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Development of Sensitive Esterase Assays Based on α-Cyano-Containing Esters

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A novel approach is reported for the development of fluorogenic esterase reporters using α-cyano-containing esters as substrates. After ester hydrolysis, the released alcohol, a cyanohydrin, rapidly eliminates HCN to yield the corresponding aldehyde resulting in strong fluorescence. The π conjugation of the resulting aldehyde also greatly enhances UV absorption and red shifts fluorescence emission relative to a corresponding alcohol or phenol. Two substrates, *R/S*-acetic acid cyano-(6-methoxynaphthalen-2-yl)-methyl ester (compound I) and *trans/cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid *R/S*-cyano-(6-methoxynaphthalen-2-yl)-methyl ester (compound II), were synthesized and evaluated as substrates. Such α-cyano substrates possess very low background fluorescence and are more stable under enzyme assay conditions than phenolic substrates due to the aliphatic cyano group. The higher molar absorbtivity and quantum yield of the aldehyde, along with its larger Stokes' shift combined with the increased stability and lower background signal of the cyanohydrin substrate, increases the utility and sensitivity of the resulting assays over current methods. Moreover, compound II showed high selectivity to pyrethroid-cleaving esterases and may provide a direct tool to monitor pyrethroid resistance in insects. © 2001 Elsevier Science

Key Words: **esterase; enzyme reporter; fluorogenic substrate; α-cyanoesters.**

Light-absorbing (colored) and fluorescent molecules play an important role in chemistry, molecular biology, and medical science. Such probes enable scientists to detect particular components in complex environmental and biological matrices, including tissues and cells, with exquisite sensitivity and selectivity. For example, carboxylesterases (EC 3.1.1.1) consist of a group of isozymes that can hydrolyze a wide range of esters, thioesters, and amides. These enzymes are known to play a key role in the metabolism of a wide range of xenobiotics such as drugs, pesticides, and endogenous compounds in insects, mammals, and most other species. Sensitive and specific substrates are of great significance for enzyme characterization, histochemistry, toxicological, and physiological studies. Common esterase substrates lack either sensitivity, such as *p*-nitrophenyl acetate (a colorimetric substrate) (1) and/or poor stability in assay systems leading to a high background signal as occurs for carboxyfluorescein diacetate (a fluorescent substrate). In addition, due to the great diversity of esterase isozymes, use of general substrates may not detect some specific isozymes, such as pyrethroid-cleaving esterases. The novel strategy described here, which was recently invented in our laboratory, will enable us to design and develop substrates to minimize these drawbacks.

Pyrethroids are fast becoming the dominant insecticide class. In both mammals and insects, pyrethroid are metabolized into an acid and an alcohol by hydrolytic ester cleavage (2–4). The hydrolysis of α-cyanopyrethroids produces an acid and a cyanohydrin that spontaneously rearrange into the corresponding *meta*-phenoxybenzaldehyde in aqueous solution (5) (Fig. 1). It was observed that this aldehyde molecule has extended π conjugation that should absorb light much more strongly than the parent compound and α-cyanophenoxybenzyl alcohol, providing the basis for a sensitive and specific assay for pyrethroid esterases. If we generalize this concept, an esterase substrate containing an α-cyano group could be designed with little or no fluorescence that is transformed to a strongly fluorescing aldehyde upon ester hydrolysis. In this paper we demonstrate this strategy for the development of novel esterase reporter substrates. To find isozyme specificity for a group of pesticides such as pyrethroid, substrate should look the same or similar to target compounds. Thus, a pyrethroid-like substrate which yields a fluorescent product upon hydrolysis was also synthesized and evaluated.

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MATERIALS AND METHODS

Reagents

Cypermethrin was obtained from Riedel de Haen (Seelze, Germany). *meta*-Phenoxybenzaldehyde, 2-naphthyl acetate (2-NA),[1] 2-naphthol, 6-methoxy-2-naphthaldehyde, 4-methyl 7-hydroxycoumarin (HO-coumarin), 4-methylumbelyl acetate, zinc iodide, trimethylsilyl cyanide (TMSCN), and other organic materials for substrate synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Lancaster Synthesis, Inc. (Windham, NH). Thin-layer chromatography (TLC) utilized 0.2-mm precoated silica gel 60 F254 on glass plates from E. Merck (Darmstadt, Germany), and detection was based on the quenching of fluorescence from ultraviolet light. Flash chromatographic separations were carried out on 40-μm average particle size Baker silica gel. Porcine liver carboxylic esterases (250 units/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO). One unit is defined as that enzyme which hydrolyzes 1.0 μmol of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25°C. Pyrethroid-susceptible and -resistant homogenates of *Heliothis virescens* were kindly provided by Dr. Ottea of Louisiana State University.

Instruments

Proton NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million (ppm) downfield from internal tetramethylsilane. Gas chromatography with mass spectral detection were performed on 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-5MS) (J & W Scientific, Folsom, CA). Fluorescent detection was performed in 4-mL quartz cuvette with a Fluoromax-2 fluorospectrometer (Instruments S. A., Inc., Edison, NJ). Kinetic study for aldehyde conversion was performed on Shimadzu RF-5301PC spectrofluorophotometer with a magnetic stirrer and injection system (Shimadzu Scientific Instruments, Kyoto, Japan). UV absorption was measured on Shimadzu UV-2101 PC spectrophotometer (Shimadzu Scientific Instruments, Inc., Pleasanton, CA).

Synthesis of (R/S)-Hydroxy-(6-methoxynaphthalen-2-yl)-acetonitrile

The hydroxy-(6-methoxynaphthalen-2-yl)-acetonitrile (assigned as cyanohydrin in the text) was prepared using the method described by Gassman and Tally (6). Zinc iodide (50 mg) was added to a solution of methoxynaphthaldehyde (1.86 g, 10 mmol) in 4.5 mL of $CHCl_3$ and stirred under N_2 with cooling in ice bath. Then one-half of 11 mmol TMSCN was injected at ambient temperature and the solution was allowed to cool slowly to ~30°C. The remainder of the TMSCN was injected after a few minutes, and the mixture was warmed to 40°C for 10 min and then stirred for 1.5 h at ambient temperature. The reaction mixture was stirred with a mix of 15 mL of ethylene glycol dimethyl ether containing 3.5 mL of 3 N HCl under fume hood (excess HCN generated!). The process was mildly exothermic. Then the mixture was stirred at ambient temperature for 30 min, diluted with water, and extracted with $CHCl_3$ twice, and the organic solvent washed with water and filtered through anhydrous Na_2SO_4 to remove any suspended water and the solvent was stripped to yield 1.91 g (91%) *R/S*-hydroxy-(6-methoxynaphthalen-2-yl)-acetonitrile. TLC R_f 0.66 (hexane:EtOAc = 4:1). ^{1}H NMR ($CDCl_3$): δ2.10 (d, 1 H, COH), 3.76 (d, 3 H, CH_3O), 5.60 (d, 1 H, CHCN), 7.02–7.75 (m, 6 H, Ar). M^+ = 214.

Synthesis of (R/S)-Acetic Acid Cyano-(6-methoxynaphthalen-2-yl)-methyl Ester (Compound I)

Esterification followed procedures employed in preceding publication (7, 8). The resulting cyanohydrin (1.06 g, 5 mmol) was dissolved in 7.5 mL of $CHCl_3$ and stirred with ice cooling as 1 mL of acetyl chloride was added rapidly followed immediately by 1.0 mL of pyridine. After 0.5–1 h, the mixture was washed twice with water, dried, and stripped to give a white solid that was flash chromatographed on silica gel (20 g) column (20% EtOAc in hexane) to recover after vacuum stripping 0.62 g (47%) of compound **I** (m.p. 84–85°C). TLC R_f 0.75 (hexane:EtOAc = 4:1). ^{1}H NMR ($CDCl_3$): δ2.20 (d, 3 H, $COCH_3$), 3.96 (d, 3 H, CH_3O), 6.56 (d, 1 H, CHCN), 7.16–7.95 (m, 6 H, Ar). M^+ = 256.

*Synthesis of trans/cis-3-(2,2-Dichloro-vinyl)-2,2-dimethylcyclopropanecarboxylic Acid Cyano-(6-methoxynaphthalen-2-yl)-methyl Ester (Compound **II**)*

(±)-*trans/cis*-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (0.75 g, 3.59 mmol) in chloroform (1 mL) containing 1 μL of dimethylformamide was treated with thionyl chloride (0.52 mL, 7.12 mmol) and stirred under N_2 in an oil bath at 65°C for 2 h. The mixture was stripped briefly of solvents, and hexane (2 mL) was added and restripped. The residue was dissolved in chloroform (3 mL) and added all at once to an ice-cooled solution of hydroxy-(6-methoxynaphthalen-2-yl)-acetonitrile (0.64 g, 3 mmol), prepared as de-

[1] Abbreviations used: 2-NA, 2-naphthyl acetate; HO-coumarin, 4-methyl 7-hydroxycoumarin; TMSCN, trimethylsilyl cyanide; TLC, thin-layer chromatography; DMSO, dimethyl sulfoxide.

scribed above, in chloroform (3 mL) and pyridine (0.348 mL), and allowed to stand at ambient temperature for 3 h. The reaction mixture was washed in order with water, 1 N HCl solution, saturated $NaHCO_3$ solution, and water and dried over Na_2SO_4. Stripping of solvent gave a pale yellow oil that was flash chromatographed on silica gel (10% EtOAc in hexane) yielding 0.72 g (60%) of compound **II**. TLC R_f 0.82 (hexane:EtOAc = 4:1). ^{1}H NMR ($CDCl_3$): δ1.12 (m, 1 H, COCH−),1.20–1.36 (q, 6 H, $2CH_3$), 1.60 (m, 1 H, C<u>H</u>CH = CCl_2), 4.14 (d, 3 H, OCH_3), 5.56 (q, 1 H, CHC<u>H</u> = CCl_2), 6.56 (d, 1 H, CHCN), 7.15–7.94 (m, 6 H, Ar). M^+ = 404.

Kinetic Studies

pH rate profile. The rate of conversion from cyanohydrin to aldehyde was characterized as a function of pH at a constant ionic strength (0.1 M) and temperature (25°C). Tris–maleate buffers with different pH values were prepared as described by Gomari (9). The reaction was initiated by the addition of 3 μL (10 mM) hydroxy-(6-methoxynaphthalen-2-yl)-acetonitrile to 3 mL of Tris–maleate buffer. The formation of 6-methoxy-2-naphthaldehyde was detected by Fluoromax-2 with excitation at 330 nm and emission at 460 nm in a time course for 20 min.

Stability comparisons. The stability of compound **I** compared to methylumbelyl acetate and 2-naphthylacetate was quantitatively evaluated in assay solutions with different pHs. The degradation rate of each compound was determined with both fluorometers and UV spectrometers. In 3 mL of buffer, 3 μL of 10 mM substrates (final concentration, 10 μM) was added and the degraded product was measured for 5 min. HO-coumarin (product of methylumbelyl acetate) was measured at excitation 360 nm and emission 450–510 nm dependent on buffer pH tested. 2-Naphthol (from hydrolysis of 2-NA) was measured by UV spectrometers at 309 nm. Methoxynaphthaldehyde (product of compounds **I** and **II**) was monitored by both fluorometers (excitation at 330 nm and emission at 460 nm) and UV spectrometers (at 320 nm).

Enzyme Dependency

Porcine esterase and substrate **I** in a pH 8.0 (0.1 M) Tris–maleate buffer system were used for this study. In 3 mL of buffer with 10 μM (final concentration) of substrate, different amounts of porcine esterase (0.06, 0.09, 0.12, 0.18, 0.30, 0.60, 0.9, and 1.2 unit of enzyme) were added to initiate the assay. The rate of hydrolysis was measured at excitation 330 nm and emission 460 nm for 10 min. A control without enzyme was used for each assay and every study was tested in triplicate with same conditions.

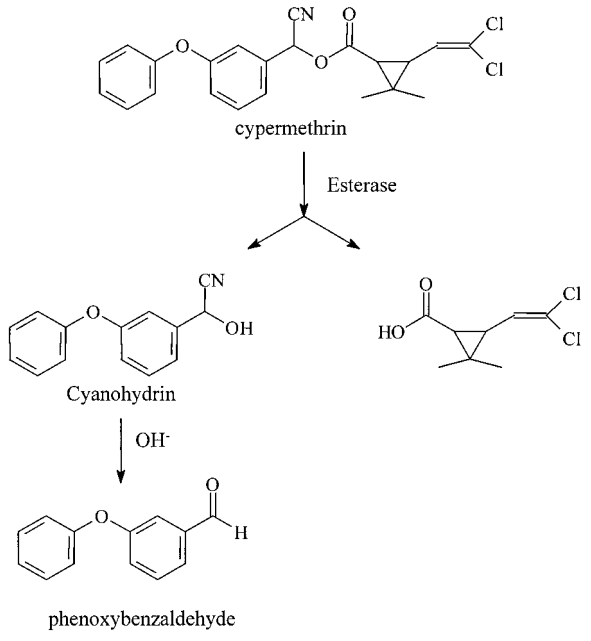

FIG. 1. Hydrolytic pathway of pyrethroids.

Enzyme Assay

Both substrate **I** and substrate **II** were tested with commercial porcine liver esterase, rat liver microsomes, and homogenates of *H. virescens*. In 3 mL of 0.1 M Tris–maleate buffer (pH 8.0), 3 μL of substrate in DMSO (10 mM) was first added (final concentration 10 μM). The reaction was initiated by the addition of 3 μL of enzyme preparations. Fluorescence intensity was measured at 460 nm and read for 1 min.

RESULTS AND DISCUSSION

Background and Rationale

Pyrethroid insecticides are generally divided into two classes: type I having no α-cyano in the alcohol portion of ester linkage and type II having an α-cyano. A major metabolism pathway of these compounds in many organisms is the enzymatic cleavage of the ester bond to form an acid and alcohol. The alcohol may be further oxidized into an acid form (2, 10). The alcohol moieties of type II pyrethroids are cyanohydrins, which are unstable and quickly and spontaneously convert to the corresponding aldehyde in basic solution (5). For example, cypermethrin can be hydrolyzed into dichlorovinyl chrysanthemic acid and phenoxybenzaldehyde cyanohydrin. The later will convert to phenoxybenzaldehyde in the aqueous solution (Fig. 1). Compared with the cyanohydrin and parent ester, the aldehyde possesses significant differences in optical properties, showing much stronger absorption at longer wavelengths (Fig. 2). The basis of this phenomenon is the

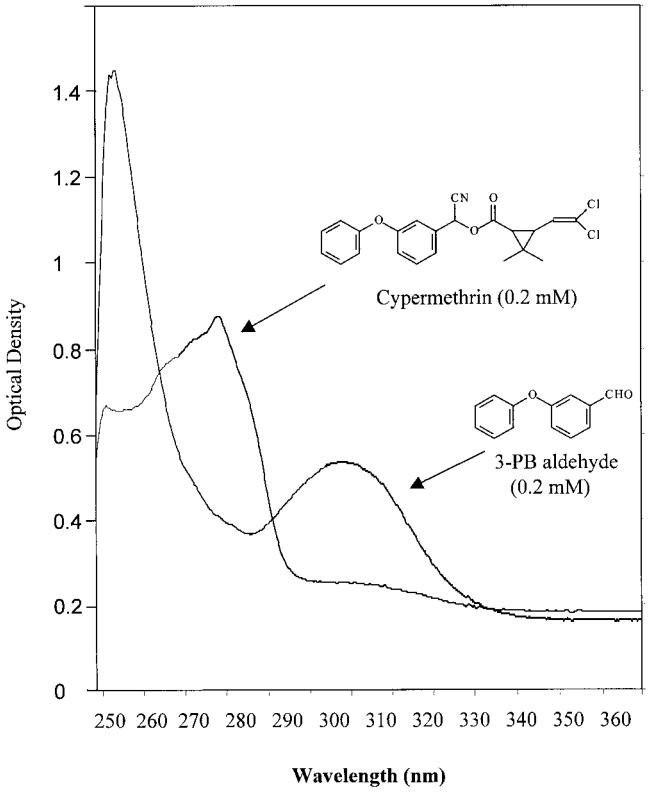

FIG. 2. UV spectra of cypermethrin and its breakdown product 3-phenoxybenzaldehyde.

formation of a C=O double bond on the phenoxyphenyl aromatic system, creating a stronger π conjugation in the product aldehyde and thus a stronger light absorption and red shift. By taking advantage of these physical properties and including aromatic groups with desirable fluorescent or chromatic properties, we hypothesized that one could design an α-cyano-containing esterase substrate with little background fluorescence. To apply this strategy, we designed and synthesized a general esterase substrate compound **I** (Fig. 3). 6-Methoxynaphthaldehyde is a known fluorescent

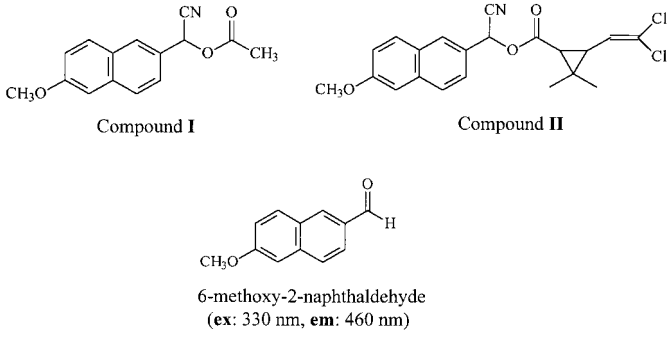

FIG. 3. Structures of fluorogenic substrates and corresponding aldehyde.

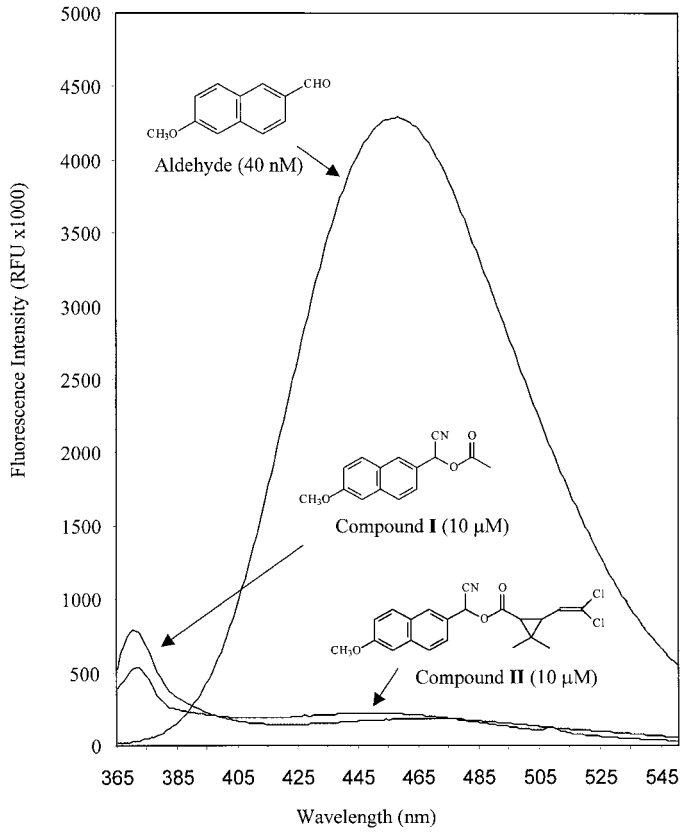

FIG. 4. Comparison of substrate and aldehyde emission curves. Compounds **I** and **II** and 6-methoxynaphthaldehyde in methanol were scanned at excitation 330 nm and emission from 360 to 550 nm. The concentrations of compounds **I** and **II** (10 μM) are 250 times higher than that of aldehyde (40 nM).

compound (excitation at 330 nm, emission at 460 nm); the interruption of the π conjugation within this molecule will greatly lower its light absorbance (11). The ester and its primary hydrolysis product, the cyanohydrin, have essentially no fluorescence emission upon irradiation with long-wavelength UV (Fig. 4), which is very important for a sensitive assay system.

In addition to general esterase reporters, the α-cyanoester to cyanohydrin to aldehyde scheme can also be used to develop isozyme class selective substrates. Pyrethroids are a group of insecticides widely used for the control of many agricultural, medical, and domestic pests (12). Esterases are a major metabolic enzyme for the detoxification of pyrethroids in animals. A rapid, sensitive, and selective assay is of importance for enzyme characterization, biological, and physiological studies and pyrethroid exposure as well as pest resistance monitoring. One of the main difficulties encountered in pyrethroid hydrolase research is the nonspecificity of current esterase assays. Crude enzyme preparations contain a large number of esterases that are capable of hydrolyzing many reporter substrates.

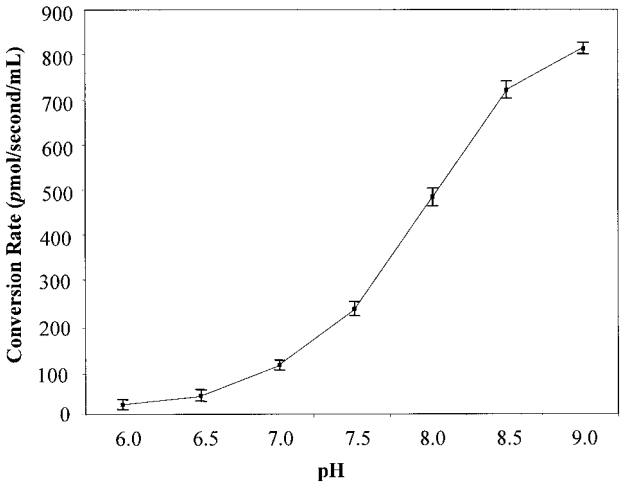

FIG. 5. Dependence of the aldehyde conversion rate on solution pH. Experimental conditions: 3 mL Tris–maleate buffer (pH 6.0–9.0) and the conversion was initiated with addition of 3 μL of 10 mM of cyanohydrin. The conversion rate, picomoles of cyanohydrin converted per second per milliliter, was measured at excitation 330 nm and emission 460 for 20 min.

This is often not an accurate reflection of an enzyme's ability to hydrolyze a given pyrethroid. Assays using the actual pyrethroid are conceptually superior, but the radiotracer, gas chromatography, and HPLC methods are slow and expensive and are point rather than continuous assays. An alternative is to use a large array of surrogate substrates of different structures, possibly with selective inhibitors, and find a combination that selectively detects the target substrate. In this article, we use an alternative approach of designing a surrogate substrate with desirable fluorescent properties which closely resembles the target substrate. Therefore, using the strategy described above, we coupled the cyanohydrin of 6-methoxynaphthaldehyde with a common pyrethroid acid (dichlorovinyl chrysanthemic acid) to yield a pyrethroid-like surrogate substrate termed compound **II** (Fig. 3) and evaluated it as a pyrethroid-selective esterase reporter.

Kinetic Investigation

To develop an efficient procedure for the esterase assay system, the effect of pH on cyanohydrin conversion to the aldehyde and assay sensitivity were studied. The mechanism of cyanohydrin conversion has been previously described by Julia (13). In the aqueous solution, the cyanohydrin is attacked by OH^-, which abstracts a proton and results in formation of a negative charge. The electron is then transferred inward to form a double bond and eliminate CN^- yielding an aldehyde. Although potentially toxic, the levels of cyanide anion released are far below the levels present in normal human urine as a result of intermediary metabolism (14). These levels of cyanide are unlikely to have effects on most enzyme reactions. Data from Fig. 5 show that the conversion from cyanohydrin to aldehyde is pH dependent and the conversion is promoted by basic conditions. The rate of aldehyde conversion increases rapidly at pH 8.0 or greater with maximum fluorescence achieved in less than 20 s. At least some of this delay may be due to mixing. This delay may be important in some very rapid kinetic experiments, but for routine enzyme monitoring and continuous processes, its effect can be ignored. At pH 8.0, the reaction rate is strongly correlated with the enzyme activity or amount of enzyme (Fig. 6) suggesting that this substrate can be used as an esterase enzyme reporter.

Substrate Stability and Comparison with Other Substrates

To further investigate the properties of the aldehyde fluorophore and substrate, we chose two widely used esterase substrates, 2-NA and methylumbelyl acetate, for comparison. Both compounds have aromatic systems similar to compounds **I** and **II** with two unsaturated aromatic rings. Data for their UV absorption and their fluorescent properties are listed in Table 1 and 2. At all pH levels tested from 6.5 to 8.5, methoxynaphthaldehyde showed a significantly higher molar absorption at long wave length (314 nm) than its parent compound **I,** while the UV absorption of 2-naphthol did not change significantly compared to 2-NA. Although an enhanced absorption peak at 309 nm was observed for 2-naphthol, the intensity is relatively low. This indicated that the conjugation of an aldehyde with the naphthyl rings can dramatically improve its chromophore properties suggesting that such a strategy may be useful for the development of other sensitive

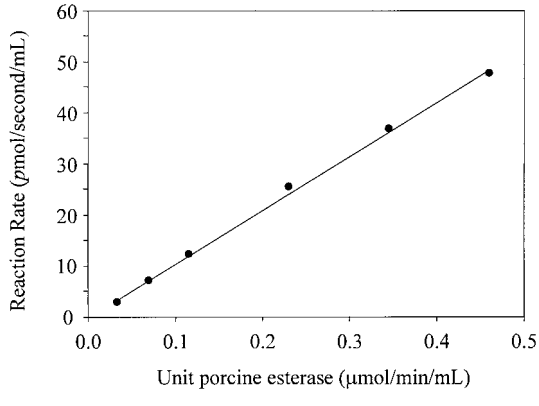

FIG. 6. Dependence of the observed rate constant on enzyme concentrations. Assay conditions: 3 mL Tris–maleate buffer (pH 8.0) with 10 μM of substrate **I,** and the assay was initiated with addition of porcine esterase (0.06–1.20 unit of enzyme). The reaction rate, picomoles of substrate converted per second per milliliter, was measured at excitation 330 nm and emission 460 for 10 min.

TABLE 1

Comparison of Optical Property of 2-NA, Compound **I,** and Their Hydrolysis Products

pH	Detection method		2-NA		Compound **I**	
			Ester	Naphthol	α-Cyanoester	Aldehyde[a]
6.5	UV[b]	λ_{max} (nm)	284	285	287	314
		OD	0.14	0.105	0.086	0.473
		ϵ[c]	3,500	2,600	3,000	11,870
7.5	UV	λ_{max} (nm)	283	284	287	313
		OD	0.141	0.155	0.137	0.468
		ϵ	3,500	3,870	3,420	11,700
8.5	UV	λ_{max} (nm)	283	283	287	314
		OD	0.133	0.170	0.142	0.465
		ϵ	3,300	4,250	3,550	11,630

[a] Aldehyde, for 6-methoxynaphthaldehyde.
[b] UV, for UV absorption, the concentration of substances used in this study is 10 μM.
[c] ϵ, molar absorbtivity.

colorimetric substrates. In terms of fluorescent properties, both HO-coumarin and methoxynaphthaldehyde have strong light emission and large Stokes' shift (Table 2). However, methoxynaphthaldehyde showed a larger red shift under all pH conditions tested, which is important for improving the sensitivity of a fluorescent assay. From pH 6.5 to 8.5, no pH effects on emission wavelength and fluorescent intensity were observed for methoxynaphthaldehyde, while for HO-coumarin these properties are pH dependent. Its maximal emission peak varied from 450 to 510 nm with the increase of assay pH, and the parent compound methylumbelyl acetate also showed a pH-dependent emission peak (from 410 to 450 nm). This small red shift will cause a high background from substrate at the wavelength used for assay, which will impact the assay sensitivity. In contrast, at all pHs tested, compound **I** had a low emission peak at 366 nm and essentially no emission at maximal emission peak of methoxynaphthaldehyde, 460 nm. Such a unique property gives this substrate an excellent signal/noise ratio over a wide range of pHs, suggesting that substrates releasing naphthaldehyde will provide superior enzyme reporters.

Substrate stability under assay conditions is an important factor for its performance. An unstable substrate will cause high background and thus limit the assay sensitivity. In comparison with 2-NA (by UV detection) and methylumbelyl acetate (by fluorescent

TABLE 2

Comparison of Optical Property of Compound **I,** Methylumbelyl Acetate, and Their Hydrolysis Products

pH	Detection method		Compound **I**		Methylumbelyl acetate	
			α-Cyanoester	Aldehyde[a]	Ester	HO-coumarin
6.5	Flu[b]	Ex[c] (nm)	330	330	360	360
		Em_{max}[d] (nm)	366	460	410	450
		RFU[e] (×1000)	2,672	18,830	241	17,783
7.5	Flu	Ex (nm)	330	330	360	360
		Em_{max} (nm)	366	460	450	485
		RFU (×1000)	1,350	19,025	581	19,250
8.5	Flu	Ex (nm)	330	330	360	360
		Em_{max} (nm)	368	460	450	510
		RFU (×1000)	1,600	19,195	3,708	19,256

[a] Aldehyde, for 6-methoxynaphthaldehyde.
[b] Flu, for fluorescence, the concentration of substances used in this study is 10 μM.
[c] Ex, optimal excitation wavelength (nm).
[d] Em_{max}, maximal emission wavelength (nm).
[e] RFU, relative fluorescent units from fluorometer.

TABLE 3

Comparison of Substrate Stability in Different Assay Systems

pH	UV absorption (mAbs/min)[a]			Fluorescence (pmol/min)[b]		
	2-NA	**I**	**II**	Methylumbelyl acetate	**I**	**II**
6	<0.01	<0.01	<0.01	14	<1	<1
7	0.05	<0.01	<0.01	20	8	<1
8	0.44	<0.01	<0.01	108	59	<1
9	0.555	0.103	0.05	830	138	10

[a] Degradation of substrates in buffer by UV detection. Assay conditions: Substrate was added (10 μM final concentration) to a Tris–maleate buffer (0.1 M) with different pH (6, 7, 8, and 9). Samples were immediately read by UV spectrometer at 309 nm for 2-NA and 320 nm for compounds **I** and **II** for 5 min.

[b] Degradation of substrates in buffer by fluorescence detection. Substrate was added (10 μM final concentration) to a Tris–maleate buffer (0.1 M) with different pH (6, 7, 8, and 9). Samples of compounds **I** and **II** were immediately read by fluorometer at excitation 330 nm and emission 460 nm for 5 min. Methylumbelyl acetate solutions were read at excitation 360 nm and different emission wavelengths for each pH buffer: 450 nm (pH 6.0), 465 nm (pH 7.0), 485 nm (pH 8.0), and 510 nm (pH 9.0).

detection), compound **I** showed much better stability than these two common substrates at all pH's tested (Table 3). Substrates such as *p*-nitrophenyl acetate, α-naphthyl acetate, and methylumbelyl acetate are excellent leaving groups from the tetrahedral transition state of either enzyme- or base-catalyzed hydrolysis. In contrast, the α-cyanoesters of compounds **I** and **II** are more reminiscent of benzyl or aliphatic esters and are much more chemically stable to hydrolysis. Compound **II** was synthesized to retain good fluorescence properties, while partially mimicking cypermethrin. This compound is similar to cypermethrin in three major ways. First, the α-cyano group in type II pyrethroids is thought to stabilize the compounds against metabolism by sterically hindering the benzylic carbon and thus the ester. The second reason is that the dichlorovinyl chrysanthemic acid is a large group identical to the acid moiety of several pyrethroids including permethrin, cypermethrin, and cyfluthrin. The acetate of compound **I** clearly is a small acid. The third reason that compound **II** mimics some pyrethroid esters is that the ester is conjugated. The cyclopropane of the dichlorovinyl chrysanthemic acid is so strained that it has aromatic character; thus electrons are delocalized from the vinyl group through the carbonyl. Thus the tetrahedral transition state leads to the acyl enzyme for enzymatic hydrolysis or an analogous tetrahedral structure from base-catalyzed hydrolysis is unfavored and the ester stabilized. In this way, compound **II** mimics some type I and type II pyrethroids, but not all. For example, the type II pyrethroid esfenvalearate does not have a resonance stabilized ester. Considering the substrate stability, aldehyde conversion rate, and the optimal enzyme activity, pH 8.0 was chosen as optimal assay pH for further studies.

Application

Substrates **I** and **II** were further evaluated with different enzyme systems. The sensitivity of an assay has many components. One issue is the stability of the substrate in buffer. As shown in Table 3, the α-cyanoesters are quite stable. With UV detection a second issue is the red shift and molar extension coefficient of the substrate vs the product. For example, Table 1 shows that there is a small red shift in λ_{max} of substrate vs product for 2-NA and a moderate increase in molar extension coefficient. In contrast, there is a red shift of approximately 90 nm between the substrate and product of compound **I** and a huge change in molar extension coefficient. Thus conjugated aldehydes formed from the corresponding chromophore should form excellent UV and colorimetric assays. Fluorescent assays depend on the above red shift between the substrate and product of emission and excitation but also on the Stokes' shift between excitation wavelength of the product and its emission. The Stokes' shift of the aldehyde is greater than that for HO-coumarin and the quantum yield similar. These data combined give an assay of very high sensitivity. In studies with porcine esterase, compound **I** could detect as low as 2×10^{-5} unit of this enzyme with fluorescence and 1×10^{-3} unit with UV detection (Table 4). Because of the large red and Stokes' shifts, even greater sensitivities could be obtained with compound **I** by using greater slit widths. This approach is of course not possible with 2-NA or methylumbelyl acetate.

The third issue is the selectivity of an assay. Enzyme homogenates from rat liver microsomes and pyrethroid-susceptible and -resistant tobacco budworm were also assayed with these two substrates (Table 5). High enzyme activities toward compound **I** were found in all preparations tested, while their catalytic activi-

TABLE 4

Sensitivity of General Esterase Substrates in Detecting Porcine Carboxylesterase

Substrate	Detection method	Sensitivity[a] (units of porcine esterase/mL)
2-NA	UV (at 310 nm)	0.002
Compound **I**	UV (at 314 nm)	0.001
Compound **I**	Fluorescence (em 330 nm; ex 460 nm)	0.00002
Methylumbelyl acetate	Fluorescence (em 360 nm; ex 485 nm)	0.0005

[a] Sensitivity is defined as the minimal amount of enzyme per milliliter used to cause at least three times greater signal than background noise from the substrate. In a test tube containing 3 mL of buffer (pH 8.0) and 10 μM of substrate, the porcine esterase was added and the UV absorption or fluorescence was measured after 1 min incubation at room temperature. 1 Unit = 1 μmol/min/mL.

ties against compound **II** are very low except for the commercial porcine esterase. Pyrethroid-selected tobacco budworm showed much higher level of activity than the susceptible strain against both compound **I** and **II.** To the general substrate compound **I,** enzyme-specific activity of homogenates from resistant strains is 1.5 times higher than the susceptible strain. A parallel assay using α-naphthyl acetate by Dr. Ottea's group of LSU (Baton Rouge, LA) showed similar results between these two strains (1.4 times). However, the resistant insects showed about 5 times higher activity than susceptible insect homogenates when tested with compound **II.** Based on a topical bioassay against cypermethrin, the pyrethroid-resistant strain is about 12 times more resistant than susceptible strain (personal communication, Dr. J. Ottea, LSU). These data suggest that elevated pyrethroid-specific esterase activity is associated with the resistance in this strain. These findings highlight the fact that enzyme assays using general substrates may fail to illustrate the real role of esterases in pyrethroid resistance mechanism; thus compound **II** or related pyrethroid mimetic substrates will be very useful for monitoring resistance mechanisms in insects. Moreover, rat liver microsomes showed lower esterase activity than the pyrethroid-selected insect strain against compound **II** indicating that these rat microsomes have lower pyrethroid-selective esterase activity.

The sensitivity of an assay depends on the optical properties of the reporter group and stability of the substrate as discussed above. It also depends in part on the interaction of the substrate with the enzyme. A kinetic evaluation of these substrates with crude enzyme preparations was not the purpose of this study. However, the maximum rate of enzymatic hydrolysis of **I** with porcine carboxyl esterase is similar to 2-NA and methylumbelyl acetate (data is not shown). The rate for **II** is reduced dramatically (Table 5), likely due to resonance stabilization of the ester and because the hydrophobic acyl enzyme have a greatly reduced k_{cat}. The cyanohydrin chemistry provides a simple, high-yield route to a variety of reporters that release aldehydes that can be detected at high sensitivity by their optical, redox, or chemical properties.

In conclusion, a novel approach was used for the development of a fluorogenic esterase reporter using an α-cyano-containing ester as substrate. Based on fluorophore spectral and fluorescent properties of 6-methoxynaphthaldehyde and the unique posthydrolysis conversion of the resulting cyanohydrin, two α-cyano-containing esterase substrates, general substrate **I** and pyrethroid-like substrate **II,** were synthesized and evaluated. These α-cyano substrates possess very low background and are more stable than phenolic substrates, in part, due to the aliphatic cyano group. After hydrolysis, the π conjugation of the resulting aldehyde results in much higher molar absorbtivity, larger Stokes' shift, and stronger and red-shifted fluorescence emission than the generation of a phenol. Following coupling of the reporter group with a pyrethroid acid, the resulting substrate, compound **II,** appeared selective for pyrethroid-cleaving esterases providing improved discrimination of pyrethroid resistance mechanisms. Such a substrate could facilitate the development of a sensitive and selective enzyme assay. The same strategy can be further applied to develop novel enzyme reporters for other enzymes such as cytochrome P450s, epoxide hydrolases, acetylcholine esterases, glucosidases, glucuronidases, and alkaline phosphatases.

TABLE 5

Specific Activity for Compounds **I** and **II** Evaluated as Substrates of Several Different Esterases

Enzyme/source	Sp act (pmol/min/mg protein)	
	Compound **I**[a]	Compound **II**[a]
Porcine esterase[b]	2,911,000 ± 1,780[c]	142,000 ± 520
Rat liver microsome[d]	39,800 ± 600	270 ± 18
H. virescens-susceptible[e]	9,700 ± 220	150 ± 9
H. virescens-resistant[f]	25,100 ± 425	840 ± 22

[a] Compound **I** is a general esterase substrate; Compound **II** is a pyrethroid-selective substrate. Assay conditions: Substrate was added (10 μM final concentration) to a Tris–maleate buffer (pH 8.0, 0.1 M) and followed by the enzyme preparation. Samples were immediately read by fluorometer at 460 nm for 1 min (ex. 330 nm).

[b] Purchased from Sigma Chemical Company.

[c] Standard error; each was from triplicates.

[d] Microsomal preparation from 8-week-old Fischer 344 rats.

[e,f] Pyrethroid-susceptible and -resistant strains of tobacco budworm obtained from Dr. J. Ottea at Louisiana State University.

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