Development of Optically Pure Pyrethroid-Like Fluorescent Substrates for Carboxylesterases

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Pyrethroids are now the world's most extensively used insecticides. One of the common metabolic routes of pyrethroid insecticides in living systems is hydrolysis by carboxylesterases, and this hydrolysis may be stereospecific since most pyrethroid insecticides have chiral centers. In previous studies, pyrethroid-like fluorescent substrates have been shown to be hydrolyzed in a fashion similar to actual pyrethroids. It is important to synthesize the stereoisomers of pyrethroid-like fluorescent substrates to study the stereointeraction between carboxylesterases and these substrates. In this study, an effective synthetic method for preparing optically enriched (*R*)- and (*S*)-α-2-hydroxy-2-(6-methoxy-2-naphthyl)acetonitrile was developed. With this alcohol, an efficient synthetic route for preparation of optically pure cypermethrin and fenvalerate analogues was provided. Identification of these stereoisomers was determined based on GC, HPLC, ^{1}H NMR, and X-ray crystallography. In addition, stereointeraction between carboxylesterases and chiral fluorescent substrates indicated that (i) stereospecificity of recombinant mouse liver carboxylesterases (NCBI accession nos. BAC36707 and NM_133960) varied significantly (up to 300-fold difference) with different stereoisomers of cypermethrin and fenvalerate analogues; (ii) on the basis of V_{max}, the sensitivity of this analytical method, using a single stereoisomer of cypermethrin analogues instead of a mixture of eight stereosiomers, could be enhanced by 4−6 times for detection of these carboxylesterases; and (iii) possible usage of these carboxylesterases for chiral synthesis is discussed.

Introduction

Currently, pyrethroids are among the most frequently used insecticides worldwide. They are used for both general plant protection and public health (*1*). Their use will probably increase further as organophosphate insecticides are phased out. Extensive applications not only result in pest resistance to these insecticides (*2*) but may lead to environmental issues and human exposure (*3*, *4*).

Stereoisomerism exists in most pyrethroid insecticides, since they possess a variety of chiral centers. For example, there are two chiral centers for permethrin, fenfluthrin, bifenthrin, and fenvalerate or three for allethrin, cypermethrin, deltamethrin, and cyfluthrin, resulting in four or eight stereoisomers, respectively. Interestingly, some pyrethroids are commercially sold as an optically pure stereoisomer (e.g., γ-cyhalothrin, esfenvalerate, and bioresmethrin), an optically rich mixture (e.g., bioallethrin, β-cyfluthrin, λ-cyhalothrin, and α-/β-cypermethrin), or a mixture of all stereosiomers (e.g., permethrin and cypermethrin).

Stereochemistry is a factor affecting not only insecticidal activity on the biochemical target but also processes such as uptake, distribution, and metabolic behavior in living systems and in the environment. After mixed stereoisomers of pyrethroids are applied in the field, pyrethroids undergo a series of biologically and environmentally mediated reactions. Biological factors may predominantly prefer one or more of several stereoisomers, resulting in stereospecific biodegradation. One of the major routes of metabolic degradation for many pyrethroids in living systems is via esterase-mediated hydrolysis. Enhanced esterase activities contribute, at least partially, to resistance in many pyrethroid resistant insects (*2*, *5*, *6*). Stereospecific hydrolysis of ester-containing pyrethroid insecticides has been reported in carboxylesterases from soil bacteria, resistant insects, and mammals (*7*−*10*). In addition, stereospecific hydrolysis of pyrethroids by carboxylesterases from murine liver (*11*, as animal models for studying the effects of xenobiotics) influences their toxicity.

From a human health perspective, we need to develop an understanding of which esterase isozymes are involved in pyrethroid metabolism and what their distribution is in human populations. Possibly pyrethroid-like substrates can aid in purification and characterization of theses enzymes as well as in their monitoring. Recently, fluorescent surrogate substrates for pyrethroids have been developed in our laboratory to facilitate the measurement of esterase-mediated hydrolysis of pyrethroid insecticides in living systems. These α-cyanoesters structurally resemble type II pyrethroid insecticides (e.g., cypermethrin, fenvalerate) with multiple stereoisomers. Carboxylesterases hydrolyze the ester bond, releasing the cyanohydrin, which quickly rearranges into the corresponding fluorescent aldehyde under basic conditions (Figure 1; *12*−*14*). These fluorescent assays with a mixture of stereoisomers are over 100 times more sensi-

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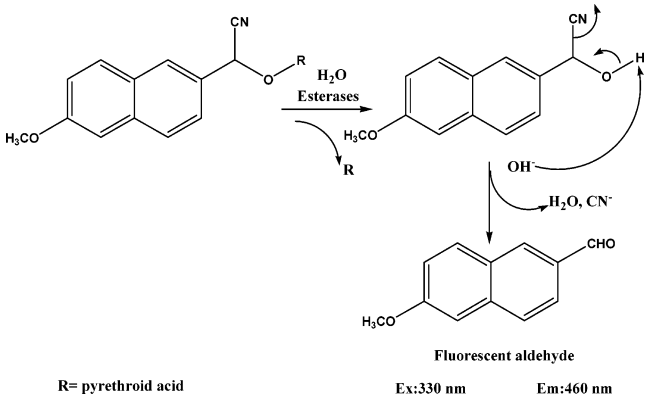

Figure 1. Mechanism of formation of a fluorescent product from pyrethroid substrates hydrolyzed by esterases.

tive than those using the normal colorimetric assays (*12*); thus, they have the potential to further enhance sensitivity through stereoisomer specification. Therefore, it is important to synthesize these isomers for the study of stereointeraction between carboxylesterases and individual stereoisomers.

The objectives of this study are to (i) synthesize and identify optically pure stereoisomers of pyrethroid fluorescent substrates (Figure 2); (ii) use recombinant mouse liver carboxylesterases to evaluate their stereoselectivity toward these substrates; (iii) compare catalytic activity with murine toxicity of the corresponding stereoisomers of cypermethrin and fenvalerate to determine whether these fluoresecent substrates can be a tool for predicting murine toxicity of new compounds; and (iv) evaluate whether the sensitivity of the monitoring tools with these fluorescent substrates can be enhanced by using a single stereoisomer instead of a mixture of all stereoisomers.

Experimental Procedures

Chemicals. The ligand (*S*)- and (*R*)-2,2′-bis(diethylaminomethyl)-substituted binaphthol (BINOLAM)[1] was kindly provided by Professor Casas from Universitat de les Illes Balears in Spain. The ligand dipeptide *cyclo*[(*S*)-Phe-(*S*)-His] was a gift from Shell Agrochemicals. The two enantiomers (*S*)-(+)- and *R*-(−)-4-chloro-α-(1-methylethyl)benzeneacetic acid (FA) were available from previous work by Shan et al. (*15*) and were approximately 96 and 98% enantiomeric excess (%ee), respectively. The methyl ester of 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (cis/trans = 40/60) and TLC plates (0.2 mm precoated silica gel 60 F_{254} on glass) were purchased from Fisher Scientific (Pittsburgh, PA). Silica gel (particle size, 32−63 μm) for chromatography was supplied by SORBTEC Co. (Atlanta, GA). 6-Methoxy-2-naphthaldehyde was obtained from AVOCADO Research Chemical Ltd. (Heysham, Lancaster, United Kingdom). (1*R*)-(+)-*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (PA) was a gift from Agricultural Chemicals Research Laboratory of Sumitomo Chemical Co. (Takarazuka City, Hyogo, Japan; 97.4%ee). All other chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). Two recombinant mouse liver carboxylesterases (NCBI accession nos. BAC36707 and NM_133960) were purified as previously reported (*14*).

Structural identification was based on data from both ^{1}H NMR and GC/MS. Proton NMR spectra were acquired from a Mercury 300 spectrometer (Varian, Palo Alto, CA). Chemical shift values are given in ppm downfield from internal standard (trimethylsilane). Melting points were determined on a Uni-Melt apparatus (Thomas Scientific, Swedesborogh, NJ) and are uncorrected. The chemical purity of the final products was verified by the spectra described above, a single spot on TLC under 254 nm wavelength, and a lack of aldehyde signal on TLC at 360 nm wavelength.

GC/MS was obtained from a Hewlett-Packard model 5890 GC equipped with a HP 5973 mass spectral detector (Agilent Corp, Arondale, PA) and a 30 m × 0.25 mm i.d. capillary column coated with a 0.25 μm film of 5:95 methylphenyl-substituted dimethylpolysiloxane (DB-5 MS) column (J&W Scientific, Folsom, CA). The DB-5 MS column was operated at 80 °C for 1 min, ramped to 300 °C (at 11 °C/min increments), and held for 5 min at this temperature. The injector port was operated in the splitless mode at 250 °C, and helium was used as the carrier gas at 0.8 mL/min. The mass spectral detector was set on full scan mode (*m/z* 50−550). Optical purity was calculated by relative peak area.

An HPLC (Hewlett-Packard series 1100) equipped with a quart HPLC pump, degasser, autosampler, and diode array detector (series 1050) was used. The chiral column (CHIRALCEL, OD-H, 4.6 mm × 250 mm, 5 μm particle size) was purchased from Chiral Technologies, Inc. (Exton, PA). The chiral column was used to identify the %ee under the following conditions: 1 mL/min of X% 2-propanol in hexane, X falls between 0.6 and 2.5, depending on the tested compound) at room temperature and maximal absorbance at 235 nm. The optical purity of stereoisomers was calculated by relative peak area.

Magic 2002 HPLC and Magic C_{18} (3 μm particle size, 100 Å pore size, and 1.0 mm × 150 mm) columns were from Michrom BioResources, Inc. (Aubum, CA) and were used to quantify the purity of PA under the following conditions: temperature (25 °C), wavelength (230 nm), mobile phase at 50 μL/min, and gradient elution (as time changes: 0−15−25−35 min, corresponding to change in solvent B of 0−80−80−0; A, H_2O; B, 10% H_2O + 90% CH_3OH and pH value of all H_2O used was 2.1 adjusted with trifluoroacetic acid). The purity of PA was calculated by relative peak area.

Preparation of HCN (Caution). Preparation of HCN followed the procedure of Fröhlich et al. (*16*, *17*) with modifications. All procedures were carried out in a well-ventilated hood using a gas mask, goggles, and impervious gloves. Water-saturated KCN (259 mmol) was added dropwise through a separatory funnel into a two-neck flask containing 60% H_2SO_4 (7.4 mL of concentrated H_2SO_4 in 9 mL of H_2O) at 80 °C. The released HCN was passed through a $CaCl_2$ filled drying tube maintained at 40 °C and collected in an ice/acetone trap, vented close to the surface of 5.25% sodium hypochlorite (i.e., household bleach, pH 10∼11 to trap any escaping HCN), which converted runoff HCN into the less toxic cyanate ion. The whole system was tightly sealed except the end of the filter funnel. After the

[1] Abbreviations: BINOLAM, 2,2′-bis(diethylaminomethyl)-substituted binaphthol; *R*- and *S*-6-MONCH(OH)CN, α-2-hydroxy-2-(6-methoxy-2-naphthyl)acetonitrile; *cyclo*[(*S*)-Phe-(*S*)-His], *cyclo*[(*S*)-phenylalanyl-(*S*)-histidyl]; FA, 4-chloro-α-(1-methylethyl)benzene acetic acid; PA, 3-(2,2-dichloro vinyl)-2,2-dimethylcyclopropanecarboxylic acid; %ee, percentage of enantiomeric excess; *R* or *S*-acetate, (*R*)- or (*S*)-α-cyano-(6-methoxynanphthalen-2-yl)methyl acetate; *R*- or *S*-butyrate, (*R*)- or (*S*)-α-cyano-(6-methoxynanphthalen-2-yl)methyl acetate; (2*R*,α*R*), (*R*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl, (2*R*)-(4-chlorophenyl)-3-methylbutanoate; (2*S*,α*S*), (*S*)-α-cyano-(6-methoxynaphthalen-2-yl) methyl, (2*S*)-(4-chlorophenyl)-3-methylbutanoate; (2*S*,α*R*), (*R*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl, (2*S*)-(4-chlorophenyl)-3-methylbutanoate; (2*R*,α*S*), (*S*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl, (2*R*)-(4-chlorophenyl)-3-methylbutanoate; (1*R cis*,α*R*), (*R*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl,(1*R*)*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; (1*S cis*,α*S*), (*S*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl, (1*S*) *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; (1*S cis*,α*R*), (*R*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl, (1*S*) *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; (1*R cis*,α*S*), (*S*)-α-cyano-(6-methoxynaphthalen-2-yl)-methyl, (1*R*) *cis*-3-(2,2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate; (1*R trans*,α*R*), (*R*)-α-cyano-(6-methoxynaphthalen-2-yl)-methyl, (1*R*) *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; (1*S trans*,α*S*), (*S*)-α-cyano-(6-methoxynaphthalen-2-yl) methyl, (1*S*) *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; (1*S trans*,α*R*), (*R*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl, (1*S*) *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; (1*R trans*,α*S*), (*S*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl, (1*R*) *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate.

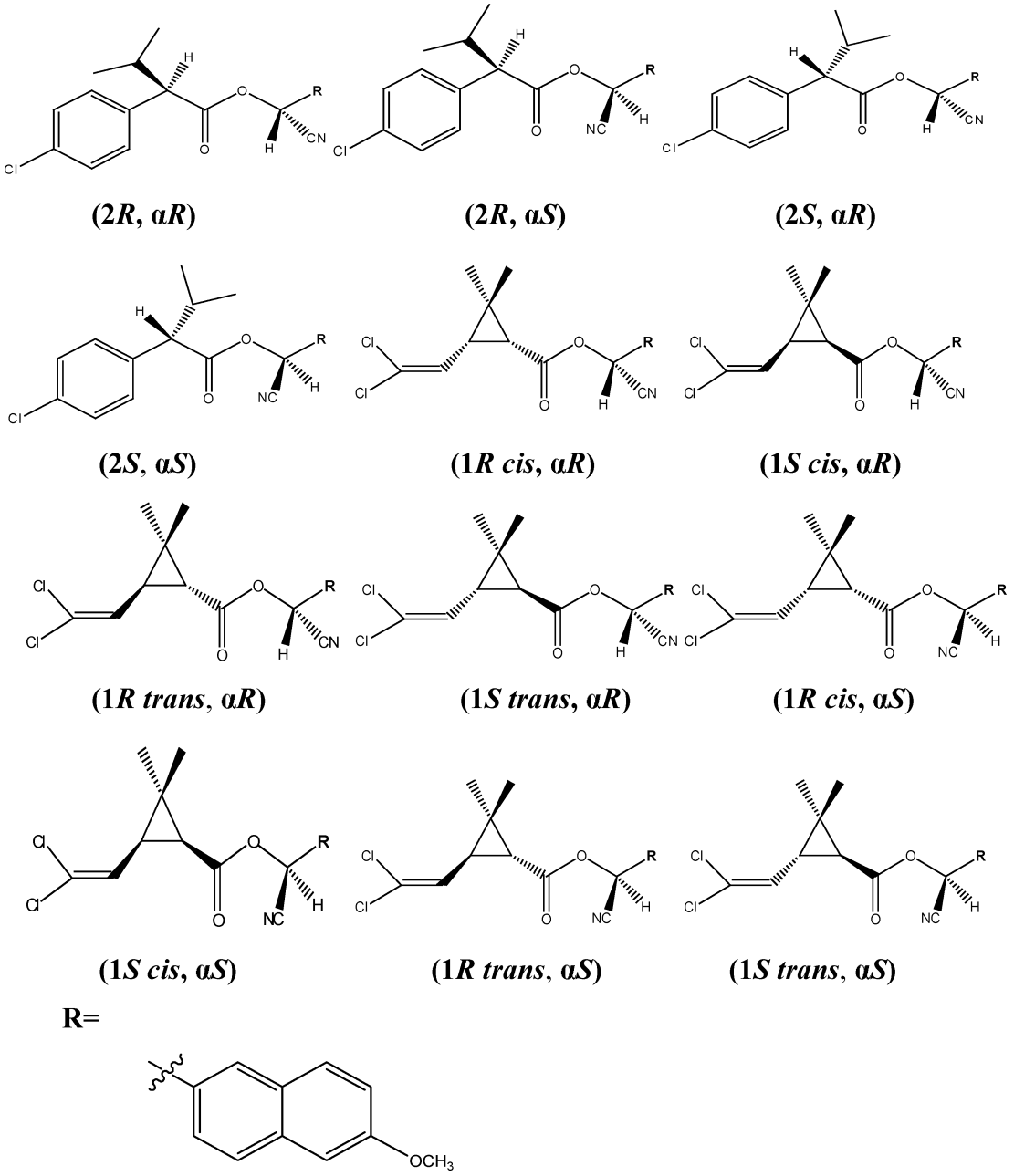

Figure 2. Structures of the synthesized target compounds.

completion of the reaction, the HCN was redistilled through a second calcium chloride drying tube (40 °C) and condensed and weighed in a tarred stoppered container and diluted with dry toluene. The HCN container was tightly sealed and put in a well-ventilated hood and well-labeled. A nitrogen blow (1−5 mL/min) was used to drive residual HCN in the reaction system into the bleach for 1 h, and then, reactant residue was washed with bleach (pH 10∼11). Finally, all equipment was washed with diluted bleach.

Preparation of PA. The preparation of PA from its methyl ester followed the procedure of Shan et al. (*18*). A mixture of the methyl ester of PA (22.32 g, 0.1 mol), NaOH (12 g, 0.3 mol), and ethanol−water (1:1, 250 mL) was refluxed for overnight, concentrated under reduced pressure, diluted with 100 mL of H_2O, and extracted with diethyl ether (3 × 35 mL). The aqueous phase was acidified with HCl (37.4%, 30 mL), and the oily precipitate was extracted into diethyl ether (3 × 30 mL), washed with H_2O (2 × 25 mL), and then dried over anhydrous $MgSO_4$ overnight. The solution was filtered, and diethyl ether was evaporated under reduced pressure to obtain a solid product, which was recrystallized from hexane/ether (1:1, v/v); yield 18.70 g (89.4%).

The *trans* and *cis* isomers of PA were separated using the method of Foggassy et al. (*19*) with modifications. A mixture of PA (20.90 g, 0.10 mol) and benzene (100 mL) was stirred at 27 °C for 5 h. The suspension was filtered, and the filter residue was recrystallized from benzene (caution, carcinogen) to give 5.2 g of *cis*-PA (purity, 99.74%; t_R, 22.940 min; 62% recovery). Similarly, a mixture of PA (22.10 g, 0.11 mol) and petroleum ether (110 mL) was stirred at 30 °C for 5 h. The suspension was filtered, and the filter residue was recrystallized from hexanes to give 4.67 g of *trans*-PA (purity > 99.9%; t_R, 21.540 min; 36.7% recovery). *cis*-PA: ^{1}H NMR (Me_2SO): δ (ppm) 12.31 (br, 1H, COO*H*), 6.31 (d, J = 8.70 Hz, 1H, C*H*=CCl_2), 1.98 (t, J = 8.70 Hz, 1H, C*H*-CH=CCl_2), 1.87 (d, J = 7.80 Hz, 1H, C*H*COOH), 1.19 (d, J = 6.30 Hz, 6H, $2CH_3$). *trans*-PA: ^{1}H NMR (Me_2SO): δ (ppm) 12.38 (br, 1H, COO*H*), 6.04 (d, J = 8.70 Hz, 1H, C*H*=CCl_2), 2.00 (dd, J_1=3.90 Hz, J_2=5.10 Hz, 1H, C*H*-CH=CCl_2), 1.79 (d, J = 5.10 Hz, 1H, C*H*COOH), 1.18 (d, J = 10.50 Hz, 6H, $2CH_3$).

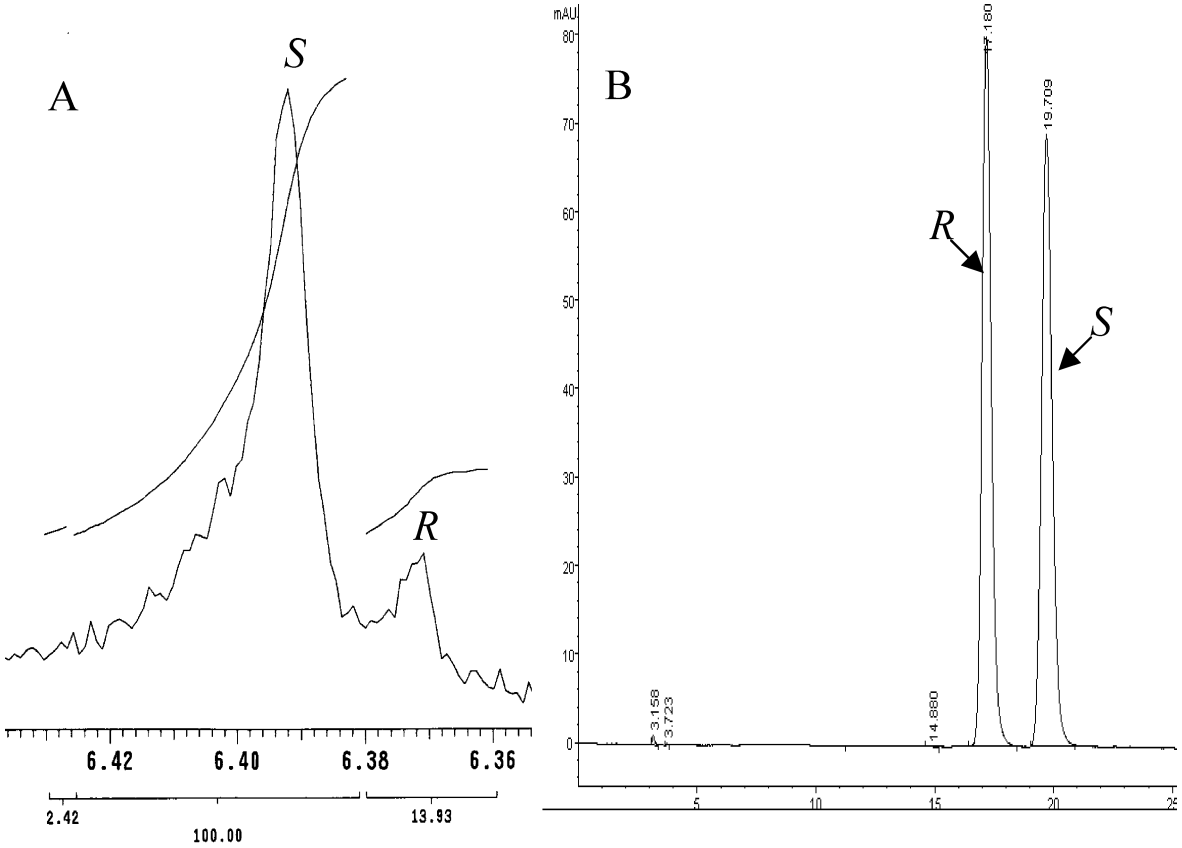

Figure 3. (A) ^{1}H NMR spectrum (Mercury 300 Hz) of cyanohydrin derivative with (1*R*,2*S*,5*R*)-(−)-menthylchloroformate showing the two peaks of the methine proton α to the cyanohydrin group. (B) HPLC chiral chromatography of *R*/*S*-acetate with the following conditions: CHIRALCEL, OD-H column (4.6 mm × 250 mm, 5 μm particle size); mobile phase, 2.5% 2-propanol in hexane at room temperature; and maximal absorbance, 235 nm.

Resolution of *cis*-PA. The resolution (1*R*)-(−)-*cis*- and (1*S*)-(+)-*cis*-PA followed the procedure of Burt et al. (*20*) with modifications. To a stirring mixture of *cis* PA (20.9 g, 0.1 mol) in benzene (250 mL) at 50 °C was added a mixture of (*R*)-(+)-α-methylbenzylamine (7.88 g, 0.065 mol) in benzene (100 mL). The mixture was cooled slowly over 3 h to give a very flocculent asbestos-like solid. Filtration gave a white solid (16.0 g, $[\alpha]_D^{25} = 16$, *c* ∼ 1.0, 95% EtOH). Recrystallization of this solid from benzene (130 mL) gave a solid (9.9 g, $[\alpha]_D^{25} = 23$, *c* ∼ 1.0, 95% EtOH). The solid was then recrystallized twice from benzene (140 mL) sequentially and gave a white solid (7.29 g, $[\alpha]_D^{25} = 28.8$, *c* 1.19, 95% EtOH, mp 160.5−162 °C) (6.98 g, $[\alpha]_D^{25} = 27.8$, *c* 1.01, anhydrous EtOH; $[\alpha]_D^{25} = 30.2$, anhydrous EtOH and four drops of water), respectively. This solid was (1*S*)-(+)-*cis*-PA.

Most of the benzene was stripped from combined filtrates given from the above procedures. To the mixture was added diethyl ether, and the mixture was acidified with 3N HCl. The organic phase was washed with diluted HCl, saturated NaCl + diluted HCl and water, sequentially, and extract washes in order with diethyl ether. The combined organic phase was dried with Na_2SO_4 and then filtered, and the solvent was stripped to give a white solid. This solid was dissolved in benzene (350 mL). To this mixture at 65 °C was added (*S*)-(−)-α-methylbenzylamine (8.4 mL, 7.88 g, 0.065 mol). The mixture was cooled to room temperature overnight and then washed with benzene, and the solid was immediately recrystallized from benzene (230 mL), allowing it to crystallize slowly. Filtration was followed by two additional recrystallizations from benzene (280 mL) to give (1*R*)-(−)-*cis*-PA as a white solid (12.6 g, $[\alpha]_D^{26} = -31.7$, *c* 1.09, 95% EtOH, mp 164−164.5 °C).

A sample (30 mg) from (1*R*)-(−)-*cis*-PA or (1*S*)-(+)-*cis*-PA was treated with ethyl acetate (1 mL) and 3 N HCl (1 mL). The organic phase was washed with diluted HCl (1 mL) and water (2 × 1 mL). The organic layer was dried by filtering through ∼200 mg of $MgSO_4$. The solvent was blown off with nitrogen, and the resulting solid was dissolved in $CHCl_3$ (0.2 mL). To this mixture was added dimethylformamide [DMF (0.01 mL)], followed by thionyl chloride (100 μL, then heated at 60 °C for 1 h). The solvent and excess thionyl chloride were briefly stripped. Hexane (200 μL) was then added and stripped again. The resultant oil was diluted with CH_2Cl_2 (0.3 mL) and then chilled in ice. To this mixture was injected all at once α-methylbenzylamine (18 mg, 99.6%ee). After 30 min at room temperature, the mixture was washed sequentially with diluted HCl (2 × 2 mL), saturated $NaHCO_3$ solution, and H_2O. The resultant organic phase was dried and blown off to give a white solid. The solid was made into a solution (1 mg/mL) and injected into the GC with 15 m DB-5 MS column under following conditions: 1 min at 50 °C, ramp from 50 to 190 at 10 °C/min, hold at 190 °C for 10 min. Complete separation of the two diasteromers was obtained. The optical purity of both (1*R*)-(−)-*cis*-PA and (1*S*)-(+)-*cis*-PA appeared to be over 99.5% without correction for the optical purity of α-methylbenzylamine.

Determination of %ee of α-Hydroxy-(6-methoxynaphthalen-2-yl)acetonitrile [6-MONCH(OH)CN]. Method I (*21*). To a solution of (1*R*,2*S*,5*R*)-(−)-menthyl chlorofomate (1 mmol) in 1,2-dichloroethane was added triethylamine (1 mmol), followed by a small portion of the obtained crude 6-MONCH(OH)-CN. After it was stirred overnight at room temperature, the mixture was run through a silica gel column. The fractions were concentrated to give a crude mixture of unreacted aldehyde and the corresponding diastereomeric carbonic esters. The diastereomer ratio was determined by ^{1}H NMR analysis, which showed two singlets at δ 6.37 ppm (*R*) and 6.39 ppm (*S*) (Figure 3A), corresponding to the methane proton α to the cyanohydrin group. The relative area of these two peaks determined the %ee.

Method II. The crude 6-MONCH(OH)CN containing the unreacted aldehyde (∼1 mmol) was dissolved in 1,2-dichloroethane (3 mL) and triethylamine (1 mmol), and then, acetyl chloride or butanoyl chloride (1 mmol) was added at ice-cooled

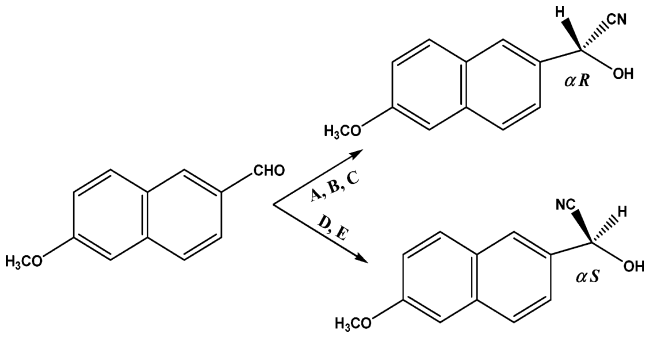

Figure 4. Scheme for synthesis of chiral α-2-hydroxy-2-(6-methoxy-2-naphthyl)acetonitrile. A−E indicate preparation methods described in the Experimental Procedures.

temperature. The mixture was stirred for 3 h at room temperature and then passed through a short silica column. The mixture was chromatographed (hexanes:ethyl acetate = 4:1), and pure α-cyano (6-methoxynaphthalen-2-yl) methyl acetate or butanoate resulted. These were chromatographed on a HPLC chiral column (2.5% 2-propanol in hexane, room temperature, and maximal absorbance at 235 nm). The relative peak area determined the %ee. *R*- or *S*-Acetate: white needles (mp 106−107 °C). ^{1}H NMR ($CDCl_3$): δ (ppm) 7.94 (s, 1H, Ar), 7.81 (d, J = 8.10 Hz, 1H, Ar), 7.78 (d, J = 7.20 Hz, 1H, Ar), 7.53 (dd, J_1 = 8.40 Hz, J_2 = 2.10 Hz, 1H, Ar), 7.21 (dd, J_1 = 9.30 Hz, J_2 = 2.10 Hz, 1H, Ar), 7.15 (dd, J = 2.40 Hz, 1H, Ar), 6.55 (s, 1H, C*H*CN), 3.94 (s, 3H, OC*H*$_3$), 2.17 (s, 3H, C*H*$_3$). GC/MS: t_R = 16.9 min, M^+ = 255. HPLC (hexane:2-propanol = 97.5:2.5): t_R = 17.18 min and 95.9% optical purity (*R*) and 19.71 min and 99.3% optical purity (*S*). *R*- or *S*-Butyrate: clear oil. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.94 (s, 1H, Ar), 7.81 (d, J = 8.10 Hz, 1H, Ar), 7.78 (d, J = 7.20 Hz, 1H, Ar), 7.52 (dd, J_1 = 8.40 Hz, J_2 = 2.10 Hz, 1H, Ar), 7.23 (dd, J_1 = 8.70 Hz, J_2 = 2.40 Hz, 1H, Ar), 7.16 (dd, J = 2.70 Hz, 1H, Ar), 6.60 (s, 1H, CHCN), 3.94 (s, 3H, OC*H*$_3$), 2.40 (h, J = 3.0−4.2 Hz, 2H, C*H*$_2$), 1.69 (h, J = 7.20 Hz, 2H, COC*H*$_2$), 0.96 (t, J = 7.20 Hz, 3H, C*H*$_3$). GC/MS: t_R = 18.4 min, M^+ = 283. HPLC (hexane:2-propanol = 97.5:2.5): t_R = 11.83 min and 93.1% optical purity (*R*) and 12.94 min and 97.6% optical purity (*S*).

Determination of the Conversion of 6-Methoxy-2-naphthaldehyde to 6-MONCH(OH)CN. The crude 6-MONCH(OH)CN containing unreacted aldehyde was run by flash chromatography (hexane:ethyl acetate = 4:1 [R_f = 0.66 on TLC], plus a drop of trifluoroacetic acid/100 mL). The weight of the white solid [6-MONCH(OH)CN] obtained after the mobile phase was stripped under reduced pressure was used to calculate the percentage conversion.

Buffer Exchange of (*R*)-Oxynitrilase from *Prunus amygdalus*. To the commercial (*R*)-oxynitrilase from *P. amygdalus* (7.5 mL) was added citrate buffer (7.5 mL, 0.1 M, and pH 5.5). The resultant mixture was centrifuged for 4 h with 15 mL centrifugal filter devices [Centriplus for regenerated cellulose, 50 kDa, Millipore Corp (Bedford, MA)] at 3500 rpm and 4 °C. After the buffer was changed twice, the solution was freshly used for enzymatic synthesis. The small proteins (less than 50 kDa) were also removed through this procedure.

Preparation of (*R* or *S*)-Cyanohydrin (Figure 4). A common procedure for all methods is below: The organic phase of 6-MONCH(OH)CN, stabilized by adding two drops of concentrated HCl, was dried over anhydrous $MgSO_4$ for 4 h, then filtered, and stripped under reduced pressure. Conversion and %ee were determined by the methods described above, and the results were shown in Table 1.

Method A. The preparation followed the procedure of Casas et al. (*22*) with some modifications. To a mixture of (*S*)-BINOLAM (0.1 mmol, 46 mg), triphenylphosphine oxide (0.4 mmol, 111 mg), 10 granules of 4 Å molecular sieves in dry toluene (2 mL), and CH_2Cl_2 (2 mL), under nitrogen, was added dimethylaluminum chloride (0.1 mmol, 100 μL), and the resulting solution was stirred for 1 h at room temperature. The reactant mixture was cooled to −17 °C, and 6-methoxy-2-naphthaldehyde (1 mmol, 0.186 g) in dry CH_2Cl_2 (2 mL) and trimethylsilyl cyanide (3 mmol) were added and the reaction mixture was stirred for 24 h at this temperature. The reaction was quenched, and the crude trimethylsilyl ether was hydrolyzed by adding HCl (12 mmol, 4 mL) and stirring for one more hour at room temperature. The resultant mixture was passed through a Celite pad, and the organic phase was washed with H_2O twice (2 × 15 mL).

Table 1. Conversion and Enantiomeric Excess of α-2-Hydroxy-2-(6-methoxy-2-naphthyl)acetonitrile Prepared by Different Methods

method[a]	predominant isomer	conversion (%)	(%ee)[c]
(*S*)-BINOLAM	*R*	62.2	43.66
cyclo[(*S*)-Phe-(*S*)-His]	*R*		
toluene:CH_2Cl_2 = 2:1		43.8	66.5
toluene:CH_2Cl_2 = 1:1		48.9	53.5
toluene:CH_2Cl_2 = 1:2		56.1	44.7
(*R*)-oxynitrilase			
pH 3.75 (HCN)	*R*	72.5	63.6
pH 5.50 (HCN)		46.0	93.2
pH 5.50 (acetone cyanohydrin)		45.0	92.9
(*R*)-BINOLAM (*S*)-oxynitrilase	*S*	55.4 (92.0)[b]	75.5 (90.0)[b]
pH 5.50 (HCN)	*S*	90.0	99.2
pH 5.50 (KCN)	*R*/*S*	53.1	0.0

[a] (*R*)-Oxynitrilase from bitter almonds (*Prunus amygdalus*); (*S*)-oxynitrilase manihot (*Manihot esculenta*). [b] Data provided by Professor Casas from Universitat de les Illes Balears in Spain under the same conditions except for −40 °C and 39 h instead of −17 °C and 24 h. [c] %ee calculated with data provided by chiral HPLC.

Method B (*21*). To a mixture of *cyclo*[(*S*)-Phe-(*S*)-His] (5.7 mg, 0.02 mmol) in dry toluene was added under nitrogen 6-methoxy-2-naphthaldehyde (1 mmol) in dry CH_2Cl_2. After the mixture was cooled to 0 °C, HCN in toluene (1.26 mmol, 0.7 mL) was added dropwise. The reaction continued for 12 h at 0 °C, and then, the reaction mixture was quenched by adding HCl (1 mmol, 1 mL), and the remaining HCN was removed under reduced pressure. The resultant mixture was washed with HCl (20 mmol, 10 mL), and the aqueous phase was extracted with diethyl ether (2 × 15 mL).

Method C (*23*). To a solution of 6-methoxy-2-naphthaldehyde (0.186 g, 1 mmol) in isopropyl ether (21 mL) was added (*R*)-oxynitrilase from *P. amygdalus* (4 mL, 38 Units/mL) and 10 mL of 0.1 M citrate buffer, pH 3.75 [for commercial (*R*)-oxynitrilase] or 5.5 [for processed (*R*)-oxynitrilase] and HCN in toluene (1 mL, 11 mmol) or acetone cyanohydrin (0.27 mL, 3 mmol). The mixture was stirred for 3 days at room temperature. Celite (1.5 g) was added into the reaction mixture to adsorb the enzymes. After 15 min, the mixture was filtered and washed twice with 3 M HCl (2 × 10 mL).

Method D. The procedure was the same as for method A except that (*R*)-BINOLAM was used in place of (*S*)-BINOLAM.

Method E. The procedure was the same as for method C except for the use of (*S*)-oxynitrilase from *Manihot esculenta* (Maniok) (120 μL, 6408 units/mL), 20 mL of 0.1 M, pH 5.5, citrate buffer, and HCN in toluene (1 mL, 11 mmol) or KCN (0.165 g, 3 mmol).

6-MONCH(OH)CN. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.94 (s, 1H, Ar), 7.81 (dd, J_1 = 10.80 Hz, J_2 = 2.1 Hz, 1H, Ar), 7.53 (dd, J_1 = 8.40 Hz, J_2 = 6.30 Hz, 1H, Ar), 7.22 (d, J = 2.70 Hz, 1H, Ar), 7.19 (d, J = 2.70 Hz, 1H, Ar), 7.15 (d, J = 2.40 Hz, 1H, Ar), 5.68 (s, 1H, C*H*), 3.94 (s, 3H, C*H*$_3$), 2.70 (m, 1H, O*H*).

Preparation of Pyrethroid Esters. Carboxylic acid (FA or PA; 1 equiv relative to aldehyde) was dissolved in $CHCl_3$ (5 mL) and then $SOCl_2$ (1.2 equiv) followed by DMF (1 μL) were added. The mixture was stirred under nitrogen in an oil bath at 50−55 °C for 3−4 h. The solvent and excess $SOCl_2$ were removed under reduced pressure, and dry 1,2-dichloroethane (2 mL) was

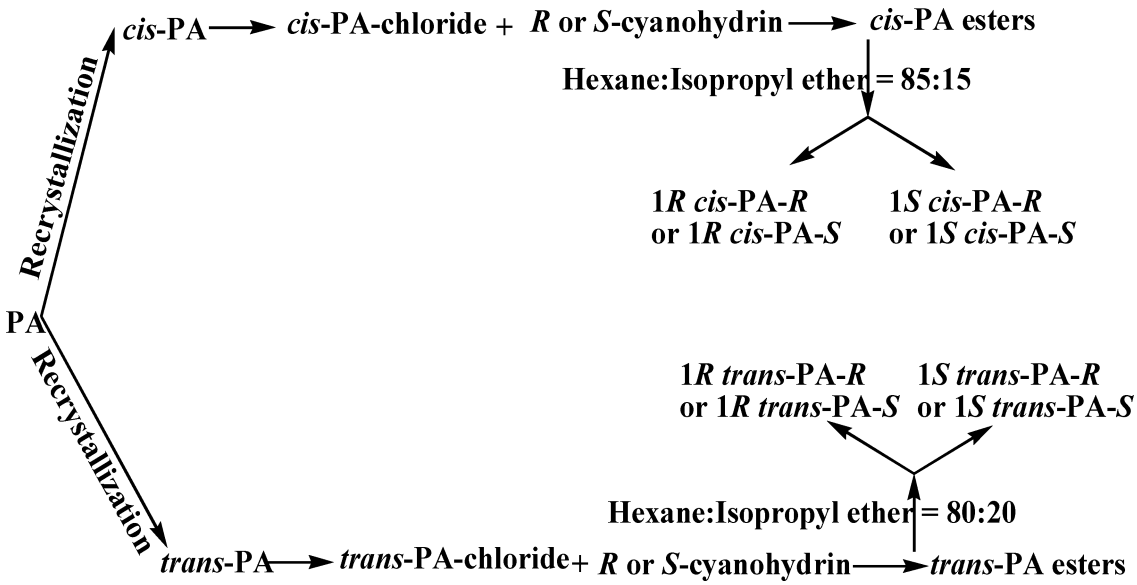

Figure 5. Scheme for synthesis of individual stereoisomers of pyrethroid fluorescent substrates with PA.

added and stripped. The residue was dissolved in dry 1,2-dichloroethane (4 mL) and added all at once to an ice-cooled solution of crude cyanhydrins in dry 1,2-dichloroethane (10 mL) and triethylamine (1 equiv). The mixture was stirred at room temperature for 2 h and filtered. The filtrate was diluted with diethyl ether (30 mL) and then washed sequentially with saturated $NaHCO_3$ solution (2 × 10 mL), 1 M HCl (2 × 10 mL), and saturated NaCl (2 × 10 mL). The organic phase was dried with $MgSO_4$ overnight, filtered, and stripped under reduced pressure. The residue was flash chromatographed (hexane:ethyl acetate = 3.5:1) for FA esters and (hexane:ethyl acetate = 5:1) for PA esters. Yields for both FA esters and PA esters depended on 6-MONCH(OH)CN (40−45% for *R*-6-MONCH(OH)CN and 85−89% for *S*-6-MONCH(OH)CN, relative to reactant 6-methoxy-2-naphthaldehyde).

In addition, individual stereoisomers of PA esters were obtained though a procedure outlined in Figure 5. After *cis*-esters or *trans*-esters were obtained as described above, *cis*-PA esters and *trans*-PA esters were flash chromatographed (hexane:isopropyl ether = 85:15 or 80:20, respectively). After the eight stereoisomers were obtained, standards of both (1*R*)-*cis*-PA and (1*R*) *trans*-PA were used to identify the absolute configuration of each individual stereoisomer.

The optical purity given in this study except for *R*/*S*-butyrate was obtained after the final products were recrystallized once from a mixture of hexane and ethyl acetate. The optical purity of substrates with FA and PA was calculated by data provided by GC with the DB-5 MS column and HPLC with the chiral column OD-H, respectively. All data from ^{1}H NMR spectra were corrected based on the chemical shift of the proton (δ_{ppm}) 7.260 in $CDCl_3$ and 2.500 in Me_2SO.

(*R*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (2*R*)-(4-Chlorophenyl)-3-methylbutanoate (2*R*,α*R*) and (*S*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (2*S*)-(4-Chlorophenyl)-3-methylbutanoate (2*S*,α*S*). White needles (mp = 75−76 °C). ^{1}H NMR ($CDCl_3$): δ (ppm) 7.88 (s, 1H, Ar), 7.77 (t, J = 8.40 Hz, 2H, Ar), 7.44 (dd, J_1 = 8.70 Hz, J_2 = 2.10 Hz, 1H, Ar), 7.31−7.15 (m, 6H, Ar), 6.53 (S, 1H, C*H*CN), 3.94 (S, 3H, OCH_3), 3.23 (d, J = 10.2 Hz, 1H, C*H*CO), 2.37−2.25 (m, 1H, C*H*(CH$_3$)$_2$), 0.95 (d, J = 6.60 Hz, 3H, CH_3), 0.70 (d, J = 6.60 Hz, 3H, CH_3). GC: t_R = 24.6 min, M^+ = 407. HPLC (hexane:2-propanol = 99.4:0.6): t_R = 15.45 min and 99.9% optical purity (2*R*,α*R*) and 16.46 min and 98.0% optical purity (2*S*,α*S*).

(*R*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (2*S*)-(4-Chlorophenyl)-3-methylbutanoate (2*S*,α*R*) and (*S*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (2*R*)-(4-Chlorophenyl)-3-methylbutanoate (2*R*,α*S*). White sponge crystal (mp = 138−139 °C). ^{1}H NMR ($CDCl_3$): δ (ppm) 7.74 (s, 1H, Ar), 7.70 (t, J = 9.60 Hz, 2H, Ar), 7.33 (dd, J_1 = 8.70 Hz, J_2 = 2.10 Hz, 1H, Ar), 7.23−7.12 (m, 6H, Ar), 6.48 (S, 1H, C*H*CN), 3.93 (S, 3H, OCH_3), 3.25 (d, J = 10.5 Hz, 1H, C*H*CO), 2.39−2.31 [m, 1H, C*H*(CH$_3$)$_2$], 1.07 (d, J = 6.60 Hz, 3H, CH_3), 0.73 (d, J = 6.90 Hz, 3H, CH_3). GC: t_R = 24.1 min, M^+ = 407. HPLC (hexane:2-propanol = 99.4:0.6): t_R = 15.29 min and 100.0% optical purity (2*S*,α*R*) and 14.51 min and 99.1% optical purity (2*R*,α*S*).

α-Cyano-(6-methoxynaphthalen-2-yl)methyl 3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate. Common ^{1}H NMR ($CDCl_3$): δ (ppm) 7.94 (s, 1H, Ar), 7.81 (t, J = 9.00 Hz, 1H, Ar), 7.53 (dd, J_1 = 8.40 Hz, J_2 = 1.80 Hz, 1H, Ar), 7.23(d, J = 2.70 Hz, 1H, Ar), 7.21 (d, J = 2.70 Hz, 1H, Ar), 7.16 (d, J = 2.40 Hz, 1H, Ar). MS EI *m*/*z* M^+ = 403.

(*R*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (1*R*) *cis*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (1*R cis*,α*R*) and (*S*)-α-Cyano-(6-methoxy naphthalen-2-yl)methyl (1*S*) *cis*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (1*S cis*,α*S*). White sponge crystals (mp = 89−90 °C). TLC: R_f = 0.67 (hexane:isopropyl ether = 50:50). ^{1}H NMR ($CDCl_3$): δ (ppm) 6.516 (s, 1H, C*H*CN), 6.200 (d, J = 8.70 Hz, 1H, C*H*=CCl$_2$), 3.944 (s, 3H, OCH_3), both 2.173 (s) and 2.111 (t, J = 8.40 Hz) for 1H in C*H*CH=CCl$_2$, 1.911 (d, J = 8.40 Hz, 1H, C*H*CO), 1.324 (s, 3H, CH_3), 1.292 (s, 3H, CH_3). GC: t_R = 23.06 min. HPLC (hexane:2-propanol = 99:1): t_R = 11.11 min and 97.7% optical purity (1*R cis*,α*R*) and 11.62 min and 99.4% optical purity (1*S cis*,α*S*).

(*R*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (1*S*) *cis*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (1*S cis*,α*R*) and (*S*)-α-Cyano-(6-methoxy naphthalen-2-yl)methyl (1*R*) *cis*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (1*R cis*,α*S*). White crystals (mp = 66−67 °C). TLC: R_f = 0.60 (hexane:isopropyl ether = 50:50). ^{1}H NMR ($CDCl_3$): δ (ppm) 6.554 (s, 1H, C*H*CN), 6.214 (d, J = 9.00 Hz, 1H, C*H*=CCl$_2$), 3.940 (s, 3H, OCH_3), both 2.173 (s) and 2.157 (t, J = 8.70 Hz) for 1H in C*H*CH=CCl$_2$, 1.915 (d, J = 8.40 Hz, 1H, C*H*CO), 1.232 (s, 3H, CH_3), 1.207 (s, 3H, CH_3). GC: t_R = 23.18 min. HPLC (hexane:2-propanol = 99:1): t_R = 12.71 min and 99.4% optical purity (1*S cis*,α*R*) and 12.15 min and 98.8% optical purity (1*R cis*,α*S*).

(*R*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (1*R*) *trans*-3-(2,2-Dichloro vinyl)-2,2-dimethylcyclopropanecarboxylate (1*R trans*,α*R*) and (*S*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (1*S*) *trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (1*S trans*,α*S*). White crystals (mp = 69−70 °C). TLC: R_f = 0.60 (hexane:isopropyl ether = 50:50). ^{1}H NMR ($CDCl_3$): δ (ppm) 6.566 (s, 1H, C*H*CN), 5.581 (d, J = 8.10 Hz, 1H, C*H*=CCl$_2$), 3.935 (s, 3H, OCH_3), 2.296 (dd, J_1 = 8.40 Hz, J_2 = 5.40 Hz, 1H, C*H*CH=CCl$_2$), 1.668 (d, J

= 5.10 Hz, 1H, C*H*CO), 1.351 (s, 3H, CH_3), 1.230 (s, 3H, CH_3). GC: t_R = 23.27 min. HPLC (hexane:2-propanol = 99:1): t_R = 13.85 min and 98.7% optical purity (1*R trans*,α*R*) and 14.16 min and 98.0% optical purity (1*S trans*,α*S*).

(*R*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (1*S*) *trans*-3-(2,2-Dichloro vinyl)-2,2-dimethylcyclopropanecarboxylate (1*S trans*,α*R*) and (*S*)-α-Cyano-(6-methoxynaphthalen-2-yl)methyl (1*R*) *trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (1*R trans*,α*S*). White needle crystals (mp = 94−95 °C). TLC: R_f = 0.55 (hexane:isopropyl ether = 50:50). ^{1}H NMR ($CDCl_3$): δ (ppm) 6.574 (s, 1H, C*H*CN), 5.611 (d, J = 8.10 Hz, 1H, C*H*=CCl_2), 3.943 (s, 3H, OCH_3), 2.335 (dd, J_1 = 8.40 Hz, J_2 = 5.40 Hz, 1H, C*H*CH=CCl_2), 1.688 (d, J = 5.40 Hz, 1H, C*H*CO), 1.257 (s, 3H, CH_3), 1.174 (s, 3H, CH_3). GC: t_R = 23.34 min. HPLC (hexane:2-propanol = 99:1): t_R = 15.44 min and 99.9% optical purity (1*S trans*,α*R*) and 14.57 min and 99.9% optical purity (1*R trans*,α*S*).

X-ray Data Collection and Structure Solution and Refinement. Diffraction experiments were performed on a Brucker SMART 1000 diffractometer, and data were collected at 90 K. The structures were solved by the use of SHELXS-97 package, and the data were corrected for absorption effects. The intensities of two standard reflections showed no significant decay during the course of data collection.

Protein Determination. Protein was determined according to the method of Smith et al. (*24*). Briefly, bicinchoninic acid (BCA) protein assay reagents A and B (Pierce, Rockford, IL) were mixed at a ratio of 50:1, and then, 180 μL of this mixture was added to 20 μL of protein sample. The mixture was incubated for 30 min at 37 °C before absorbance was measured at 562 nm. On the basis of a standard curve of bovine serum albumin, absorbance was converted into protein concentration.

Fluorescent Assays. Assays were performed by the methods of Wheelock et al. (*13*). In short, fluorescent assays were conducted with a Spectrafluor Plus (Tecan, Research Triangle, NC). Activities were measured in black 96 well polystyrene clear flat-bottomed microtiter plates (Corning, Inc., New York, NY) at 30 °C. Substrates were prepared in ethanol (10 mM, except for fenvalerate analogues, which also contained 10% Me_2SO). The reaction mixture contained (total volume, 201 μL): 20 μL of protein solution, 180 μL of 20 mM Tris/HCl buffer (pH 8.0), and 1 μL of substrate solution. The reaction was initiated by the addition of 1 μL of substrate solution (final concentration, 50 μM) followed by shaking for 5 s. Three replicates were conducted for each substrate. The fluorescence was monitored with excitation at 330 nm (band-pass, bp 35) and emission at 465 nm (bp 35). Assays for chiral pyrethroid substrates were conducted with five flashes (15 cycles) to give a ~10 min linear assay, respectively. The amount of protein in each assay varied with the substrate and was adjusted so that no more than 5% of the substrate was hydrolyzed over the reported time. A standard curve of dependence of aldehyde fluorescence response on protein concentration was generated by adding an equivalent amount of each protein sample as used in enzymatic assays to explain the protein-induced aldehyde quenching.

Kinetic Assays. Fluorescent assays for both BAC36707 and NM_133960 carboxylesterases were conducted according to procedure described above, except for a 5 min incubation at 30 °C before adding substrates. The concentrations of cypermethrin and fenvalerate analogues were 9.0, 4.5, 2.7, 1.8, 0.9, 0.45, 0.225, and 0.0225 and 1.8, 0.9, 0.45, 0.225, and 0.0225 (μM final concentration), respectively. Three replicates were conducted for each concentration. Reported kinetic data (K_m and V_{max}) were calculated based on the Michaelis−Menten equation and three replicates.

Results and Discussion

Generation of Enantiomeric Excess of 6-MONCH(OH)CN. Cyanohydrins are highly versatile synthetic intermediates, which are precursors to a great number of synthetic targets such as α-hydroxyl acids, α-hydroxyl carboxamides, α-amino acids, and β-amino alcohols (*25*). They are also necessary components of agriculturally important compounds including type II pyrethroid insecticides (*12*) and their analogues (*18*). Because of their importance, considerable attention has recently been focused on the preparation of nonracemic cyanohydrins. Several useful classes of catalysts for the asymmetric addition of cyanides to aldehydes have been reported as follows: synthetic peptides (e.g., *cyclo*[(*S*)-Phe-(*S*)-His]) and chiral transition metal complexes (e.g., complexes of BINOLAM and aluminum) (*26*) and enzymes (e.g., oxynitrilases).

In this study, all three types of catalysts used for asymmetric addition have been tried using 6-methoxy-2-naphthaldehyde as a substrate. The methods used for activation of the dipeptide, *cyclo*[(*S*)-Phe-(*S*)-His], made little difference in conversion and %ee. For example, activation by freeze-drying a water solution, crystallization from methanol and ether, or removal of acetic acid from the acetate salt (i.e., by addition of dipeptide to acetic acid) by distillation of toluene through a fractionation column gave almost the same low conversions and %ee (data not shown). However, solvent composition gave minor differences in the enantiomeric excess (Table 1). Residual water in the HCN solution might contribute to the low %ee. However, Tanaka et al. (*21*) obtained high conversion (76%) and %ee (93%) using HCN in water and solvent (toluene and dichloromethane = 1:2). The use of chiral transition metal complexes gave similar results, i.e., low conversion and %ee using *R*- or *S*-BINOLAM as a substrate for metal complexes. The data (Table 1) provided by Prof. Casas were obtained under conditions of lower temperature (−40 °C) and extended time (39 h). We could not maintain such conditions in our laboratory. In contrast, the use of oxynitrilase gave much better results. Optimal conditions for the highest activity of *R*-oxynitrilase were 0.1 M, pH 3.75, citrate buffer, and this enzyme is sold in such a buffer. The first trial with 0.1 M, pH 3.75, citrate buffer resulted in very high conversion (72.5%, even though this is a reversible enzyme) and moderate %ee (63.6%). Buffer exchange resulted in very high %ee (93%) but only moderate conversion (46%). The source of cyanide (e.g., HCN or acetone cyanide) did not affect %ee. However, for *S*-oxynitrilase, the source of cyanide was critical (99.2%ee and 90.0% conversion for HCN and 0.0%ee and 53.1% conversion for KCN). The difference in conversion between *R*- and *S*-oxynitrilase results from the fact that the former is a reversible enzyme, but the latter is not.

Determination of Conversion and %ee. The methods for direct (e.g., HPLC with chiral column) and indirect (e.g., formation of derivatives) determination of conversion from aldehydes to cyanohydrins and %ee of cyanohydrins are available in the literature (*21*, *27*). Indirect determination of %ee of 6-MONCH(OH)CN by synthesis of a derivative with (1*R*,2*S*,5*R*)-(−)-menthylchloroformate and then ^{1}H NMR was known to determine absolute configuration of *R* and *S* isomers (*21*). In this study, the same method was adopted to determine absolute configuration. In ^{1}H NMR analysis, the two peaks of the methine proton α to the cyanohydrin group were not completely separated (less than 0.03 ppm or 9 Hz, Figure 3A), resulting in imprecise integration and estimation of

%ee. However, conversion to *R*- and *S*-FA esters gave complete separation (0.05 ppm or 15 Hz interval). Similarly, using carboxylic acids with a chiral center at the 2-position [e.g., α-methoxy-2-naphthylacetic acid, α-methoxy-α-(trifluoromethyl)phenylacetic acid, and (*S*)-2-(6-methoxynaphth-2-yl)propionyl chloride] have been reported (*21*, *28*, *29*). In chiral HPLC separation, two peaks of *R*- and *S*-cyanohydrin acetate or butanoate were completely separated (Figure 3B) allowing precise measurement.

The traditional method used for synthesizing optically pure stereoisomers of pyrethroid insecticides or analogues with PA was based on a reaction between optically enriched PA chloride and alcohols or cyanohydrins (*18*, *20*). In this study, this traditional method was adopted at the beginning with optically enriched 1*R cis*-PA. One minor product (5−10%) was obtained and could be separated from the major product with hexane and isopropyl ether on a silica column. Similar phenomena were observed when optically enriched 1*S cis*-PA was used. However, when optically pure *1R trans*-PA was used, no minor product was obtained. This led to adopting procedures to synthesize individual stereoisomers with the method as outlined in Figure 5. The individual stereoisomers were obtained effectively. After individual stereoisomers were obtained, standard 1*R cis*-PA and 1*R trans*-PA were used to determine the absolute configuration of each individual stereoisomer. However, using a mixture of *cis*- and *trans*-PA was not successful because of similar R_f values between 1*R trans*-PA and 1*S cis*-PA with *R*-6-MONCH(OH)CN or 1*R cis*-PA and 1*S trans*-PA with *S*-6-MONCH(OH)CN.

Identification of Pyrethroid Fluorescent Substrates. Not surprisingly, each pair of enantiomers of substrates with FA or PA shared the same chemical and physical properties such as melting point, ^{1}H NMR spectra, and retention time on an achiral GC column. In contrast, different diastereomers showed different chemical and physical properties. These properties were also utilized for identification. For example, when optically pure *R*- or *S*-FA was used, the optical purity of pyrethroid substrates with FA was assessed by GC based on different retention times or by ^{1}H NMR based on the different chemical shifts of the two diastereomers. Similarly, these two methods were also used for substrates with PA, but substrates with PA had to be recrystallized once from hexane and ethyl acetate because each individual stereoisomer that was obtained by the method described in Figure 5 contained a small amount of its corresponding enantiomer, depending on the enantiomeric excess of 6-MONCH(OH)CN. After recrystallization, optical purity by GC or ^{1}H NMR analysis was closer to expected values. In addition, the optical purity of the four stereoisomers of FA esters was determined with chiral HPLC unless the optical purity was very high because of the close retention time of two diasteromers (15.45 min for 2*R*,α*R* and 15.29 min for 2*S*,α*R*) using a mobile system of 0.6% 2-propanol in hexane. When the percentage of 2-propanol was decreased to 0.5%, the compounds did not come off after 2 h. In contrast, the optical purity of substrates with PA could be determined with chiral HPLC.

X-ray Crystallography. To further verify our targets and synthetic routes, a pair of enantiomers of the cypermethrin analogues (1*S trans*,α*R* and 1*R trans*,α*S*) and one of the fenvalerate analogues (2*R*,α*S*) were identified by X-ray crystallography. The results (Figure 6) indicated that our synthetic route and our targets were correct.

Specific Activities of Carboxylesterases toward Chiral Fluorescent Substrates. Hydrolytic enzymes such as carboxylesterases have received considerable attention in the last two decades. One of the main reasons is that carboxylesterases often exhibit high enantioselectivity (*30*, *31*), which can be used for enzymatic synthesis of chiral acids or alcohols or their derivatives. Carboxylesterases do not always show satisfying performance in terms of activity, stability, and most importantly stereoselectivity. However, these properties could be optimized by alteration of substrates or the reaction system or by protein engineering techniques (*32*). In this study, synthesized chiral pyrethroid fluorescent substrates were used to evaluate the stereoselectivity of two purified murine liver carboxylesterases under the same conditions. The cDNA for these enzymes were cloned from murine liver, and both were shown to hydrolyze pyrethroid insecticides when expressed in the baculovirus system (*14*).

Pyrethroid Fluorescent Substrates (Table 2). Significant differences were shown in specific activities of each carboxylesterase toward individual stereoisomers of both cypermethrin and fenvalerate analogues. For cypermethrin analogues, the two chiral centers in the acidic moiety produced larger effects on hydrolysis than the chiral center in the alcoholic moiety (cyanohydrin). In general, the stereoisomer (*1S trans*,α*S*) displayed the highest activities and its corresponding diastereomer (*1S trans*,α*R*) showed 1−2-fold lower activities for both carboxylesterases. However, a different pattern in substrate preference of each carboxylesterase was shown. For this study, we consider the enzyme to show a preference if one stereoisomer is hydrolyzed over five times faster than the least favored isomer in a single series. For example, purified BAC36707 (apparently the major single enzyme hydrolyzing cypermethrin in murine liver) preferred five stereoisomers (1*R trans*,α*R*; 1*S trans*,α*R*; 1*R cis*,α*R*; 1*R trans*,α*S*; and 1*S trans*,α*S*) to the other three (1*R cis*,α*S*; 1*S cis*,α*R*; and 1*S cis*,α*S*). However, purified NM_133960 only favored three (1*S trans*,α*R*; 1*R cis*,α*S*; and 1*S trans*,α*S*). Although there has not been a previous report on the spectrum of stereospecific hydrolysis of eight stereoisomers of cypermethrin by mammalian carboxylesterases, similar stereoselective hydrolysis by carboxylesterases from soil microorganisms has been reported (*33*).

It is well-known (*34*) that (1*R cis*,α*S*) and (1*R trans*,α*S*) are the most toxic stereoisomers of cypermethrin, and enantiomer pairs (1*R cis*,α*S*; 1*S cis*,α*R*) and (1*R trans*,α*S*; 1*S trans*,α*R*) are much more toxic than the enantiomers (1*R cis*,α*R*; 1*S cis*,α*S*) and (1*R trans*,α*R*; 1*S trans*,α*S*). It is interesting that the most toxic isomers are among those isomers most resistant to hydrolysis by BAC36707 and NM_133960. Moreover, the isomer (1*R cis*,α*S*) of cypermethrin, like deltamethrin, has the optimal configuration at each chiral center for the main target site (i.e., voltage sensitive sodium channel) of the central nervous system as well as the highest insecticidal activity and highest toxicity to mammals (*35*). Surprisingly, the patterns of rapid hydrolysis of the least toxic isomers of these pyrethroids are in accordance with the patterns of hydrolysis of the surrogate fluorescent substrates by BAC36707. This recombinant enzyme comprises less

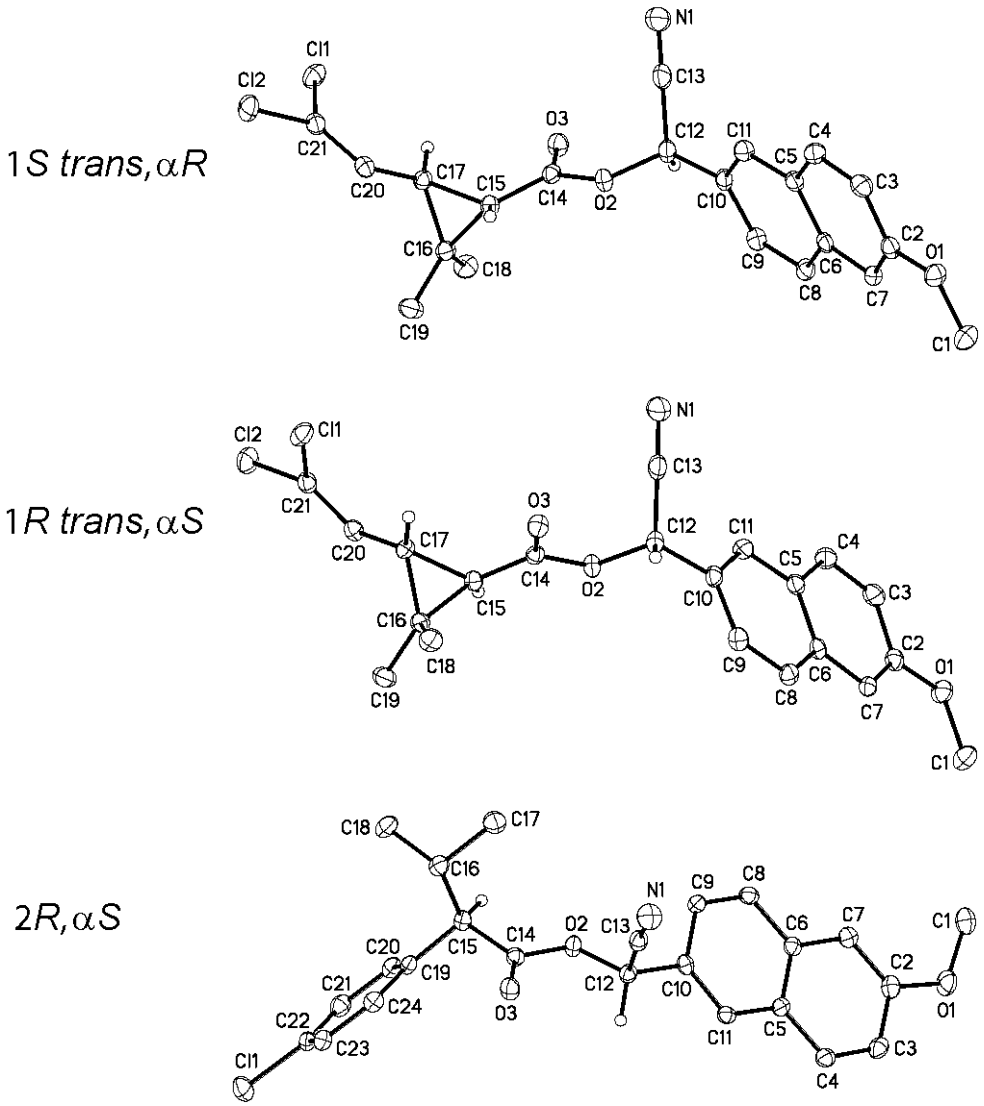

Figure 6. Computer-generated drawing of (1*S trans*,α*R*), (1*R trans*,α*S*), and (2*R*,α*S*) with the atom-labeling scheme. Hydrogen atoms except for those located at the chiral center are omitted for clarity.

Table 2. Specific Activities of Carboxylesterases toward Chiral Fluorescent Substrates Mimicking Cypermethrin and Fenvalerate

substrates	NM_133960 (nmol/min/mg)	BAC36707 (nmol/min/mg)
1*R cis*,α*R*	3.94 ± 0.37	19.74 ± 2.18[b]
1*S cis*,α*R*	7.44 ± 0.66	8.44 ± 0.37
1*R trans*,α*R*	7.63 ± 0.27	26.24 ± 2.80[b]
1*S trans*,α*R*	85.14 ± 3.23[b]	280.69 ± 23.64[b]
1*R cis*,α*S*	20.32 ± 0.07[b]	2.28 ± 0.31
1*S cis*,α*S*	8.69 ± 0.85	6.69 ± 1.06
1*R trans*,α*S*	7.34 ± 0.76	14.58 ± 1.19[b]
1*S trans*,α*S*	155.09 ± 1.87[b]	326.55 ± 25.31[b]
2*R*,α*R*	10.27 ± 0.44[b]	40.25 ± 2.62[b]
2*R*,α*S*	15.18 ± 0.25[b]	54.17 ± 3.44[b]
2*S*,α*R*	0.63 ± 0.05	NM[a]
2*S*,α*S*	0.79 ± 0.11	NM[a]

[a] NM, not measurable. [b] Indicates preference for a stereoisomer: The preference was considered if the activity of the carboxylesterase toward one stereoisomer was over 5-fold higher than the lowest activity of eight stereoisomers of cypermethrin analogues or four stereoisomers of fenvalerate analogues.

than 0.5% of the total hepatic protein, but it accounts for approximately 30−40% of total cypermethrin hydrolysis activity in murine liver (*14*). Undoubtedly the stereospecific interaction of the pyrethroid with the target site is the major factor determining pyrethroid toxicity. However, the stereospecificity of hydrolysis may play an additional role.

For fenvalerate analogues, the acidic moiety with the 2*R* form showed the highest activity for both carboxylesterases (BAC36707 and NM_133960). This preference is similar to the result that two pairs of diastereomers of fenvalerate analogues [(α*R*/*S*)/(2*R*) and (α*R*/*S*)/(2*S*)] are hydrolyzed by both purified carboxylesterases (*14*) and mouse liver microsomes (*13*). Interestingly, all four isomers of fenvalerate are mainly hydrolyzed in murine liver, and the liver microsomes hydrolyzed the (2*R*,α*R*) and (2*R*,α*S*) isomers of fenvalerate to a larger extent and faster than their corresponding enantiomers (*36*). This indicates that fenvalerate and its fluorescent analogues share the same stereospecific hydrolysis by the recombinant enzymes tested here and by microsomes. However, differential stereoselectivity between (2*R*,α*R*) and (2*R*,α*S*) existed in fenvalerate and its analogues. With BAC36707, there was at least a 10-fold preference for the (2*R*,α*R*) isomer of fenvalerate as compared to its corresponding diastereomer (2*R*,α*S*) (*14*) but a significant preference for the (2*R*,α*S*) isomer of fenvalerate fluorescent substrates.

Table 3. Kinetic Data of Recombinant Mouse Liver Carboxylesterase (BAC36707 and NM_133960) toward Pyrethroid Fluorescent Substrates

enzyme	substrates	K_m (μM)	V_{max} (nmol/min/mg)
BAC36707	mix[a]	2.20 ± 0.23	155.75 ± 7.22
	1*R cis*,α*S*[b]	1.70	1.95
	1*S trans*,α*R*	4.45 ± 0.42	560.96 ± 44.34
	1*S trans*,α*S*	4.36 ± 0.34	736.30 ± 121.73
	2*R*,α*R*	0.57 ± 0.03	49.54 ± 1.40
	2*R*,αS	0.60 ± 0.03	57.12 ± 1.73
NM_133960	mix[a]	4.86 ± 0.18	33.20 ± 3.13
	1*S trans*,α*R*	3.13 ± 0.20	124.00 ± 6.73
	1*S trans*,α*S*	7.30 ± 0.32	199.26 ± 12.67
	2*R*,α*R*	2.80 ± 0.15	21.02 ± 1.37
	2*R*,α*S*	1.84 ± 0.13	28.82 ± 1.64

[a] Mix = eight stereoisomers of PA esters. [b] 1*R cis*,α*S* = only one replicate was measured.

This suggests that a different alcoholic moiety (cyanohydrin) also affects its preference for stereoselectivity. In addition, in vitro stereospecificity of the four isomers of fenvalerate is strongly dependent on tissues (*36*). Whether the stereospecificity of the four isomers of fenvalerate fluorescent analogues also shows such a dependence in other tissues needs further experimentation. Thus, these and other surrogate substrates for pyrethroids may be useful in determining the contribution of esterases to toxicity in both target and nontarget species (*37*, *38*).

The stereospecific hydrolysis of CEs could greatly change the toxicity of pyrethroids. For example, esfenvalerate is the most toxic isomer among four fenvalerate isomers and consists of 25% of the total mass in the fenvalerate mixture. The acute oral toxicity of fenvalerate to rats (LD_{50}, 370 mg/kg) is about one-quarter to that of esfenvalerate to rat (87 mg/kg, *39*), suggesting that the acute toxicity of fenvalerate largely results from esfenvalerate. This implies that the stereospecific actions of CEs effectively reduce the body burden of the less toxic isomers much faster than the toxic isomer.

In addition, these two recombinant CEs may be used in industrial synthesis. There was at least 10- or 20-fold difference between 1*R trans*,α*R* and 1*S trans*,α*R* or 1*R trans*,α*S* and 1*S trans*,α*S*, respectively, indicating that these two carboxylesterases could be used to resolve *trans*-PA. Similarly, both BAC36707 and NM_133960 prefer (at least 10 times) 2*R* isomers of fenvalerate and its analogues, suggesting that these carboxylesterases could be used to resolve α-chiral acids with similar structures to FA. For example, antiinflammatory drugs such as ibuprofen [(*R*,*S*)-2-(4-isobutylphenyl)propionic acid], naproxen [(*R*,*S*)-2-(6-methoxy-2-naphthyl)propionic acid], and ketoprofen [(*R*,*S*)-2-(3-benzophenyl)propionic acid] are structurally similar to FA, belonging to α-chiral acids. Their *R*-counterparts are biologically inactive or have negative side effects (*40*, *41*). Thus, their active forms (*S*-isomers) may be obtained by enantio/stereospecific hydrolysis of their racemates by both BAC36707 and NM_133960.

Kinetic Data. One of the main objectives of this study was to know whether fluorescent pyrethroid substrates could be used to improve the sensitivity of the carboxylesterase assays used to monitor hydrolysis of pyrethroids. Data (Table 3) indicated that K_m and V_{max} [k_{cat} could be calculated by using the molecular mass of both BAC36707 and NM_13960 (62.5 kDa)] varied significantly with stereoisomers and carboxylesterases. On the basis of V_{max}, the sensitivity of this analytical method, using a single stereoisomer (e.g., 1*S trans*,α*S*) of cypermethrin analogues instead of a mixture of eight stereoisomers, could be enhanced by 4−6 times for both BAC36707 and NM_133960. Remarkably, with carboxylesterase BAC36707, there was at least 300-fold difference in specific activity between the most insensitive stereoisomer (1*R cis*,α*S*) and the most sensitive one (1*S trans*,α*S*). In comparing K_m, there was a 1−2-fold difference among different stereoisomers in both carboxylesterases.

The cyanohydrin reporters offer many advantages over classical fluorescent reporter groups such as coumarin or resorufin in terms of lower background, better quantum yield, and larger Stoke's shifts when one uses them for phosphatase, P450, esterase, or other assays (*12*). With esterases in particular, they offer a much lower hydrolytic background. The α-cyano group appears to make them also more selective substrates for esterases involved in the hydrolysis of type II pyrethroids. Their ease of use and sensitivity as compared to assays involving actual pyrethroids make them attractive as well. However, a serious caution with the use of these compounds is that they are in fact only surrogates. As with all surrogate substrates, results with these substrates should be confirmed with the actual target compound. For example, in a recent study with murine enzymes, a surrogate substrate for cypermethrin successfully predicted the major single cypermethrin hydrolyzing esterase in hepatic homogenates (BAC36707) (*14*). Surprisingly, this was the major single esterase involved in the hydrolysis of a variety of ester-containing pyrethroids. The surrogate substrate was an essential tool in the purification, cloning, and expression of this enzyme. However, a caution is that less than 10% of the metabolism of this surrogate substrate was associated with a mixture of esterases, which collectively account for the metabolism of 50−60% of cypermethrin in a homogenate (*14*).

Conclusions. An effective synthetic method for preparation of enantiomerically enriched *R*- and *S*-6-MONCH-(OH)CN from 6-methoxy-2-naphthaldehyde has been developed comparing three classes of chiral catalysts: enzymes (e.g., oxynitrilases), synthetic peptides {e.g., *cyclo*[(*S*)-Phe-(*S*)-His]}, and chiral transition metal complexes (e.g., complexes of BINOLAM and aluminum). Methods for synthesis of derivatives for the determination of enantiomeric excess were developed. Moreover, an efficient synthetic route for obtaining individual stereoisomers of substrates with both PA and FA was developed, using achiral GC and chiral HPLC chromatography, ^{1}H NMR, and X-ray crystallographic analyses.

Determination of specific activity of carboxylesterases toward these chiral fluorescent substrates showed that a very high stereoselectivity (up to 300 times) existed among different stereoisomers of cypermethrin and fenvalerate analogues. The stereoselective pattern of the same substrates also varied greatly between carboxylesterases. In addition, on the basis of V_{max}, the sensitivity of this analytical method, using a single stereoisomer (e.g., 1*S trans*,α*S*) of cypermethrin analogues instead of a mixture of eight stereoisomers, could be enhanced by 4−6 times for both mouse liver carboxylesterases (BAC36707 and NM_133960).

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Supporting Information Available: X-ray crystallography in CIF file for structure determination of (MN 1671, 1*S trans*,α*R*), (MN 1672, 1*R trans*,α*S*), and (MN 1674, 2*R*,α*S*).

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