JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of Chiral α-Cyanoesters as General Fluorescent Substrates for Screening Enantioselective Esterases

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Esterases play a crucial role in industrial chemical synthesis, maintaining normal physiological metabolism and detoxifying exogenous ester-containing toxicants. To meet the rapidly increasing industrial need for all kinds of esterases, especially enantioselective esterases used to generate highly pure chiral compounds, general substrates are necessary for rapid screening, monitoring, purification, and characterization. In this study, general fluorescent substrates including phenolic derivatives and α-cyanoesters were evaluated for sensitivity in detecting esterases in buffer systems. Results with two different esterases and different incubation times suggested that the α-cyanoesters examined were significantly more sensitive at detecting esterases than the corresponding tested phenolic derivatives. More importantly, α-cyanoesters, containing a secondary alcohol, possess at least one chiral center; thus, they are tools to screen for enantioselective hydrolysis. Results indicated that the enantioselectivity of esterases toward general α-cyanoesters strongly depended on the esterase and the substrate, but the majority of esterases examined preferred *S*-isomers to their corresponding *R*-enantiomers. Most appealing was the very high enantioselectivity displayed in cytosolic esterases of the house fly. The potential utility of such esterases is discussed. In addition, the use of α-cyanoesters as chiral fluorescent substrates was demonstrated for monitoring in enantioselective esterases.

KEYWORDS: Chiral fluorescent substrates; esterases; enantioselectivity, partial purification

INTRODUCTION

Esterases play an important role in maintaining normal physiology and metabolism, detoxifying various drugs and environmental toxicants in living systems (*1*−*4*), and are increasingly important for chemical synthesis in industry (*5*). Esterases catalyze the hydrolysis of a variety of ester-, thioester- and amide-containing chemicals. Interestingly, esterases hydrolyze some substrates regio-, enantio-, or stereospecifically (*5*). This characteristic can be used to design chiral drugs, for industrial chemical synthesis, and for metabolic studies with chiral xenobiotics (*6*, *7*).

To meet the rapidly increasing demand for the optically pure form of chiral compounds including drugs, pesticides, fine chemicals, and even plastics (*8*), chiral general substrates, which can be used as colorimetric or fluorescent assays in a 96 or 384 well plate or other high throughput format, will aid in rapid screening, purification, and characterization of enantioselective esterases. Most currently available general substrates for esterases are phenolic substrates [e.g., *para*-nitrophenyl acetate, 1-naphthyl acetate (1-NA), or 7-acetoxy-4-methylcoumarin (7-AC-4-MC)]. Novel fluorescent substrates (called α-cyanoesters) have been developed in our laboratory to facilitate the measurement of esterase-mediated hydrolysis of ester-containing xenobiotics (e.g., pyrethroid insecticides; *9*). Esterases hydrolyze the ester bond, releasing the α-cyano alcohol (or cyanohydrin), which quickly rearranges into the corresponding fluorescent aldehyde under neutral or basic conditions (*9*). As compared with phenolic substrates, these α-cyanoesters possess additional advantages including a larger Stokes' shift, lower intrinsic fluorescence of the substrate, high quantum yield, and slower chemical hydrolysis in buffer system (*9*). More importantly, phenols themselves are not chiral and do not yield chiral substrates unless they are coupled with optically active chiral acids (*10*), whereas α-cyanoesters are derivatives of a secondary alcohol (i.e., cyanohydrin) and they contain at least two enantiomers in their ester derivatives. Thus, these fluorescent esters may be used as a tool for evaluation of the enantioselectivity of various esterases. Actually, optically pure pyrethroid-like fluorescent ester substrates have been used to show that carboxylesterases hydrolyze the α-cyanohydrin enantiospecifically (*6*, *11*). These studies provided us an impetus for a further investigation into whether general α-cyanoesters (e.g., acetic acid or butanoic acid derivatives) can be used as tools for rapid screening and purification of enantiospecific esterases. Thus, the major objectives of this study were (i) to further compare the sensitivity of general fluorescent substrates in detecting esterases in buffer system, (ii) to evaluate the enantioselectivity

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10.1021/jf0526083 CCC: $33.50
Published on Web 01/17/2006

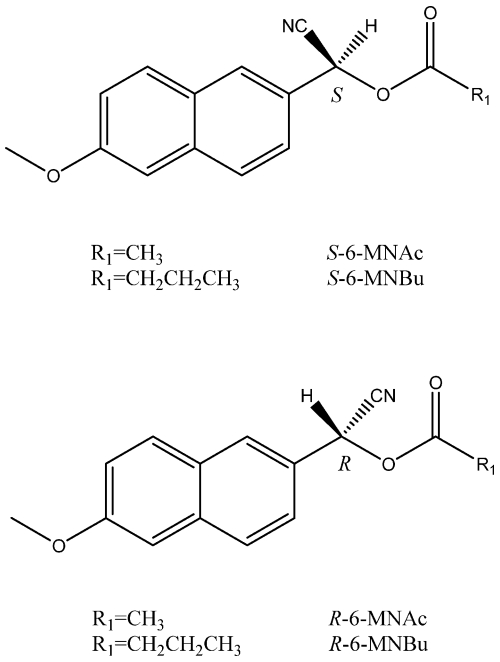

Figure 1. Structures and labels of chiral α-cyanoesters.

of commercial esterases, (iii) to screen enantioselective esterases from insects, and (iv) to demonstrate crude purification of enantiospecific esterases.

MATERIAL AND METHODS

Chemicals. Chiral fluorescent substrates in **Figure 1** were synthesized and characterized in previous work (*6*). They are (*R*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl acetate (*R*-6-MNAc), (*S*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl acetate (*S*-6-MNAc), (*R*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl butanoate (*R*-6-MNBu), and (*S*)-α-cyano-(6-methoxynaphthalen-2-yl)methyl butanoate (*S*-6-MNBu). The chemical purity of all of these compounds was over 99%, and the optical purities of *R*-6-MNAc, *S*-6-MNAc, *R*-6-MNBu, and *S*-6-MNBu were 95.9, 99.3, 93.1, and 97.6%, respectively (*6*). These compounds contained none of the corresponding aldehyde. 1-Phenyl-2-thiourea (PTU, 97%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). DL-Dithiothreitol (DTT, 99%), ethylenediaminetetraacetic acid (EDTA), and bovine serum albumin (approximately 99%) were obtained from Sigma Chemical Co. (St. Louis, MO). The percentage of enantiomeric excess (ee%) was calculated by $100 \times (R_{activity} - S_{activity})/(R_{activity} + S_{activity})$.

Esterases (See Table 1). Esterases from porcine liver (catalog no. E2884; porcine CarE), rabbit liver (catalog no. E-9636; rabbit CarE), and acetyl cholinesterase from electric eel (catalog no. C-2626; AChE) were purchased from Sigma Chemical Co. Esterases from *Bacillus stearothermophilus* (catalog no. 46051; BST), *Saccharomyces cerevisiae* (catalog no. 46071; SC), *Thermoanaerobium brockii* (catalog no. 46061; TB), *Mucor miehei* (catalog no. 46059; MM), *Bacillus* sp. (catalog no. 46062; BS), and *Streptomyces diastatochromogenes*, recombinant from *E. coli* (catalog no. 78042; SDREC) were obtained from Fluka (Industriestrasse 25, CH-9471 Buchs SG, Switzerland).

Insects (See Table 1). Pyrethroid-susceptible (CS and SRS) and -resistant (LPR, YPER, and NG98; *12*−*14*) strains of house fly, *Musca domestica*, were kindly provided by Dr. Jeffrey G. Scott from Cornell University (Ithaca, NY). Pyrethroid-susceptible (S1) and permethrin-resistant (PER; *15*) strains of Colorado potato beetle, *Leptinotarsa decemlineata* (Say), were generously offered by Dr. Marshall J. Clark from the University of Massachusetts (Amherst, MA). Pyrethroid-susceptible (ACY; *16*) and -resistant strains of German cockroaches, *Blattella germanica* (L.), were generous gifts from Dr. Nannan Liu from Auburn University (Auburn, AL). Pyrethroid-susceptible (S), -resistant (Super-kdr), and diazinon-resistant (LSU) strains of horn fly were generously provided by Dr. Allen J. Miller from the U.S. Department of Agriculture (Kerrville, TX).

Table 1. Sources and Abbreviations of Esterases Used in the Text

abbreviation	name and source
	commercial esterases
AChE	acetyl cholinesterase from electric eel
BS	esterase from *Bacillus* sp.
BST	esterases from *B. stearothermophilus*
MM	esterase from *M. miehei*
porcine CarE	carboxylesterases from the porcine liver
rabbit CarE	carboxylesterases from the rabbit liver
SC	esterase from *S. cerevisiae*
SDREC	esterase from *S. diastatochromogenes*, recombinant from *E. coli*
TB	esterase from *T. brockii*
	esterases from insects
CS, SRS	esterases from pyrethroid-susceptible strains of house fly
YPER, LPR, and NG98	esterases from pyrethroid-resistant strains of house fly
S	esterases from a pyrethroid-susceptible strain of horn fly
LSU	esterases from a diazinon-resistant strain of horn fly
super-kdr	esterases from a pyrethroid-resistant strain of horn fly
S1	esterases from pyrethroid-susceptible strain of Colorado potato beetle
PER	esterases from a permethrin-resistant strain of Colorado potato beetle
ACY	esterases from pyrethroid-susceptible strain of German cockroach
R	esterases from a pyrethroid-susceptible strain of German cockroach
CYP	esterases from a cypermethrin-resistant strain of tobacco budworm

Preparation of Cytosols. Third day adults of house fly (∼60 individuals/strain), fourth instar larvae of Colorado potato beetle (∼20 individuals/strain), and adults of horn fly (∼50 individuals/strain) were homogenized. Last stage nymph or adults of German cockroaches (∼30 individuals/strain) were decapitated and homogenized. Each strain of different insects was separately homogenized in 24 mL of 0.1 M ice-cold sodium phosphate buffer (pH 7.6; 0.1 mM PTU, 1 mM DTT, and 1 mM EDTA) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at speed 4−7 for 20 s. Multiple homogenizations were carried out if necessary, but the homogenate was cooled between homogenizations. The homogenate was then centrifuged at 4 °C and 10000*g* for 15 min, and the supernatant was filtered through glass wool and centrifuged at 4 °C and 100000*g* for 1 h. The 100000*g* supernatants were stored at −80 °C until used (less than 5 days). Protein was determined according to the method of Smith et al. (*17*). On the basis of the standard curve of bovine serum albumin, absorbance was converted into protein concentration.

Fluorescent Assays. Assays were performed by the method of Wheelock et al. (*18*). In short, fluorescent assays were conducted with a Spectrafluor Plus (Tecan, Research Triangle Park, NC). Activities were measured in black 96 well polystyrene clear flat-bottomed microtiter plates (Corning, Inc., New York, NY) at 30 °C for all esterases except that AChE was measured at 37 °C. Substrates were prepared in ethanol (10 mM). The reaction mixture contained (total volume, 201 μL) 20 μL of protein solution, 180 μL of 20 mM Tris/HCl buffer (pH 8.0) for α-cyanoesters or 180 μL of 100 mM sodium phosphate buffer (pH 7.0) for 7-AC-4-MC, and 1 μL of substrate solution. The reaction was initiated by adding 1 μL of substrate solution (final concentration, 50 μM) followed by shaking for 5 s. Three replicates were conducted for each substrate. Fluorescence was monitored with excitation at 330 nm (band-pass, bp 35) and emission at 465 nm (bp 35) for α-cyanoesters, or excitation at 330 (bp 35) and emission at 450 nm (bp 35) for 7-AC-4-MC. Assays for substrates were performed with 10 cycles (i.e., the number of cycles is the number of

actual measurement steps. It takes approximately 4 min for the Spectrafluor Plus to conduct 10 actual measurement steps) to give a ~4 min linear assay when enantioselectivity was evaluated. The amount of protein in each assay varied with the substrate and was adjusted so that the hydrolysis of the substrate was not more than 5% over the reported time. To correct for protein-induced aldehyde quenching, a standard curve of the fluorescent aldehyde is constructed in the presence of the same protein concentration as used for the assay of that enzyme.

Assays were performed with 10 cycles (~4 min) and 30 cycles (~13 min) when the sensitivity was evaluated. The experimental conditions were exactly the same as described as above except for protein concentrations. Sensitivity of general fluorescent substrates in detecting esterases in buffer was considered as the minimal amount of enzyme/mL used to cause at least three times greater signal than background noise from the substrate.

Crude Purification of Hydrolases by Ion-Exchange Column. An ion-exchange column (HiTrap DEAE FF, 5 mL) was sequentially washed with water (25 mL) and then starting buffer (10 mL, 20 mM, pH 8.0, Tris/HCl) at a speed of 1 mL/min. Cytosol (3 mL, 59.1 mg of protein) from pyrethroid-resistant house fly was loaded on the column by pump at 1 mL/min. The elution (16 mL of the total volume for each concentration) was done sequentially with starting buffers (20 mM, pH 8.0, Tris/HCl) containing different concentrations of sodium chloride (25, 50, 75, 100, 125, 150, 175, and 200 mM). Finally, the column was washed with starting buffer (30 mL) containing 1 M sodium chloride.

Statistical Treatment of the Results. Student's *t*-test was used to evaluate whether a difference between two means was significant at the given probability level. Analysis of variance (ANOVA)−Tukey's HSD test ($P \leq 0.01$) was used to analyze and compare the values from different strains of house fly or horn fly in **Table 4**.

RESULTS AND DISCUSSION

Sensitivity of General Fluorescent Substrates in Detecting Esterases in Buffer. Sensitivity used in this study was considered as the minimal amount of enzyme/mL used to cause at least three times greater signal than background noise from the substrate. The sensitivity of a fluorescent assay is determined by many components such as the fluorescence background of a potential substrate, stability of a substrate in experimental buffer, and the red and Stokes' shifts. Low background, higher stability in buffer, and larger Stokes' and red shifts make α-cyanoesters (e.g., 6-MNAc) as fluorescent substrates commonly better than phenolic substrates (e.g., 7-AC-4-MC) (*9*). Possibly of greater significance, these cyano aliphatic substrates are chemically quite distinct from phenolic substrates. Properties of the enzyme used and incubation time length (or the running time) may also determine the sensitivity of a fluorescent assay. These factors are crucial for purification of protein of low concentration or low activity. To determine these two factors, porcine CarEs and SDREC in this study were chosen under different running times. The former represented highly active esterases (184 units/mg protein), and the latter represented moderately active esterases (56 units/mg protein). Under optimized conditions for both 7-AC-4-MC and 6-MNAc, the sensitivity of these fluorescent substrates decreased with increased running time (**Table 2**). There was a 2-fold decrease from 10 cycles (approximately 4 min) to 30 cycles (approximately 13 min). Moreover, the sensitivity of both 7-AC-4-MC and 6-MNAc depended on the esterase used. There was at least a 30-fold difference in sensitivity between porcine CarEs and SDREC. However, the sensitivity of 6-MNAc was statistically (Student's *t*-test, $P \leq 0.002$) higher than that of 7-AC-4-MC (**Table 2**). Thus, the sensitivity of α-cyanoesters examined (e.g., 6-MNAc) as general fluorescent substrates was significantly higher than that of the tested phenolic fluorescent substrates (e.g., 7-AC-MC) in the system examined.

Table 2. Sensitivity of General Substrates in Detecting Porcine CarE and SDREC[a,b]

		ng/mL	
esterases	time (min)	7-AC-4-MC	6-MNAc
porcine CarE	4	1.01 (0.012)[c]	0.52 (0.002)
	13	2.05 (0.026)[c]	1.03 (0.001)
SDREC	4	40.3 (1.46)[c]	20.2 (0.16)
	13	60.5 (2.28)[c]	40.4 (2.17)

[a] Sensitivity is defined as the minimal amount of enzyme/mL used to cause at least three times greater signal than background noise from the substrate, and data (±SE) are represented as ng protein/mL based on triplicate assays. The final concentration of both 7-AC-4-MC and 6-MNAc in 96 well plate was 50 μM. The buffer conditions for 6-MNAc and 7-AC-4-MC were 20 mM Tris/HCl buffer (pH 8.0) and 100 mM phosphate buffer (pH 7.0), respectively. The enzymes were analyzed at 30 °C. [b] Porcine CarE and SDREC, porcine CarE and porcine liver carboxylesterases; SDREC, esterase from *S. diastatochromogenes*, recombinant from *E. coli*. [c] Stands for significance for a two-tailed distribution when two samples were assumed with equal variances. *p* values of ≤0.05 were considered a significant different level.

Table 3. Enantioselectivity of Commercial Esterases toward Chiral α-Cyanoesters

esterases[a]	isomers	6-MNAc[b]	*E*[c]	6-MNBu[b]	*E*[c]
BS	*R*	138 (11)	1.0	289 (37)	1.0
	S	755 (25)[d]	5.4	3080 (220)[d]	10.6
BST	*R*	468 (50)	1.0	673 (49)	1.0
	S	893 (84)[d]	1.9	2200 (113)[d]	3.3
MM	*R*	5.9 (0.2)	1.0	62 (12)	1.0
	S	13.1 (1.5)[d]	2.2	450 (45)[d]	7.3
SC	*R*	78.7 (11.9)	1.0	32.7 (2.6)	1.0
	S	309 (5)[d]	3.9	89.2 (7.2)[d]	2.7
TB	*R*	90 (23)	1.0	194 (16)	1.0
	S	313 (21)[d]	3.5	886 (94)[d]	4.6
SDREC	*R*	486 (8)	1.0	3.9 (0.2)	1.0
	S	3310 (135)[d]	6.8	203 (16)[d]	52.0
rabbit CarE	*R*	173 (123)	1.0	3010 (154)	1.0
	S	995 (55)[d]	5.7	7360 (146)[d]	2.4
porcine CarE	*R*	897 (32)	1.0	21000 (1980)	1.0
	S	3860 (42)[d]	4.3	48000 (1550)[d]	2.1
AChE	*R*	16.5 (1.0)	1.0	NM[e]	
	S	4.7 (0.8)[d]	0.3	NM[e]	

[a] Esterases: BS, BST, MM, SC, SDREC, TB, porcine CarE, rabbit CarE, and AChE. [b] Substrates: 6-MNAc and 6-MNBu. The unit for data values (±SD) in the table for BS, BST, MM, SC, TB, and SDREC is pmol min^{-1} mg $prot^{-1}$, and for all other esterases, it is nmol min^{-1} mg $prot^{-1}$. [c] *E*: enantioselectivity is the rate of the activity of *S*-isomer over that of its enantiomer. [d] Denotes a value that is significantly different ($P \leq 0.05$) from the corresponding value for the *R*-enantiomer when Student's *t*-test was used with two-tailed distribution. [e] NM: no activity was measurable at 8 μg/mL.

Enantioselectivity of Commercial Esterases and Insect Cytosolic Esterases toward General α-Cyanoesters. An esterase was considered enantioselective (*E*) in this study if the activity toward the *S*-isomer was 2-fold higher ($E > 2$) or lower ($E < 0.5$) than the activity of the esterase toward its enantiomer. In terms of this standard, all commercial esterases displayed enantioselectivity (**Table 3**). All esterases except for AChE from electric eel preferred the *S*-isomer of these general fluorescent substrates to their corresponding *R*-isomers. This preference varied among esterase and substrate combinations, up to 52 times (e.g., SDREC toward 6-MNBu). For a given esterase, this preference also depended on the acyl chain length. For example, this preference in BS, BST, MM, TB, and SDREC increased with an increase of acyl chain length ($C_2 \rightarrow C_4$) but decreased in SC, rabbit CarE, and porcine CarE. This confirmed earlier work in which BST (*19*) preferred longer chain acyl groups (C2→C6),

Table 4. Specific Activity of Insect Cytosolic Esterases toward Chiral α-Cyanoesters[a,b]

insect	strain[c]	*R*-6-MNAc	*S*-6-MNAc	*E*[d]	*R*-6-MNBu	*S*-6-MNBu	*E*[d]
house fly	CS	NM[e]	0.35 (0.05) A		NM[e]	0.41 (0.05) A	
	SRS	NM[e]	0.98 (0.20) B		0.11 (0.01)	1.35 (0.08) B	12
	YPER	NM[e]	1.77 (0.05) C		NM[e]	2.99 (0.29) D	
	NG98	NM[e]	1.19 (0.02) B		NM[e]	1.16 (0.07) B	
	LPR	NM[e]	2.07 (0.16) C		NM[e]	1.70 (0.17) C	
horn fly	S	0.09 (0.01) A	1.56 (0.07) C	17.3	0.65 (0.04) A	6.52 (0.06) C	10.0
	LSU	0.09 (0.01) A	0.74 (0.08) A	8.2	0.56 (0.03) A	2.83 (0.24) A	5.1
	super-kdr	0.08 (0.03) A	1.19 (0.11) B	14.9	0.65 (0.07) A	5.10 (0.08) B	7.8
Colorado potato beetle	S1	4.54 (0.26)	4.54 (0.16)	1.0	8.71 (0.07)	9.84 (0.33)	1.1
	PER	3.21 (0.22)*	3.61 (0.26)*	1.1	5.74 (0.33)*	8.46 (0.19)*	1.5
cockroach	ACY	1.07 (0.02)	0.47 (0.02)	0.4	1.51 (0.12)	2.30 (0.16)	1.5
	R	1.53 (0.06)*	0.80 (0.09)*	0.5	2.20 (0.07)*	4.00 (0.07)*	1.8
tobacco budworm	CYP	3.26 (0.15)	10.6 (0.52)	3.3	5.25 (0.10)	21.4 (0.3)	4.1

[a] Substrates: *R*-6-MNAc, *S*-6-MNAc, *R*-6-MNBu, and *S*-6-MNBu. [b] Values stand for mean activities (nmol min^{-1} mg $prot^{-1}$; ±SD) based on triplicate assays with cytosol from each strain. For comparing activities, values with the same capital letters are not significantly different (ANOVA–Tukey's HSD test; $P \leq 0.01$). Similarly, values with a star are significantly different (Student's *t*-test; $P \leq 0.01$) from the corresponding values from the susceptible strain. [c] Strain: House fly: CS and SRS are pyrethroid-susceptible strains, and YPER, LPR, and NG98 are pyrethroid-resistant strains. Horn fly: S is a pyrethroid-susceptible strain, LSU is diazinon-resistant strain, and super-kdr is a pyrethroid-resistant strain. Colorado potato beetle: S1 is a pyrethroid-susceptible strain, and PER is a permethrin-resistant strain. Cockroach: ACY is a pyrethroid-susceptible strain, and R is a pyrethroid-resistant strain. CYP, cypermethrin-resistant tobacco budworm. [d] *E*: enantioselectivity is the rate of the activity of *S*-isomer over that of its enantiomer. [e] NM: no activity was measurable under 10-fold protein as compared with its corresponding enantiomer.

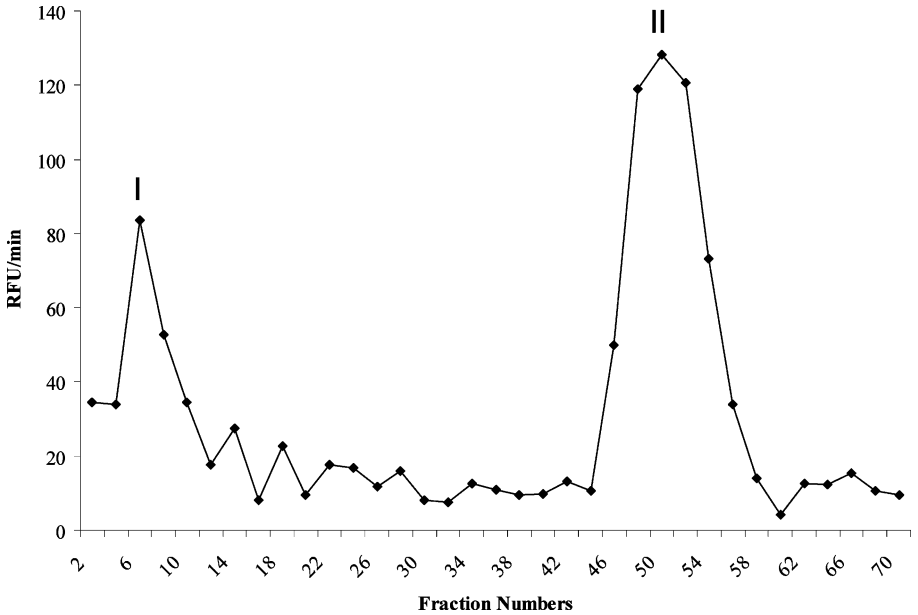

Figure 2. Evaluation of crude purification of cytosolic esterases from pyrethroid-resistant house fly (LPR strain) with *S*-6-MNac. The salt concentrations for elution of peaks I and II were 25 and 150–175 mM, respectively. Conditions at which *S*-6-MNAc was monitored are as follows: excitation at 330 nm (band-pass, bp 35), emission at 465 nm (bp 35), 50 μM substrate (final concentration), and 20 μL of elution solution in a total volume of 200 μL/well.

but SDREC (*20*) favored shorter chain acyl groups (C2→C6). AChE from electric eel was unique in this study, which statistically preferred *R*-isomer of α-cyanoesters [60% enantiomeric excess (ee%) for 6-MNAc]. This is very similar to the results from the previous studies in which AChE from electric eel stereoselectively (up to 96% ee) hydrolyzed the ester bond in the 3-postion of *cis*-3,5-diacetylpent-1-ene (*21*, *22*).

Similar to commercial esterases, most insect cytosolic esterases also preferred *S*-isomers of these α-cyanoesters to their corresponding enantiomers (**Table 4**). This may be useful for insects to quickly detoxify the most toxic isomers of type II pyrethroids (i.e., in terms of type II pyrethroids, the isomers with *S*-configuration in α-carbon with a cyano group are more toxic than the isomers with *R*-configuration). Most appealing was the very high enantioselectivity of cytosolic esterases from house fly toward both 6-MNAc and 6-MNBu (**Table 4**). High enantioselectivity was displayed by both pyrethroid-susceptible and pyrethroid-resistant strains, suggesting that pyrethroid selection might not change the enantioselectivity of the cytosolic esterases but rather change the efficiency of the cytosolic esterases to hydrolyze pyrethroids. This high enantioselectivity may be one of the explanations why house flies develop resistance so quickly to type II pyrethroids (*23*). Enantioselectivity of esterases from the LSU strain of horn fly (selected by diazinon, an organothiophosphorus insecticide) toward these general cyanoester substrates dramatically decreased as compared with those in the corresponding susceptible strain. This phenomenon may be explained by a similar enantioselectivity of above-described acetyl cholinesterases from electric eel because the targets of organophosphates are acetyl cholinesterases (*24*), which also play a critical role in insect resistance to organophosphates (*25*).

To demonstrate whether chiral α-cyanoesters (e.g., 6-MNAc or 6-MNBu) can be used to purify enantioselective esterases, we chose cytosol from pyrethroid-resistant strains of house fly (LPR strain) as an example. We found that there were two peaks

containing esterases that hydrolyzed *S*-6MNAc. These two peaks corresponded to salt concentrations of 25 mM and from 150 to 175 mM (**Figure 2**), respectively. The protein in peak I was too low to evaluate the hydrolysis of chiral substrates. Esterases concentrated from peak II, consisting of 15% of the total proteins, only showed specific activity toward *S*-isomers of α-cyanoesters [*S*-6MNAc (10.4 ± 1.1 nmol/min/mg); *S*-6-MNBu (13.3 ± 0.5 nmol/min/mg)]. The specific activities of these partially purified esterases toward *S*-6MNAc and *S*-6-MNBu were increased 4.88 and 7.84 times as compared to the original cytosol, respectively. Because of limited materials, identification of specific esterases in peak II could not be done. However, this interesting finding will encourage exploration of new enantioselective esterases from insects, especially house flies, which are easily reared in large-scale in the laboratory.

Higher enantioselectivity of cytosolic esterases of house fly toward both 6-MNAc and 6-MNBu may be useful for optical resolution of α-chiral alcohols and acids. For example, cyanohydrins, a class of α-chiral alcohols, are necessary components of agriculturally important compounds including type II pyrethroid insecticides (*26*) and their analogues (9). They are also highly versatile synthetic intermediates that can be converted into a great number of synthetic targets such as α-hydroxyl acids, α-hydroxyl carboxamides, α-amino acids, and *β*-amino alcohols (*27*). 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 α-chiral acids. Their *R*-counterparts are biologically inactive or have negative side effects (*28*, *29*).

It may be possible to use α-cyanoesters as general substrates for screening esterases that are enantioselective for one isomer of esters containing α-chiral alcohols or acids. Results in this study and other studies may provide an insight to this question. BST displayed not only enantiospecificity toward α-chiral alcohols [e.g., cyanoesters (*E*: 3.3 for 6-MNBu, **Table 3**), (*R*,*S*)-1-phenylethyl acetate (*E*: 8 favoring *R*-isomer), and (±)-menthyl acetate (*E*: >100 favoring *R*-isomer; *30*] but also toward α-chiral acids [e.g., (*R*,*S*)-ketoprofen ethyl ester (*E*: >200 favoring *S*-isomer; *28*). Similarly, SDREC also showed enantiospecificity in both α-chiral alcohols [e.g., α-cyanoesters (*E*: 52 for 6-MNBu, **Table 2**); (*R*,*S*)-1-phenylethyl acetate (*E*: 3.3 favoring *S*-isomer; *31*), (10*R*)-3-acetoxy-11-hydroxy-6,10-dimethyl-5-undecen-5-one (*E*: >100, favoring *S*-isomer; *32*)] and α-chiral acids [e.g., methyl 3-bromo-2-methylpropionate (*E*: 5.6 favoring *S*-isomer; *20*)]. However, enantioselectivity was dependent on the three-dimensional structures involved, and this can vary considerably due to the intermolecular interactions and intramolecular motions. For example, it is well-known that AChE displayed high stereoselectivity toward substrates and/or inhibitors (*33*−*35*). However, this stereoselectivity is strongly dependent on the source of AChE and the structure of substrates or inhibitors. For example, the (−)-enantiomer of Sarin reacts 4200 times faster with oxerythrocyte AChE than does the (+)-enantiomer (*33*, *34*). Conversely, in the inhibition of rat brain AChE by ethyl *S*-2-chloroethylphosphonothiolate, the (+)-enantiomer of Sarin reacts twice as fast as the (−)-enantiomer (*35*). Thus, it is necessary to optimize when these substrates are used for screening enantioselective esterases for special substrates.

Conclusions. The examined α-cyanoesters (e.g., 6-MNAc) as general fluorescent substrates for esterase were better than the tested phenolic esters (e.g., 7-AC-4-MC) when considering the sensitivity and enantioselectivity. Enantioselectivity of esterases toward α-cyanoesters strongly depended on the esterases and substrates, but most esterases examined preferred *S*-isomers to their corresponding enantiomers. Interestingly, SDREC and cytosolic esterases from house fly and horn fly displayed very high enantioselectivity. In addition, α-cyanoesters as general fluorescent substrates were demonstrated in partially purified insect esterases.

ABBREVIATIONS USED

1-NA, 1-naphthyl acetate; 7-AC-4-MC, 7-acetoxy-4-methylcoumarin; 6-MNAc, α-cyano(6-methoxynaphthalen-2-yl)methyl acetate; 6-MNBu, α-cyano(6-methoxynaphthalen-2-yl)-methyl butanoate; PTU, 1-phenyl-2-thiourea; DTT, DL-dithiothreitol.

ACKNOWLEDGMENT

We acknowledge Drs. J. G. Scott, M. J. Clark, N. Liu, and A. J. Miller for generously providing pyrethroid-resistant and -susceptible insects.

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Received for review October 20, 2005. Revised manuscript received November 21, 2005. Accepted December 2, 2005. This project was funded partially by NIEHS R37ES02710, NIEHS Superfund Basic Research Program, P42 ES04699, NIEHS Center P30 ES05707, UC Systemwide Biotechnology Research and Education Training Grant #2001-07, NIEHS Center for Children's Environmental Health & Disease Prevention, 1 P01 ES11269, NIH/NHLBI R01 HL59699-06A1, and U.S. Army Medical Research and Material Command DAMD 17-01-1-0769.

JF0526083