stained with silver stain according to Yan et al. [35] or developed with α-naphthyl acetate [36], and scanned in a UMAX Powerlook III flatbed scanner (UMAX Technologies, Inc., Dallas, TX, USA).

One-dimensional native PAGE was performed as described for the second dimension above, except that gels were incubated with the esterase substrates for 1–2 min, and fluorescence was measured for 2–10 s. Data were quantified using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Results and discussion

Two aldehydes were used in this work to prepare the described substrates: 6-methoxynaphthaldehyde, first reported by Shan and Hammock [15], and 2-naphthaldehyde. We examined a number of additional aldehydes for use in substrate synthesis and chose

2-naphthaldehyde for substrate development for multiple reasons: increased solubility, large Stokes' shift, production of more "pyrethroid-like" surrogates, and decreased cost relative to 6-methoxynaphthaldehyde.

Stability studies

Physical parameters (filter width, buffer, pH). The first variable examined was the effect of filter bandwidth upon the signal/noise ratio. We examined two configurations of bandwidths for excitation and emission, a narrow bandwidth (20 nm) and a broad bandwidth (35 nm). All permutations for excitation and emission were examined for 6-methoxynaphthaldehyde and it was determined that a 35-nm bandwidth for both excitation and emission was preferable because it increased the sensitivity of the assay (data not shown). This filter configuration with the broader bandwidth was used for all assays with both aldehydes. 6-Methoxynaphthaldehyde consistently gave a stronger fluorescent response on a molar basis compared to 2-naphthaldehyde (~3× greater).

Aldehyde stability assays. Studies were undertaken to determine whether the fluorescent aldehyde produced in this assay was sufficiently stable to be used in a continuous assay format.

Aldehyde stability studies were first performed in two different buffer systems (sodium phosphate and Tris/HCl) to examine for buffer effects. No differences were observed between these two buffers, and the remainder of the stability assays were conducted with Tris/HCl buffer (data not shown). The rates of aldehyde loss at two different concentrations of aldehyde were also examined for concentration effects. We observed no differences in the degradation rates at 5.0 and 0.5 nmol/well, when expressed as a function of total aldehyde. Subsequent aldehyde stability assays were performed at 0.5 nmol/well.

We examined ionic strength effects in 20, 50, and 100 mM Tris/HCl buffer. 2-Naphthaldehyde showed no effects of ionic strength or pH upon degradation rates (data not shown), whereas 6-methoxynaphthaldehyde exhibited increased degradation (~1–5 pmol/min) at higher pH. However, it should be noted that the decreased fluorescence observed with 2-naphthaldehyde over 6-methoxynaphthaldehyde may be responsible for this observation. Degradation rates for 6-methoxynaphthaldehyde were not significant at 20 mM and were slow at higher ionic strengths, indicating that it is possible to run these assays at higher ionic strength and pH if necessary. It is important to point out that degradation of the aldehyde during most esterase assays will be so minor that it is negligible (all assays in this study exhibited <1% aldehyde loss over the length of the assay). However, for samples with low esterase activity resulting in high protein concentrations and long incubation times, or in assays in the presence of cofactors or living cells, loss of aldehyde may become significant.

Effects of protein concentration. Another potential source of interference is the amount of protein added to the assay. We found significant variations in the effects of protein concentration upon aldehyde fluorescence, both in terms of aldehyde response and quenching. The effect on both aldehydes was similar in that fluorescence quenching increased with protein concentration from 0.002 to 1000 μg protein/well (data not shown). Significant quenching in terms of relative fluorescence units/mole (RFU/mol) was observed at protein concentrations greater than 1 μg/well, which is below the level at which many esterase assays are performed (~5–50 μg/well). The rate of aldehyde loss was constant for all three "proteins" up to 100 μg/well at which point the rate of loss increased dramatically for rat liver microsomes, but not for ovalbumin or BSA (data not shown). It appears that aldehyde loss is dependent upon both protein type and concentration. The fact that noncatalytically active protein did not affect aldehyde degradation suggests that the loss might be due to enzymatic activity in the microsomal preparation such as aldehyde dehydrogenase (ALDH). However, because no cofactors were added, it is unlikely that aldehyde loss is due to either ALDH or P450 activity. We determined standard curves from 0 to 100 μg protein/well and observed a significant difference in the slopes of the resulting trend lines (Fig. 3). These

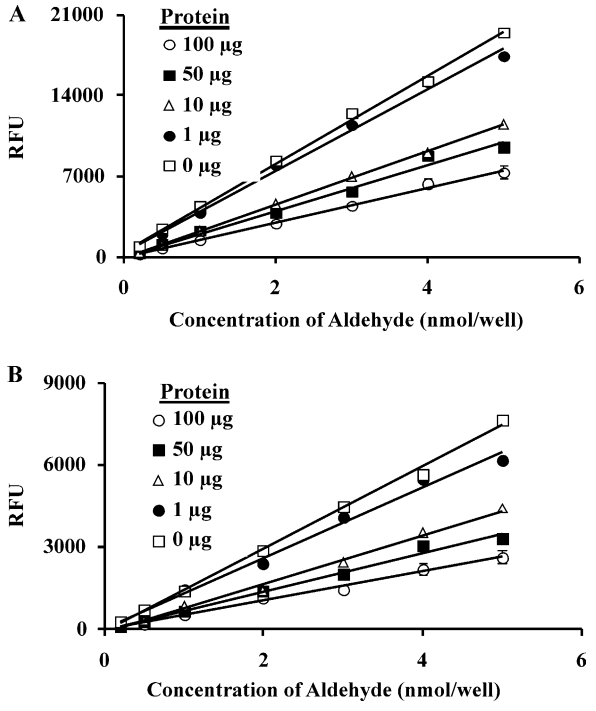


Fig. 3. Dependence of aldehyde fluorescence response and linearity upon protein concentration. Both 6-methoxynaphthaldehyde (A) and 2-naphthaldehyde (B) were incubated with rat liver microsomes at a range of concentrations (0–100 μg/well). Assays were performed in Tris/HCl buffer (pH 8.0, 20 mM) at 37 °C. All trend lines for both A and B have r^2 values $\geqslant 0.99$. Data are the averages ± the standard deviations of four replicates.

assays therefore require the use of appropriate standard curves, which contain an equal amount of similar protein to correct for degradation and quenching effects.

We originally hypothesized that the quenching effect of protein upon aldehyde fluorescence was due to Schiff's base formation between free amino groups on the protein and the reporter aldehydes. The effect of amines upon aldehyde fluorescence using two different amines, 3-amino-propanol and lysine, were therefore determined. We examined the effects of amine concentrations varying from 0.002 to 1000 μg/well and saw no effect with either amine (data not shown). It therefore appears that the mechanism by which the protein quenches aldehyde fluorescence is not through direct reaction with amine residues.

Substrate stability and solubility. We compared the hydrolysis of PNPA and compound **1** from pH 7.0 to 9.0, which encompasses the range over which esterase assays are typically conducted [1]. PNPA was hydrolyzed 17× faster than compound **1**, with a background hydrolysis of 243 ± 20 pmol/min as opposed to 14.3 ± 0.5 pmol/min for compound **1** (Fig. 4). These effects remained similar with increasing pH. The increased stability of these new substrates is partly due to the presence of the α-cyano group, which stabilizes the ester. It is also possible that steric effects are important. We compared the signal/noise ratio of two α-cyanoester substrates versus PNPA and α-naphthyl acetate, and found that the α-cyanoesters consistently had increased signal/noise ratios over a range of enzyme concentrations (Fig. 5). These results demonstrate the increased sensitivity of these α-cyanoesters versus PNPA and α-naphthyl acetate.

Solubility is an important component of the assay and is of special consideration when synthesizing pyrethroid analogs. Most synthetic pyrethroids are very insoluble and have log *P* values on the order of 6–7 [37]. It can therefore be difficult to achieve a high enough substrate concentration in an assay to ensure that the enzyme is operating at V_{max} conditions. The use of more water-soluble aldehydes to synthesize these substrates should increase their water solubility. However, care must be taken to prevent a subsequent loss in fluorescence. We found that 2-naphthaldehyde was ~2× as soluble as 6-methoxynaphthaldehyde, 1.0–1.5 mM versus 0.50–0.75 mM, respectively (Fig. 6). In situations in which the substrate was very hydrophobic, the difference in aldehyde solubilities could be very important in increasing the substrate solubility.

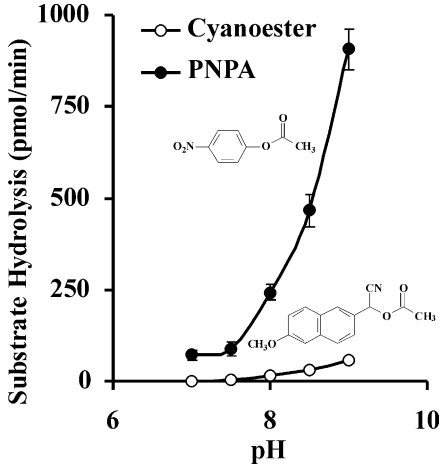


Fig. 4. The pH dependence of hydrolysis of (*R*/*S*)-α-cyano-(6-methoxy-2-naphthyl)methyl acetate (cyanoester, compound **1**) and *p*-nitrophenyl acetate (PNPA). Substrates were incubated in 100 mM Tris/HCl buffer at each pH. Hydrolytic activity was monitored for each substrate as described in the text. Data are the averages ± the standard deviations of three replicates.

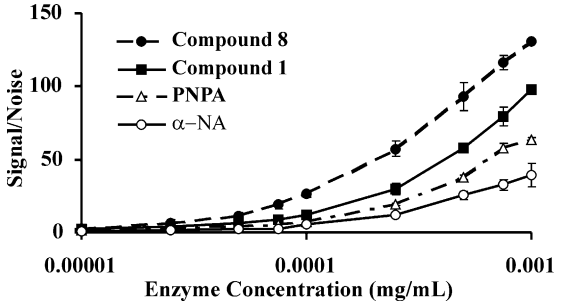


Fig. 5. The variance of signal-to-noise ratio of different substrates with decreasing enzyme concentration. Porcine esterase was incubated with each substrate in Tris/HCl buffer (pH 7.4, 20 mM) as described in the text. Data are the averages ± the standard deviations of six replicates.

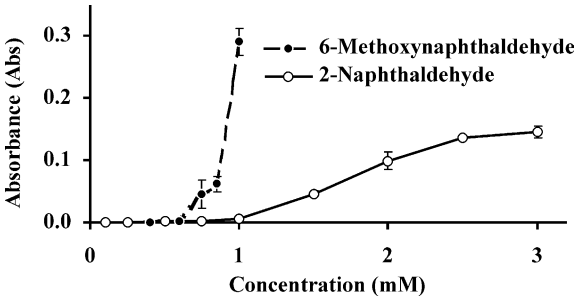


Fig. 6. Determination of the water solubility of 6-methoxynaphthaldehyde (0.50–0.75 mM) and 2-naphthaldehyde (1.0–1.5 mM) in sodium phosphate buffer (pH 7.4, 100 mM) at 25 °C. Data are the averages ± the standard deviations of three replicates.

Summary of stability studies. Based upon these studies, it was concluded that 6-methoxynaphthaldehyde is superior to 2-naphthaldehyde. 6-Methoxynaphthaldehyde has higher RFU/mol than 2-naphthaldehyde (Fig. 3), decreased background, and decreased rate of aldehyde loss with protein concentration. However, in situations in which substrate solubility is important, 2-naphthaldehyde could be more useful.

Enzyme assays

To evaluate the hydrolysis activity of these new substrates, they were compared against standard

substrates using a commercial porcine esterase preparation. Table 2 provides the porcine esterase specific activity for all substrates synthesized in this study as well as those for the standard substrates, PNPA, α-naphthyl acetate, and 4-methylumbelliferyl acetate. The α-cyanoesters were hydrolyzed slower than the reference substrates and the pyrethroid analogs were predictably hydrolyzed slower than the alkyl-substituted α-cyanoesters.

In order to further examine the utility of these new substrates for measuring esterase activity, a set of 10 human liver samples was examined (Table 3). Hydrolysis activity of the α-cyanoesters was compared against PNPA and α-naphthyl acetate. PNPA is a standard substrate used to monitor esterase activity and has been extensively used in the literature [1,20]. The main advantages in working with this substrate are that it is easily synthesized, very soluble, and the excellent leaving group makes it readily hydrolyzed. It is subsequently considered to be a broad reporter of esterase activity [1], but it is unlikely that this single substrate provides an accurate assessment of relative esterase abundance when multiple isozymes are present. The values for PNPA hydrolysis in Table 3 showed a fivefold range in activity and are in agreement with those reported by Hosokawa et al. of 2.02 μmol/min/mg protein as the average of 12 individuals [38]. The specific activity in mouse liver mi-

Table 2
Porcine esterase hydrolysis of substrates

Compound	Specific activity (nmol/min/mg)[a]
Acetates	
1	5130 ± 340
2	2040 ± 200
PNPA[b]	$119,000 \pm 7500$[c]
α-NA[d]	$150,000 \pm 1340$[c]
4-MU acetate[e]	$42,000 \pm 3500$[f]
Cyclopropane carboxylates	
3	33 ± 3
4	4.5 ± 0.4
Permethrin	390 ± 20
Cypermethrin	70 ± 1
Isovalerates	
5	5.4 ± 0.4
6	3.1 ± 0.3
7	5.6 ± 0.2
5 and **7**[g]	4.9 ± 0.4
Fenvalerate	2.7 ± 0.1
Esfenvalerate	2.5 ± 0.1
Butyrate	
8	$10,530 \pm 400$

[a] Data are reported as the averages ± the standard deviations of at least three replicates.
[b] *p*-Nitrophenyl acetate.
[c] Activity data from Huang et al. [39].
[d] α-Naphthyl acetate.
[e] 4-Methylumbelliferyl acetate.
[f] Activity data from Shan and Hammock [15].
[g] Assay was conducted with a 50:50 mix of compounds **5** and **7**.

Table 3
Carboxylesterase specific activity in human liver microsomes (nmol/min/mg)[a]

No.	*p*-Nitrophenyl acetate	α-Naphthyl acetate	Compound **1**	Compound **2**	Compound **3**	Compound **7**	Compounds **5** and **7**[b]	Compound **8**
1	1980 ± 50	3300 ± 120	248 ± 44	176 ± 27	1.87 ± 0.18	0.77 ± 0.08	0.41 ± 0.03	373 ± 68
2	3240 ± 220	2520 ± 10	325 ± 48	143 ± 26	1.71 ± 0.11	0.81 ± 0.05	0.32 ± 0.05	448 ± 54
3	1680 ± 80	2730 ± 400	158 ± 9	76 ± 12	1.16 ± 0.12	0.55 ± 0.03	0.27 ± 0.04	217 ± 41
4	2040 ± 20	4610 ± 860	236 ± 23	173 ± 31	1.30 ± 0.09	0.44 ± 0.03	0.21 ± 0.02	444 ± 66
5	2230 ± 20	2100 ± 300	283 ± 29	120 ± 21	3.01 ± 0.58	0.73 ± 0.02	0.37 ± 0.03	472 ± 58
6	2720 ± 40	7000 ± 760	123 ± 14	74 ± 13	2.20 ± 0.07	0.92 ± 0.11	0.41 ± 0.03	293 ± 47
7	5306 ± 90	7370 ± 690	348 ± 52	86 ± 15	1.81 ± 0.23	0.55 ± 0.09	0.24 ± 0.03	350 ± 56
8	4660 ± 90	3380 ± 290	736 ± 128	216 ± 38	1.52 ± 0.18	0.49 ± 0.02	0.20 ± 0.01	605 ± 83
9	4170 ± 130	6220 ± 510	399 ± 70	164 ± 28	2.83 ± 0.23	1.21 ± 0.11	0.50 ± 0.02	548 ± 39
10	8520 ± 380	7250 ± 370	662 ± 45	137 ± 23	2.96 ± 0.31	1.30 ± 0.12	0.53 ± 0.05	517 ± 34
Average[c]	3700 ± 2100	4650 ± 2120	345 ± 188	137 ± 48	2.04 ± 0.68	0.78 ± 0.30	0.35 ± 0.12	427 ± 125
% RSD	58%	46%	57%	35%	34%	38%	34%	28%
Pooled[d]	1540 ± 70	3420 ± 660	298 ± 51	167 ± 15	1.70 ± 0.13	0.61 ± 0.07	0.32 ± 0.05	136 ± 30
Murine[e]	8150 ± 390	5650 ± 460	527 ± 27	358 ± 52	0.97 ± 0.11	0.40 ± 0.07	0.30 ± 0.04	772 ± 104

[a] All samples were run in at least triplicate. Compounds **4**, **5**, and **6** had no detectable hydrolysis activity in either human or murine microsomes. Assay precision with the α-cyanoester substrates always exhibited <1% variability.
[b] Assays were run with a 50:50 mixture of compound **7** (*R*/*S*, *R* isomer) and compound **5** (*R*/*S*, *S* isomer).
[c] Average of all 10 human samples.
[d] Commercial sample of 16 pooled human livers.
[e] Mouse liver microsomes.

crosomes was roughly 4× that of human liver microsomes.

The hydrolysis results for α-naphthyl acetate were on average ~30% greater than those for PNPA, with human and mouse microsomes displaying similar activities. These results are slightly higher than those reported by Huang et al. who reported that average human liver microsome hydrolysis activity was 1.07 μmol/min/mg protein and that of mouse was 1.04 μmol/min/mg [39]. The similarity of PNPA and α-naphthyl acetate in detecting a broad range of esterases was reflected in their correlation values (Table 4; $r = 0.62$, $p < 0.05$).

As one would expect for aliphatic in contrast to aromatic esters, all of the α-cyanoesters were hydrolyzed at a lower rate than PNPA and α-naphthyl acetate. The relative rates of ester hydrolysis in human blood are butyl > propyl > ethyl > methyl [40]. This trend held true for hydrolysis data in liver microsomes for compound **8** (propyl) versus compound **2** (methyl), with compound **8** having from two- to fourfold greater activity than compound **2**. Structurally, these two substrates are similar as evidenced by their identical ranges of activity (threefold) and high degree of correlation ($r = 0.79$, $p < 0.01$). Compound **1** and compound **2** have quite different activity profiles, yet differ only by the presence of the 6-methoxy group on compound **1**. Compound **1** exhibited a sixfold range in activity, suggesting that a different isozyme(s) may be involved in compound **1** hydrolysis compared to compound **2**. The hydrolysis of these two compounds is not correlated at the 95% confidence level ($r = 0.59$, $p > 0.05$); however, they are correlated at 90% ($p < 0.1$). These data suggest that the 6-methoxy substituent affects the K_m and/or k_{cat} of some esterase isoforms. Results for the pyrethroid surrogates are discussed under *Pyrethroid hydrolysis assays*.

The range of esterase activities observed for the carboxylesterase substrates examined in this work was low. Lund-Pero et al. [41] reported an 18-fold range in activity for a steroidal esterase in mammary tissue among 16 women. Additionally, Hosokawa and co-workers reported a broad range of esterase activities for a number of substrates, with a 33-fold range for PNPA and a 45-fold range for *p*-nitrophenylpropionate [38]. Leng et al. [12] reported that the $t_{1/2}$ for cyfluthrin, a Type I pyrethroid hydrolyzed by esterases, varied over 46-fold among eight different agricultural workers who had been exposed to the pesticide. It appears that interindividual variation in carboxylesterase activity can be large across different groups and that any attempt to map the full range of activity needs to include a much larger data set with a variety of substrates. Substrates with unsubstituted naphthyl rings, α-naphthyl acetate, compound **2**, and compound **8**, had identical activity ranges (threefold), whereas those substrates that contained a substituent, PNPA and compound **1**, had similar activity ranges (five- and sixfold, respectively). These observations suggest that similar groups of isozymes hydrolyze the two different substrate groups.

No strong correlations were observed between esterase activity and either P450 or mEH activity (Table 5). However, the significance of these observations is limited by the small sample size (Table 6).

Pyrethroid hydrolysis assays

One of the main goals for this work was to evaluate these novel α-cyanoesters as pyrethroid surrogates with carboxylesterases. The hydrolysis rates of four different pyrethroids were measured for comparison: permethrin, a Type I pyrethroid; and cypermethrin, fenvalerate, and esfenvalerate, all Type II pyrethroids. These new fluorescent substrates all contain an α-cyano group and are more appropriate surrogates for Type II pyrethroids than Type I. Compounds **3** and **4** are cypermethrin analogs and compounds **5**, **6**, and **7** are fenvalerate analogs (Table 1). The stereochemistry of these compounds is important as each compound contains multiple stereocenters, one on the α-carbon of the alcohol and one or two in the acid. For the cyclopropane carboxylates, stereochemistry is defined by giving the *R*/*S* configuration of the 1-carbon and whether the substituted vinyl is *cis* or *trans* to the acid across the planar cyclopropane ring. Thus, cypermethrin has eight possible isomers and fenvalerate has four, with the in-

Table 4
Correlation table for esterase activity[a]

	PNPA[b]	α-NA[c]	**1**[d]	**2**	**3**	**7**	**8**
PNPA		0.62	0.78	0.10	0.45	0.54	0.48
α-NA			0.15	−0.28	0.32	0.47	0.00
1				0.59	0.24	0.24	0.80
2					−0.06	−0.05	0.79
3						0.79	0.41
7							0.28

[a] Correlation data are for 10 human liver microsome samples (Table 3).
[b] *p*-Nitrophenyl acetate.
[c] α-Naphthyl acetate.
[d] Refers to compound numbers in Table 1.

Table 5
Xenobiotic metabolizing enzyme activity in human liver microsomes[a]

No.	P450 isoforms (pmol/mg protein)			P450-associated activities (pmol/min/mg protein)					Total P450[g]	mEH[h]
	1A2	2E1	3A4	MEPH[b]	BUFH[c]	EROD[d]	ECOD[e]	TEST[f]		
1	40.2	72.3	53.7	99.5	25.0	186	359	1200	182	14.5
2	14.0	62.3	36.0	280	197	72.0	373	5100	243	19.4
3	41.2	39.7	51.0	38.8	98.4	195	151	1600	190	11.3
4	28.3	67.0	68.7	N.D.[i]	176	142	371	3800	171	28.0
5	56.8	67.0	148	98.3	125	168	465	11,000	409	22.3
6	27.5	56.7	144	N.D.	60.5	140	213	12,200	173	16.9
7	42.5	60.7	114	254	98.8	241	279	9100	250	15.7
8	37.2	42.7	54.0	93.4	77.1	185	158	2900	197	29.7
9	33.7	69.7	108	49.0	26.0	194	597	29,000	884	64.8
10	32.5	38.3	48.7	159	28.7	223	150	12,900	370	23.2
Average	35.4	57.6	82.6	134	91.3	1745	312	8880	307	24.6
StDev	11.3	12.9	42.0	90.0	60.8	48	150	8329	219	15.3
%RSD	32%	22%	51%	67%	67%	27%	48%	94%	71%	62%

[a] Data are the means of two replicates.
[b] *S*-Mephenytoin 4′-hydroxylase.
[c] Bufuralol 1′-hydroxylase.
[d] 7-Ethoxyresorufin *O*-deethylase.
[e] 7-Ethoxycoumarin *O*-deethylase.
[f] Testosterone 6-β-hydroxylase.
[g] Activity is reported as pmol/mg protein.
[h] Microsomal epoxide hydrolase, specific activity reported as nmol/min/mg protein (data are the means of three replicates).
[i] Not detected.

Table 6
Liver donor information[a]

No.	Sex	Age	Cause of death	Smoker
1	M	17	MVA[b]	No
2	M	36	ICH[c]	No
3	M	45	MVA	Yes
4	F	53	ICH	Yes
5	M	23	MVA	Unknown
6	F	36	MVA	No
7	F	44	PA[d]	No
8	M	64	ICH	Yes
9	M	73	ICH	Yes
10	F	71	ICH	Unknown

[a] All donors were described as Caucasian.
[b] Motor vehicle accident.
[c] Intracranial hemorrhage.
[d] Pulmonary arrest.

dividual *S*, α*S*-isomer of fenvalerate (esfenvalerate or A alpha) marketed alone due to its increased potency. Note that the most insecticidal 1*R*-cyclopropane carboxylate ester isomers have the same absolute configuration at this carbon as 1*S*-isovalerate esters. The stereocenter at the α-cyanoester was not controlled in the compounds synthesized here and was always *R*/*S*. Compounds **3** and **4** were therefore a mixture of all eight 1*R*/*S* *cis*/*trans* cypermethrin α*R*/*S* analogues. Compounds **5** and **6** were a mixture of two isomers (*S*, α*R*/*S*) synthesized from the *S*-isomer of the fenvalerate acid and are more appropriate analogs of esfenvalerate, whereas compound **7** (*R*, α*R*/*S*) was prepared from the *R*-isomer. The corresponding *R*-isomer with 2-naphthaldehyde was not synthesized, as it was not expected to be hydrolyzed in human liver microsomes given the lack of observed hydrolysis of compounds **4** and **6**.

Porcine liver esterase pyrethroid assays. The commercial preparation of porcine esterase is essentially one solubilized enzyme [42] and was used in all studies to compare the surrogate substrates with commercial pyrethroid hydrolysis (Table 2). Both compounds **3** and **4** were excellent surrogates for cypermethrin hydrolysis. Compound **3** was slightly superior to compound **4** because it was hydrolyzed ∼2× slower than cypermethrin, as opposed to compound **4**, which was hydrolyzed ∼15× slower. The Type I pyrethroid permethrin was hydrolyzed ∼12× faster than compound **3** and ∼86× faster than compound **4**, illustrating that these α-cyanoesters are better surrogates for Type II pyrethroids.

The fenvalerate analogs were hydrolyzed significantly slower than the cypermethrin analogs. The 2-naphthaldehyde derivative, compound **6**, was hydrolyzed at essentially the same rate as both fenvalerate and esfenvalerate, whereas compounds **5** and **6** with the 6-methoxynaphthaldehyde were hydrolyzed ∼2× faster. However, all of these surrogates are excellent reporters of fenvalerate hydrolysis activity. No stereoselectivity between the *R* and the *S* isomers was observed with the porcine enzyme, with compounds **5** and **7** having the same hydrolysis rates. A 50:50 mixture of **5** and **7** did not significantly affect hydrolysis rates.

Human liver microsome pyrethroid assays. After verifying that these surrogates were appropriate reporters

for pyrethroid hydrolysis, we examined hydrolysis activity in both human and murine liver microsomes (Table 3). Hydrolysis of compounds **4**, **5**, and **6** was not detectable at the highest concentration of human and murine microsomes examined (~45 μg protein/well). However, the hydrolysis rates for compound **3** (the 6-methoxynaphthaldehyde cypermethrin analog) and compound **7** (the 6-methoxynaphthaldehyde fenvalerate analog, *R*-isomer) were detectable in both systems. The observed hydrolysis was much lower than that for the other esterase substrates and did not correlate with them (Table 4). This observation provides further evidence that standard esterase substrates are not appropriate indicators of pyrethroid hydrolysis activity. However, compounds **3** and **7** correlated well with each other ($r = 0.79$, $p < 0.05$), indicating that similar isozymes are likely responsible for hydrolysis of these Type II pyrethroids.

We then examined the activity of a 50:50 mixture of compounds **5** and **7** for effects upon catalytic activity. The hydrolysis rate of compound **7** was reduced by ~50% with the addition of compound **5**, suggesting that the kinetics were dependent upon the stereochemistry. This observation is in agreement with the observed increased toxicity of esfenvalerate versus the racemic mixture [37]. These data raise the concern that esfenvalerate is potentially interacting with esterases in a mechanism similar to that of carbamates in which the compound forms an acyl intermediate with the enzyme that is not hydrolyzed, effectively inhibiting the enzyme. Given the implications of this effect for human toxicity following exposure to esfenvalerate, it is important to discern how this isomer interacts with the enzyme. These effects were not observed with the porcine system, further illustrating the fact that other mammalian systems are not perfect models for human enzyme activity. The rate of hydrolysis by the murine enzyme exhibited an effect between the porcine and the human systems, with the *S*-isomer reducing the hydrolysis rate of the *R*-isomer by 25%. These data suggest that murine microsomes, while not ideal, may be a more appropriate model for study of the human enzyme than the porcine system.

The results of this work indicate that these new fluorescent substrates can be appropriate surrogate substrates for some Type II pyrethroids. Further development of these substrates should focus on using 6-methoxynaphthaldehyde or test the utility of other aldehydes. It would be desirable to have an aldehyde with a greater Stokes' shift, particularly if it was further red-shifted, which could significantly reduce background in biological systems.

In-gel hydrolytic activity assays

Many studies report the utility of using in-gel assays for esterase activity with a number of different substrates: α-naphthyl acetate coupled to a diazonium salt [16], 4-methylumbelliferyl acetate [17], and fluorescein diacetate [43]. We compared the response of 4-methylumbelliferyl acetate (another fluorescent substrate) and α-naphthyl acetate in in-gel activity assays with our α-cyanoester substrates. These new substrates visualized hydrolysis activity in minutes and were consistently quicker than α-naphthyl acetate. Another advantage with these α-cyanoesters is that it is possible to further stain the gels with α-naphthyl acetate, silver stain, or Coomassie brilliant blue after the aldehyde activity stain. In fact, we found that both aldehydes seemed to sensitize proteins for silver staining relative to control silver-stained gels (data not shown). The pyrethroid surrogates (compounds **3**, **4**, **5**, **6**, and **7**) were hydrolyzed so slowly that it was not possible to visualize activity in a gel with any of the esterase systems used in this study. The data for compound **2** were very similar to those of compound **1**, except that they were less intense due to the decreased fluorescence of the produced aldehyde, and are therefore not displayed.

We determined the linearity of substrate response with protein concentration from 5 to 40 μg protein/lane and found that it varied with substrate, with compound **1** providing the most linear response followed by compounds **2** and **8** (Fig. 7). Both α-naphthyl acetate and 4-methylumbelliferyl acetate exhibited a high degree of linearity ($r^2 = 0.95$ and 0.98, respectively). An important quality of these α-cyanoester substrates for the continuous assay format is that the aldehyde hydrolysis product and the substrate itself are both water soluble (unlike the α-naphthyl acetate hydrolysis product, which couples to a diazonium salt). Unfortunately, due to the water solubility of the hydrolysis product, the fluorescent response diffuses over time and in-gel hydrolysis quantification becomes diffusion limited after ~15 min. The differences in aldehyde solubility were reflected in the quality of gel response. In both one and two dimensions, 6-methoxynaphthaldehyde consistently provided a more intense and less diffuse band or spot, which was most likely due to its decreased water solubility. Therefore limited water solubility of the product may be an asset in designing in-gel activity substrates, while increased solubility of the substrate is likely to be an advantage. Additional studies could attempt to derivatize the aldehyde to render it insoluble, thereby potentially increasing the sensitivity of the assay. However, caution needs to be exercised, because loss of fluorescence is possible.

The activity staining was useful for comparisons of human interindividual esterase activity. We quantified band intensity for compounds **1**, **2**, and **8** and compared them to 4-methylumbelliferyl acetate and α-naphthyl acetate (Fig. 8). Slight selectivity was observed with the substrates used in terms of the activity bands visualized. Compound **1** mainly visualized one intense band,

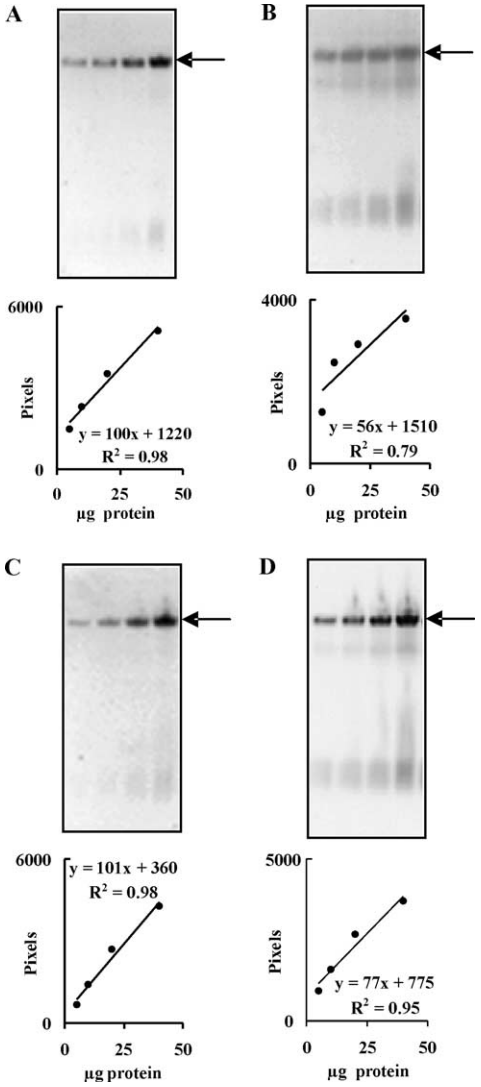


Fig. 7. Linearity of a commercial sample of 16 pooled human liver microsomes from 5 to 40 µg protein/lane. Native PAGE gels were run and analyzed as described in the text. The position of the arrow indicates the band used for quantification. (A) (*R*/*S*)-Cyano(6-methoxy(2-naphthyl))methyl acetate (compound **1**), (B) (*R*/*S*)-cyano-2-naphthylmethyl butanoate (compound **8**), (C) 4-methylumbelliferyl acetate, (D) α-naphthyl acetate. The r^2 value for substrate B increases to 0.99 if the 5-µg protein point is removed. (*R*/*S*)-Cyano-2-naphthylmethyl acetate (compound **2**) was not included as it provided a response similar to that of compound **1** ($y = 25x + 352$, $r^2 = 0.84$).

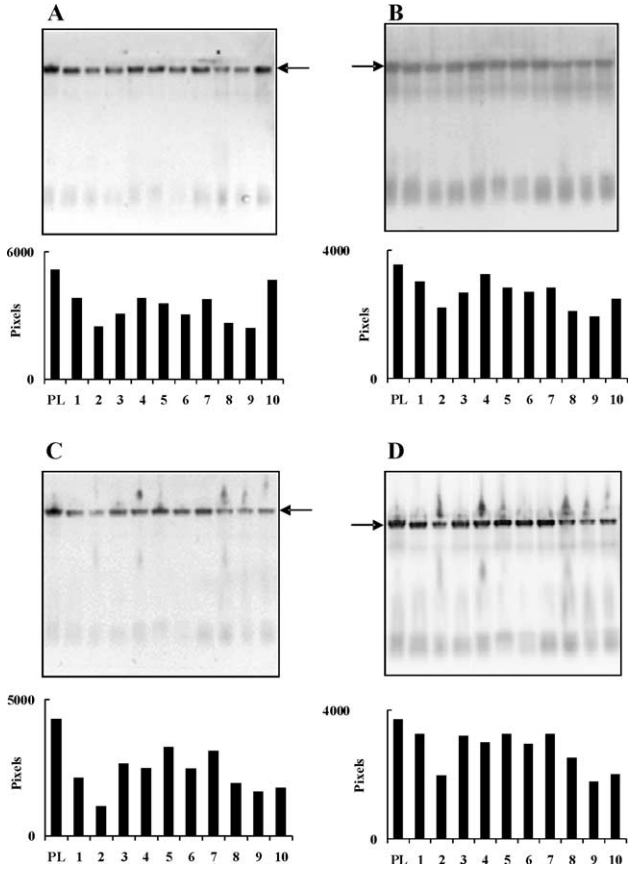


Fig. 8. Interindividual variation in carboxylesterase activity. Native PAGE gels were run and analyzed as described in the text. Arrows indicate the band used for quantification. (A) (*R*/*S*)-Cyano(6-methoxy(2-naphthyl))methyl acetate (compound **1**), (B) (*R*/*S*)-cyano-2-naphthylmethyl butanoate (compound **8**), (C) 4-methylumbelliferyl acetate, (D) α-naphthyl acetate. The numbers on each graph refer to each individual human liver sample and are consistent throughout the text. PL is a commercial pooled sample of 16 human livers. (*R*/*S*)-Cyano-2-naphthylmethyl acetate (compound **2**) was not included as it provided a response very similar to that of compound **1**, except that the fluorescence was reduced (max pixels 2000).

whereas compound **8** and 4-methylumbelliferyl acetate visualized two weaker bands and numerous smaller bands that appeared as streaking in the gel. Compound **1** provided the strongest signal, in terms of RFU/sample, whereas more interindividual variation was observed with 4-methylumbelliferyl acetate. Overall, the level of interindividual variation was rather low for all three substrates varying from 16 to 30% RSD, which agreed fairly well with the amount of variation noted with the other esterase substrates (PNPA and α-naphthyl acetate). It appears that the amount of interindividual variation remains constant with a range of esterase substrates. However, individual differences vary with specific substrates, for example, individual 10 has the highest activity for compound **8**, but not for compound **1**, thus demonstrating that there are isozyme-specific differences in esterase abundance between individuals.

Two-dimensional electrophoresis revealed additional substrate selectivity (Fig. 9). Compound **1** visualized one main spot (spot 1, p*I*∼5), whereas compound **8** strongly visualized two additional trains of spots (spots 2, p*I*∼5, and 3, p*I*∼6). 4-Methylumbellyl acetate visualized spots 1 and 3, but spot 2 was very faint. Activity staining with α-naphthyl acetate visualized spot 1 strongly and spot 2 much more weakly, and spot 3 was very faint. It is uncertain exactly how many individual esterases constitute each individual spot and it is very likely that there are multiple isozymes present and/or a varied degree of posttranslational modification of each isozyme. However, we can see selectivity among these different sub-

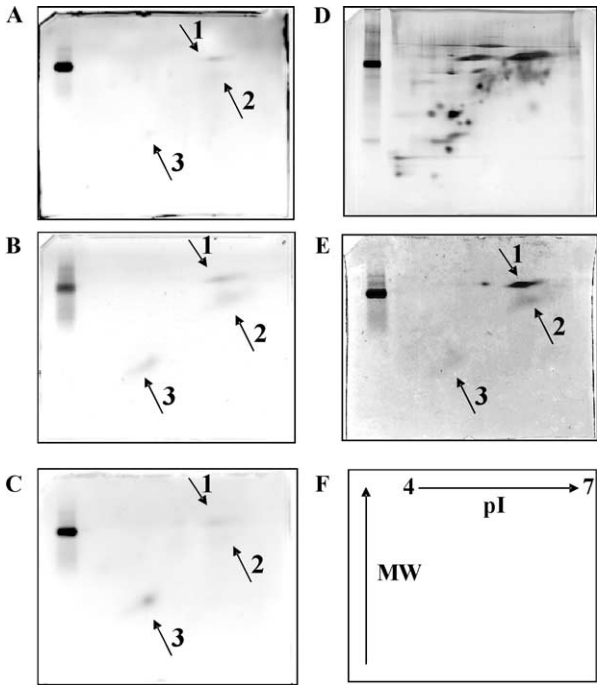


Fig. 9. Two-dimensional electrophoresis analysis of a commercial pooled sample of 16 human livers. Both first and second dimensions were run and analyzed as described in the text. (A) (*R*/*S*)-Cyano(6-methoxy(2-naphthyl))methyl acetate (compound **1**), (B) (*R*/*S*)-cyano-2-naphthylmethyl butanoate (compound **8**), (C) 4-methylumbelliferyl acetate, (D) silver stain, (E) α-naphthyl acetate, (F) legend for the first and second gel dimensions. The numbers and arrows on each graph refer to specific spots for comparison purposes. (*R*/*S*)-Cyano-2-naphthylmethyl acetate (compound **2**) was not included as it provided a response very similar to that of compound **1**, except that the fluorescence was reduced. Porcine esterase (200 ng/lane; Sigma Chemical Co.) was used as a positive control in the left-hand side of each gel.

strates and it appears that compound **8** is the most "general" substrate in that it visualizes the greatest amount of activity. This response is logical given that hydrolysis rates of the propyl moiety should be faster than those of the methyl, thus correspondingly increasing the signal [40]. These data demonstrate the utility of these substrates for in-gel activity assays. A potential application would be in proteomic analyses for studies interested in monitoring changes in esterase levels and activity. We have shown that they perform at least as well as a common fluorescent commercial substrate, 4-methylumbelliferyl acetate, in terms of fluorescent response. With certain analogs (compound **8**), we can detect more esterase activity as evidenced by the strong visualization of spot 2. Additionally, compound **8** provides a much stronger response than α-naphthyl acetate.

Conclusion

We have developed new esterase substrates and shown their application in detecting both general and pyrethroid-selective hydrolysis activity in human liver microsomes. Results showed that the hydrolytic profile was dependent upon the substrate structure and that these compounds are appropriate surrogates for pyrethroid hydrolysis activity. However, only those pyrethroid surrogates containing the 6-methoxy substituent had hydrolysis activity that could be detected in human liver microsomes. This effect could be due to the increased fluorescence of the 6-methoxynaphthaldehyde versus the 2-naphthaldehyde and/or effects upon K_m and/or k_{cat}. The pyrethroid surrogates had similar ranges of interindividual variability similar to those of the other esterase substrates, but did not correlate well with them. This observation suggests that "general" esterase substrates are not appropriate reporters of pyrethroid hydrolysis activity. In addition, we were able to use these substrates to visualize activity in one- and two-dimensional native gels. Results showed that we could detect a profile of hydrolytic activity different from those of the commercial substrates, α-naphthyl acetate and 4-methylumbelliferyl acetate.

These new substrates can be easily synthesized and this chemistry can be adapted to a wide variety of carboxylic acids. Another advantage is that a range of aldehyde reporters with varying Stokes' shifts could be used to assay multiple substrates simultaneously. In addition, these reporters can readily be formatted for high-throughput assays. The aldehydes used have a large Stokes' shift allowing the use of wide bandpass filters for high sensitivity. The α-cyanoesters have lower fluorescence background than other commonly used fluorescent esters and lower hydrolysis background due to greater stability. Like other fluorescent reporters, they offer a wider dynamic range of detection than colorimetric substrates. The reporter for PNPA, *p*-nitrophenol, was linear over three orders of magnitude, whereas 2-naphthaldehyde and 6-methoxynaphthaldehyde were linear over four and five orders of magnitude, respectively.

The pyrethroid surrogates have many applications, including monitoring of pyrethroid-resistant insect strains. Many species of insects have developed pyrethroid resistance by increasing their endogenous esterase levels, thus increasing their ability to detoxify pyrethroids [44]. Shan and Hammock showed that compound **3** was useful for detecting esterase activity in pyrethroid-resistant strains of the tobacco budworm [15]. Another potential application will be in monitoring the effects of organism exposure to agrochemicals. Both organophosphate and carbamates are carboxylesterase inhibitors and can have synergistic effects upon pyrethroid toxicity [11]. These new substrates could be useful in determining whether a specific organophosphate or carbamate inhibits the isozyme(s) responsible for pyrethroid (i.e., cypermethrin or fenvalerate) hydrolysis and enable the prediction of synergistic toxicity.

These new tools will aid in the study of carboxylesterase-mediated pyrethroid hydrolysis and provide information about interindividual variability in carboxylesterase activity. These results imply that different isozymes are responsible for the hydrolysis of a range of substrates and that isozyme abundance is not directly related to "total esterase activity," which is often reported as PNPA activity. Therefore caution needs to be exercised in extending the results of hydrolysis activity assays to general carboxylesterase activity, as it appears that a true general esterase substrate has not yet been discovered.

Acknowledgments

The authors thank Dr. John Newman for many useful discussions and Dr. Katja Dettmer for assistance with development of the GC/MS pyrethroid assay. In addition we thank Dr. Guomin Shan for assistance in project development. C.E.W. was supported by a UC TSR&TP Graduate Fellowship and a University of California Systemwide Biotechnology Research Program Fellowship in Structure Assisted Drug Discovery (Grant 2001-7). This work was supported in part by NIEHS Grant R37 ES02710, NIEHS Superfund Grant P42 ES04699, and NIEHS Center for Environmental Health Sciences Grant P30 ES05707, and UC-Systemwide Mosquito Research Program #01-017-2-1.

References

[1] T. Satoh, M. Hosokawa, Annu. Rev. Pharmacol. Toxicol. 38 (1998) 257–288.
[2] T. Satoh, P. Taylor, W.F. Borsron, S.P. Sanghani, M. Hosokawa, B.N. La Du, Drug Metab. Dispor. 30 (2002) 488–493.
[3] P.D. Senter, K.S. Beam, B. Mixan, A.F. Wahl, Bioconjugate Chem. 12 (2001) 1074–1080.
[4] P.D. Senter, H. Marquardt, B.A. Thomas, B.D. Hammock, I.S. Frank, H.P. Svensson, Cancer Res. 56 (1996) 1471–1474.
[5] C.S. Cook, P.J. Karabatsos, G.L. Schoenhard, A. Karim, Pharm. Res. 12 (1995) 1158–1164.
[6] H. Van de Waterbeemd, B. Testa, C. Marrel, D.R. Cooper, P. Jenner, C.D. Marsden, Drug Des. Discovery 2 (1987) 135–143.
[7] J.E. Casida, D.W. Gammon, A.H. Glickman, L.J. Lawrence, Annu. Rev. Pharmacol. Toxicol. 23 (1983) 413–438.
[8] T.J. Wallace, S. Ghosh, W.M. Grogan, Am. J. Respir. Cell. Mol. Biol. 20 (1999) 1201–1208.
[9] R.C. Gupta, W.D. Dettbarn, Chem.–Biol. Interact. 87 (1993) 295–303.
[10] L. Epstein, S. Bassein, F.G. Zalom, Calif. Agric. 54 (2000) 14–19.
[11] M.A. Sogorb, E. Vilanova, Toxicol. Lett. 128 (2002) 215–228.
[12] G. Leng, J. Lewalter, B. Rohrig, H. Idel, Toxicol. Lett. 107 (1999) 123–130.
[13] P.W. Riddles, H.J. Schnitzerling, P.A. Davey, Anal. Biochem. 132 (1983) 105–109.
[14] W. Butte, K. Kemper, Toxicol. Lett. 107 (1999) 49–53.
[15] G. Shan, B. Hammock, Anal. Biochem. 299 (2001) 54–62.
[16] M.-B.A. Ashour, L.G. Harshman, B.D. Hammock, Pestic. Biochem. Physiol. 29 (1987) 97–111.
[17] E.V. Pindel, N.Y. Kedishvili, T.L. Abraham, M.R. Brzezinski, J. Zhang, R.A. Dean, W.F. Borson, J. Biol. Chem. 272 (1997) 14769–14775.
[18] G. Shan, D.W. Stoutamire, I. Wengatz, S.J. Gee, B.D. Hammock, J. Agric. Food Chem. 47 (1999) 2145–2155.
[19] C.E. Wheelock, M.E. Colvin, I. Uemura, M.M. Olmstead, Y. Nakagawa, J.R. Sanborn, A.D. Jones, B.D. Hammock, Use of ab initio calculations to predict carboxylesterase inhibitor potency, J. Med. Chem. 45 (2002) 5576–5593.
[20] C.E. Wheelock, T.F. Severson, B.D. Hammock, Chem. Res. Toxicol. 14 (2001) 1563–1572.
[21] P.G. Gassman, J.J. Talley, Tetrahedron Lett. 40 (1978) 3773–3776.
[22] H. Nellaiah, C. Morisseau, A. Archelas, R. Furstoss, J.C. Baratti, Biotechnol. Bioeng. 49 (1996) 70–77.
[23] M.B. Bradford, Anal. Biochem. 72 (1976) 248–254.
[24] A. Ljungquist, K.B. Augustinsson, Eur. J. Biochem. 23 (1971) 303–313.
[25] K. Van Asperen, J. Insect. Physiol. 8 (1962) 401–416.
[26] U. Laemmli, Nature 227 (1970) 680–685.
[27] H. Towbin, T. Staehlin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[28] T. Omura, R. Sato, J. Biol. Chem. 239 (1964) 2370–2378.
[29] W. Greenlee, A. Poland, J. Pharmacol. Exp. Ther. 205 (1978) 596–605.
[30] R.J. Pohl, J.R. Fouts, Anal. Biochem. 107 (1980) 150–155.
[31] T. Kronbach, D. Mathys, J. Gut, T. Catin, U. Meyer, Anal. Biochem. 162 (1987).
[32] J. Goldstein, M. Faletto, M. Romkes-Sparks, T. Sullivan, S. Kitareean, J. Raucy, J. Lasker, B. Ghanayem, Biochemistry 33 (1994) 1743–1752.
[33] R.E. Pearce, C.J. McIntyre, A. Madan, U. Sanzgiri, A.J. Draper, P.L. Bullock, D.C. Cook, L.A. Burton, J. Latham, C. Nevins, A. Parkinson, Arch. Biochem. Biophys. 331 (1996) 145–169.
[34] R.N. Wixtrom, B.D. Hammock, in: D. Zakim, D.A. Vessey (Eds.), Biochemical Pharmacology and Toxicology, vol. 1: Methodological Aspects of Drug Metabolizing Enzymes, Wiley, New York, 1985, pp. 1–93.
[35] J.X. Yan, R. Walt, T. Berkelman, R.A. Harry, J.A. Westbrook, C.H. Wheeler, M.J. Dunn, Electrophoresis 21 (2000) 3666–3672.
[36] T.L. Huang, S.A. Villalobos, B.D. Hammock, J. Pharm. Pharmacol. 45 (1993) 458–465.
[37] J.H. Davies, in: J.P. Leahey (Ed.), The Pyrethroid Insecticides, Taylor & Francis, Philadelphia, 1985, pp. 1–41.
[38] M. Hosokawa, T. Endo, M. Fujisawa, S. Hara, N. Iwata, Y. Sato, T. Satoh, Drug Metabol. Dispor. 23 (1995) 1022–1027.
[39] T.L. Huang, T. Shiotsuki, T. Uematsu, B. Borhan, Q.X. Li, B.D. Hammock, Pharmaceu. Res. 13 (1996) 1495–1500.
[40] P. Buchwald, N. Bodor, Pharmazie 57 (2002) 87–93.
[41] M. Lund-Pero, B. Jeppson, B. Arneklo-Nobin, H.O. Sjogren, K. Holmgren, R.W. Pero, Clin. Chim. Acta. 224 (1994) 9–20.
[42] M.B.A. Ashour, B.D. Hammock, Biochem. Pharmacol. 36 (1987) 1869–1879.
[43] G. Lomolino, A. Lante, A. Crapisi, P. Spettoli, A. Curioni, Electrophoresis 22 (2001) 1021–1023.
[44] S.P. Foster, A.L. Devonshire, Pestic. Sci. 55 (1999) 810–814.