Structure-Activity Relationships for Substrates and Inhibitors of Mammalian Liver Microsomal Carboxylesterases

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Purpose. Carboxylesterases are important in the detoxification of drugs, pesticides and other xenobiotics. This study was to evaluate a series of substrates and inhibitors for characterizing these enzymes.

Methods. A series of novel aliphatic esters and thioesters were used in spectral assays to monitor human, murine and porcine esterases. A series of transition state mimics were evaluated as selective esterase inhibitors.

Results. Several α-alkyl thioacetothioates were found to be ~2 to 11-fold superior to commonly used substrates for monitoring carboxylesterase activity. Insertion of a heteroatom in the acid portion of these esters in the β or γ position relative to the carbonyl had a dramatic effect on enzyme activity with S or O substituents often improving the k_{CAT}/K_M ratio of the substrate and N decreasing it. Several α,α′-bis(2-oxo-3,3,3-trifluoropropylthio)alkanes proved to be potent selective transition state mimics of the esterase activity with IC_{50}'s from 10^{-5} to 10^{-9}M.

Conclusions. This library of substrates and inhibitors are useful research tools for characterizing the numerous isozymes of carboxylesterases present in mammalian tissues.

KEY WORDS: carboxylesterases; mammalian liver; thioester substrates; trifluoromethylketone inhibitors; structure-activity relationships.

INTRODUCTION

Mammalian liver microsomal carboxylesterases (EC 3.1.1.1) are a group of isozymes located mainly in the endoplasmic reticulum. These enzymes are involved in the metabolism of a wide variety of xenobiotics containing ester, thioester and amide functional groups (1–5).

In the present study, a series of methyl and phenyl α-alkyl thioacetothioates and related compounds were developed as selective, spectrophotometric substrates of mammalian liver microsomal carboxylesterases. In order to study substrate selectivity adequately, it is important to characterize the substrate selectivity of carboxylesterase in the crude as well as the purified state. The substrates reported in this study are potentially useful diagnostic tools for monitoring and distinguishing the different isozymes of carboxylesterase in mammalian tissues. One of these substrates, methyl β-(1-pentylthio)propiothioate (MBPTP, 15), is a potentially useful diagnostic substrate for mammalian serum carboxylesterase (7), and has been used to monitor carboxylesterase activity in the affinity purification of mammalian liver carboxylesterases (8).

In a previous study (9), we reported the inhibition of mammalian liver carboxylesterases by a series of potent selective substituted trifluoromethylketones (TFKs) as "transition state" mimics. These compounds have proven very useful in studying the catalytic mechanism and biological roles of a variety of esterases (9–12). To further characterize the important hydrolytic carboxylesterase enzymes, we investigated the inhibitory effects of a series of α,α′-bis(2-oxo-3,3,3-trifluoropropylthio)alkanes (bis-TFKs) due to their potency as transition state inhibitors and suspected higher water solubility than the monosubstituted TFKs as discussed previously (9).

MATERIALS AND METHODS

Reagents

The synthesis and characterization of the substrates reported in this study are described in McCutchen *et al.* (6). Technical grade (95%) malathion, obtained from American Cyanamid Corp. (Princeton, NJ) was purified by thin layer chromatography. *p*-Nitrophenol, *p*-nitrophenyl acetate (*p*-NpAc) were purchased from Aldrich Chemical Co. (Milwaukee, WI). α-Naphthol, α-naphthyl acetate (α-NA), *p*-iodonitrophenyltetrazolium violet (INT), alcohol dehydrogenase, NAD and NAD diaphorase, bovine serum albumin (fraction V), n-octyl-β-D-glucopyranoside (octyl glucoside), and porcine liver carboxylesterase (EC 3.1.1.1) with 200 units/mg protein were purchased from Sigma Chemical Co. (St. Louis, MO). BCA reagent was purchased from Pierce Chemical Co. (Rockford, IL). 3-Octylthio-1,1,1-trifluoro-propanone (OTFP) and α,α′-bis-(2-oxo-3,3,3-trifluoropropylthio)alkanes were available or prepared according to previously reported methods (9,11).

Preparation of Liver Microsomes

Liver microsomal fractions from human and mouse were prepared as described previously (2,7). Microsomes were solubilized in 0.1 M Tris-HCl buffer, pH 7.5 containing 1% octyl glucoside, at a final protein concentration of 4.0 mg/ml. The mixtures were gently shaken for 1 hr on a rotating wheel at approx. 50 rpm and afterwards centrifuged at 100,000 *g* for 1 hr. The supernatant fractions were collected and dialyzed against the same buffer to obtain the solubilized microsomes, which was used for enzyme assays.

Esterase Assays with Spectrophotometric Substrates

The rates of hydrolysis of the thioesters, 1–21, by the various mammalian liver carboxylesterases were measured in a continuous assay using 96-well microtiter plates (Dynatech Laboratories, Inc., Virginia, VA) with a V_{max}^{TM} plate reader (Molecular Devices, Palo Alto, CA).

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Unless otherwise specified, the buffer used in the assay of porcine liver carboxylesterase was 0.1 M Tris-HCl, pH 7.5 containing 50 μg/ml BSA and for the assay of human and mouse liver carboxylesterases, the buffer used was 0.1 M Tris-HCl, pH 7.5. The esterase assays with the thioesters are based on a modification of the Ellman method (13) by colorimetric determination at 405 nm of the free chromophoric product (5-thiolate-2-nitro benzoic acid) formed after reacting with 0.015% 5,5′-dithiobis-(2-nitro benzoic acid) (DTNB). In a typical assay, 278 μl of 0.015% DTNB in buffer and 20 μl of the enzyme solution were added to individual wells. In the nonenzymatic reaction, 20 μl buffer instead of the enzyme solution was added. The reaction was started by the injection of 2 μl of the substrates (in ethanol) to give a final concentration of 2×10^{-4} M. The hydrolytic rates were monitored at 405 nm for 2 mins at 23°C and corrected for nonenzymatic rates.

Previous reported assay procedures were followed for the assays of substrates 22–28 (12), *p*-NpAc and α-NA (2,7), and malathion (2,7). The enzyme assays were carried out under conditions where the initial hydrolytic rates were linear with time for the protein and substrate concentrations used. The assay was carried out in quadruplicate. The measured rates were corrected for the spontaneous hydrolysis of the substrate where this was significant. Specific activities were determined using a substrate concentration which was >10 times the K_m for the compounds. Protein concentrations were determined using the standard protocol version of the Pierce BCA assay modified for use in a 96-well plate reader. BSA was used as a protein standard.

Determination of Kinetic Constants

The apparent K_m and V_{max} were calculated as described previously (2). Eleven substrate concentrations (5×10^{-6} – 2×10^{-4} M) were used with the partially purified porcine liver carboxylesterase. The maximal velocities (V_{max}) are expressed in micromoles of substrate hydrolyzed per mg protein in 1 min under the conditions described.

Optimum pH of Porcine Liver Esterase

Buffers (286 μl at pH 6.0–10.0 in 0.2 or 0.5 pH unit increments) were added to 10 μl of porcine esterase and incubated at room temperature for 10 min in individual wells. In control samples, the final pH of the reaction mixture was verified using a pH meter. DTNB (2 μl solution of a 2.25% in acetone, w/v) was injected to give a final concentration of 0.015%. The esterase activity was measured after injecting 2 μl of the substrates (in ethanol) to give a final concentration of 2×10^{-4} M. Nonenzymatic rates for each substrate were subtracted from the reported enzymatic rates. The ionic strength of all buffers was 0.1 M. A phosphate buffer was used for pH 6.0–8.0, Tris-HCl for pH 8.0–9.0, and glycine-NaOH for 9.5–10.0.

Inhibition Studies

The inhibition of carboxylesterase activity by the TFKs was determined according to previously described procedures (9). For measuring the inhibition of malathion carboxylesterase, the inhibitors (in acetone) were added in 1.0 μl to wells containing 100 μl enzyme solutions. After preincubation of the enzyme with the inhibitor for 10 min at 23°C, the reagent mixture (200 μl) was added followed by injection of 1.0 μl of an acetone solution of malathion to give a final concentration of 3×10^{-4} M. For measuring the inhibition of carboxylesterase acting on *p*-NpAc and 15, the inhibitors (in acetone or ethanol) were added in 1.0 μl to wells containing 300 μl enzyme solutions diluted in 0.1 M Tris-HCl buffer pH 7.5, or the same buffer containing 0.015% DTNB reagent, respectively. After preincubation of the enzyme with the inhibitor for 10 min at 23°C, the substrate *p*-NpAc or 15 in 2.0 μl was added and the hydrolytic rates were monitored for 2 min at 405 nm. Separate experiments indicated that the solvents used (<1% of acetone or ethanol) had little effect on enzyme activity.

The inhibitors were screened at concentrations ranging from 1×10^{-4} to 1×10^{-10} M. For determination of the concentration required for 50% inhibition of the enzyme, triplicate incubations at four or five different concentrations of the inhibitor were used. The IC_{50} values were calculated from semi-log plots using the linear portion of the curve by least-squares regression analysis.

RESULTS AND DISCUSSION

Substrate Selectivity of Mammalian Liver Microsomal Carboxylesterases

Significant differences in the substrate selectivity of liver microsomal carboxylesterases of human, porcine and mouse were observed (Table I). This may be due to species differences and/or an unequal quantities of specific isozymes involved in the hydrolysis of these substrates. The higher specific activity exhibited by the porcine liver carboxylesterase acting on these substrates probably indicate the greater purity of this enzyme preparation.

Among a homologous series of unbranched aliphatic thioesters (1–12) (Table I), the mammalian liver carboxylesterases showed a preference for substrates with shorter acyl chains 1 or 2 (i.e. C_5 or C_7). Substitution of a S or O in the β position of the acyl group (9–12), did not significantly alter the hydrolytic rates compared to the corresponding carbon analogues, although the ether analog (14), was about 2.6-fold better than its carbon analog (3) when tested with the human enzyme. However, in terms of kinetic characteristics, the heteroatom (S or O) in the β position of the acyl group of 10 and 14, lowered the K_ms of the substrates by about 5-fold when compared to substrate 2 (Table II). An increased binding to the enzyme resulted in decreased turnover rates (lower V_{max}s) for 10 and 14. Unlike the methyl thioesters, the insertion of a S or O in the β position of the acyl moiety in the phenyl thio-and carboxyl esters significantly enhanced the hydrolytic rates catalyzed by the mammalian liver carboxylesterases e.g. 18 and 26 (Table I). However, 18 and 26, have different binding affinities and turnover rates (Table II). Although 26 has a very high specificity (V_{max}/K_m) ratio, its usefulness as a substrate may be limited due to its high lability under basic conditions (Table III). Compound 26 is the most labile to base hydrolysis among the substrates tested.

The presence of a S in the γ position of the acyl moiety in the methyl (15) and phenyl γ thioethers (21 and 28) significantly enhanced the substrate specificity profile of these compounds. Compound 15 was approximately 2.3 to 2.6-fold more active

Table I. Specific Activities of Solubilized Human and Mouse Liver Microsomal Carboxylesterases, Partially Purified Porcine Liver Carboxylesterase, and Horse Serum Butyrylcholinesterase with Spectrophotometric Substrates[a]

Compd. No.	Substrates		Specific Activity (μmol/min/mg protein)			
Methyl aliphatic thioesters		*n*	*HUMAN*	*PORCINE*	*MOUSE*	*HORSE*
1	CH_3-$(CH_2)_n$-C(O)-SCH_3	4	8.23 ± 0.28	98 ± 3.9	2.79 ± 0.04	25.8 ± 0.7
2		6	7.32 ± 0.23	203 ± 3.3	2.89 ± 0.22	9.9 ± 0.4
3		7	3.62 ± 0.15	109 ± 2.4	2.04 ± 0.15	8.6 ± 1.0
4		8	1.93 ± 0.08	73 ± 2.5	0.91 ± 0.04	29.3 ± 2.4
5		9	0.75 ± 0.05	32 ± 1.9	0.41 ± 0.04	3.6 ± 0.2
6		10	0.51 ± 0.02	13 ± 0.4	0.19 ± 0.01	6.5 ± 0.8
7		11	1.49 ± 0.17	6.2 ± 0.9	0.18 ± 0.02	5.7 ± 0.7
8		13	0.33 ± 0.01	0.2 ± 0.001	0.10 ± 0.01	5.8 ± 0.2
9	CH_3-$(CH_2)_n$$SCH_2$-C(O)-$SCH_3$	4	3.64 ± 0.09	80.4 ± 1.9	2.86 ± 0.03	1.5 ± 0.1
10		5	2.45 ± 0.10	72.9 ± 4.3	2.40 ± 0.06	<0.1
11		6	1.77 ± 0.12	50.7 ± 1.6	1.10 ± 0.02	<0.1
12		7	1.15 ± 0.08	37.4 ± 2.5	0.90 ± 0.06	5.8 ± 1.1
13	CH_2-$(CH_2)_5$-NH-CH_2-C(O)-SCH_3		<0.1	<0.1	<0.1	<0.1
14	CH_3-$(CH_2)_5$-O-CH_2-C(O)-SCH_3		9.50 ± 0.23	60 ± 1.6	2.02 ± 0.07	7.5 ± 0.9
15	CH_3-$(CH_2)_4$-S-CH_2CH_2-C(O)-SCH_3		8.19 ± 0.10	278 ± 11	5.40 ± 0.17	2.1 ± 0.4
Phenyl aliphatic thioesters & thiocarbonate						
16	CH_3-$(CH_2)_4$-C(O)-S-Ph		6.82 ± 0.29	121 ± 17	1.27 ± 0.11	22.5 ± 2.6
17	CH_3-$(CH_2)_3$-O-C(O)-S-Ph		<0.1	<0.1	<0.1	<0.1
18	CH_3-$(CH_2)_2$-O-CH_2-C(O)-S-Ph		9.81 ± 0.57	369 ± 12	1.75 ± 0.09	75.2 ± 4.3
19	CH_3-$(CH_2)_2$-S-CH_2-C(O)-S-Ph		8.48 ± 0.16	201 ± 15	1.35 ± 0.16	57.0 ± 5.9
20	CH_3-CH_2-O-$(CH_2)_2$-C(O)-S-Ph		1.96 ± 0.15	118 ± 4.6	0.36 ± 0.02	3.7 ± 0.3
21	CH_3-CH_2-S-$(CH_2)_2$-C(O)-S-Ph		10.3 ± 0.43	366 ± 20	1.68 ± 0.15	23.3 ± 1.5
Phenyl carboxylesters, carbonate, thiocarbonate						
22	CH_3-$(CH_2)_4$-C(O)-O-Ph		10.5 ± 0.03	209 ± 4.3	7.88 ± 0.36	82.3 ± 5.3
23	CH_3-$(CH_2)_3$-S-C(O)-O-Ph		0.23 ± 0.04	5.08 ± 1.3	<0.1	1.0 ± 0.1
24	CH_3-$(CH_2)_3$-O-C(O)-O-Ph		1.12 ± 0.04	14.8 ± 3.4	1.02 ± 0.01	9.9 ± 0.1
25	CH_3-$(CH_2)_2$-O-CH_2-C(O)-O-Ph		8.10 ± 0.51	171 ± 5.6	9.21 ± 0.07	52.6 ± 2.0
26	CH_3-$(CH_2)_2$-S-CH_2-C(O)-O-Ph		10.2 ± 0.24	299 ± 3.0	10.1 ± 0.30	88.3 ± 5.9
27	CH_3-CH_2-O-$(CH_2)_2$-C(O)-O-Ph		5.05 ± 0.16	127 ± 5.1	2.20 ± 0.07	8.9 ± 0.5
28	CH_3-CH_2-S-$(CH_2)_2$-C(O)-O-Ph		12.3 ± 0.31	286 ± 2.8	7.88 ± 0.17	N.D.
	p-Nitrophenyl acetate		0.85 ± 0.04	119 ± 7.5	2.25 ± 0.10	12.4 ± 0.3
	α-Naphthyl acetate		3.89 ± 0.20	150 ± 1.34	3.56 ± 0.03	121 ± 0.6

[a] Assay conditions are described under materials and methods. Each value represents the mean ± SD of four determinations. Protein concentrations for human, porcine, mouse and horse were 37.8 μg/ml, 0.475 μg/ml, 71 μg/ml, and 0.9 μg/ml, respectively. The final substrate concentrations were 0.5 mM for *p*-nitrophenyl acetate and α-naphthyl acetate, 0.2 mM for the other substrates. N.D. indicates not determined.

Table II. Kinetic Parameters of the Hydrolysis of Selected Substrates by Porcine Liver Carboxylesterases[a]

Compound No.	Substrate	Range of Substrate concentration (μM)	K_m (μM)	V_{max} (μmol/min/mg protein)	$\frac{V_{max}}{K_m}$
2	CH_3-$(CH_2)_6$-C(O)-SCH_3	5.0–200	43.0 ± 6.3	232 ± 26	5.39
10	CH_3-$(CH_2)_5$-SCH_2-C(O)-SCH_3	5.0–200	9.79 ± 0.8	75.1 ± 1.7	7.67
14	CH_3-$(CH_2)_5$-OCH_2-C(O)-SCH_3	5.0–200	8.01 ± 0.6	42.9 ± 0.9	5.36
15	CH_3-$(CH_2)_4$-SCH_2CH_2-C(O)-SCH_3	5.0–200	38.7 ± 3.7	425 ± 19	10.98
18	CH_3-$(CH_2)_2$-OCH_2-C(O)-SPh	5.0–200	268 ± 33	616 ± 41	2.30
21	CH_3-CH_2-S$(CH_2)_2$-C(O)-SPh	5.0–200	69.9 ± 19	360 ± 45	5.15
26	CH_3-$(CH_2)_2$-SCH_2-C(O)-OPh	5.0–200	40.1 ± 5.9	433 ± 26	10.80
28	CH_3-CH_2-S$(CH_2)_2$-C(O)-OPh	5.0–200	53.4 ± 6.1	422 ± 21	7.90
	p-Nitrophenyl acetate	5.0–200	174 ± 15	170 ± 15	0.98
	α-Naphthyl acetate	5.0–200	126 ± 13	250 ± 15	1.98

[a] Assay conditions are described under materials and methods. Each value represent the means ± SD of three determinations of each substrate concentration.

Table III. Base-catalyzed Hydrolysis of Selected Substrates in 0.02M NaOH at 23°C[a]

Compound No.	Substrates	Apparent first order rate constant ($min.^{-1}$)	Relative rates (%)
2	CH_3-$(CH_2)_6$-C(O)-SCH_3	0.001	0.059
4	CH_3-$(CH_2)_8$-C(O)-SCH_3	0.0008	0.047
10	CH_3-$(CH_2)_5$-SCH_2-C(O)-SCH_3	0.062	3.68
11	CH_3-$(CH_2)_6$-SCH_2-C(O)-SCH_3	0.071	4.22
14	CH_3-$(CH_2)_5$-OCH_2-C(O)-SCH_3	0.216	12.8
15	CH_3-$(CH_2)_4$-SCH_2CH_2-C(O)-SCH_3	0.027	1.60
18	CH_3-$(CH_2)_2$-OCH_2-C(O)-SPh	1.055	62.7
21	CH_3-CH_2-S$(CH_2)_2$-C(O)-SPh	0.270	16.0
26	CH_3-$(CH_2)_2$-SCH_2-C(O)-OPh	2.440	145
28	CH_3-CH_2-S$(CH_2)_2$-C(O)-OPh	0.289	17.2
	p-Nitrophenyl acetate	1.683	100
	α-Naphthyl acetate	0.400	23.8

[a] The final concentrations of the substrates were 0.2 mM. The apparent first order rate constant was calculated by recording the absorbance of the reaction at 405 nM at regular intervals of time according to the assay procedure described in materials and methods. The relative rates of these substrates are expressed as percentages of the rate of *p*-NpAc.

than its corresponding carbon analogue (3), for the mammalian liver microsomal carboxylesterases (Table I). When tested with the porcine liver carboxylesterase, the methyl γ thioether (15), exhibited a V_{max}/K_m ratio of 10.98 which is the highest among the substrates tested (Table II). This substrate is also the least labile among the oxy- and thioether series to base hydrolysis (Table III). The γ thioether (15) is clearly a superior substrate for mammalian liver carboxylesterases. The V_{max}/K_m ratios for 21 and 28 are about 5- and 8-fold greater than the widely used substrate *p*-NpAc. The kinetics of porcine liver carboxylesterase

Table IV. Inhibitory Potencies of α,α[1]-Alkanebis (thiotrifluoropropanones) and OTFP on the Hydrolysis of *p*-nitrophenyl Acetate (*p*-NpAc), Methyl β-(1-pentylthio) Propiothionate (MBPTP) and Malathion by Solubilized Human Liver Microsomal Carboxylesterase

Compound No.	Inhibitors	IC_{50}[a] (M)		
		p-NpAc	MBPTP	Malathion
	$CF_3C(O)CH_2S$-$(CH_2)_n$-$SCH_2C(O)CF_3$			
I	n: 3	1.06×10^{-8}	1.28×10^{-8}	7.07×10^{-7}
II	4	2.96×10^{-8}	2.18×10^{-8}	6.93×10^{-7}
III	5	2.06×10^{-8}	1.81×10^{-8}	4.66×10^{-7}
IV	6	8.10×10^{-9}	7.50×10^{-9}	2.93×10^{-7}
V	7	7.29×10^{-8}	5.45×10^{-8}	3.89×10^{-6}
VI	8	2.60×10^{-7}	1.47×10^{-7}	[b]
VII	9	1.23×10^{-7}	1.03×10^{-7}	9.25×10^{-6}
VIII	10	6.85×10^{-8}	1.08×10^{-7}	7.62×10^{-6}
IX	11	1.86×10^{-6}	3.89×10^{-6}	7.56×10^{-5}
X	12	3.22×10^{-7}	1.63×10^{-7}	4.40×10^{-5}
	$CF_3C(O)CH_2S$-R-$SCH_2C(O)CF_3$			
XI	R: -$(CH_2)_2$-O-$(CH_2)_2$-	3.50×10^{-8}	2.94×10^{-8}	6.04×10^{-7}
XII	-$(CH_2)_2$-S-$(CH_2)_2$-	2.26×10^{-8}	2.23×10^{-8}	4.40×10^{-7}
XIII	-CH(CH_2)$_2$- | CH_3	5.19×10^{-7}	4.80×10^{-7}	[b]
XIV	-CH(CH_2)$_3$- | CH_3	2.86×10^{-7}	2.18×10^{-7}	7.56×10^{-6}
XV	OTFP[c]	2.90×10^{-9}	1.70×10^{-9}	2.17×10^{-7}

[a] IC_{50} values were obtained as the means of three determinations. The concentrations of *p*-NpAc, MBPTP and malathion were 0.5 mM, 0.2 mM and 0.3 mM, respectively. Protein concentrations were 76 ug/ml for *p*-NpAc, 38 ug/ml for MBPTP and 380 ug/ml for malathion.

[b] IC_{50} was not determined.

[c] OTFP (1-octylthio-3,3,3,-trifluoropropan-2-one) was used as a reference.

Table V. Inhibitory Potencies of α,α^1-Alkanebis (thiotrifluoropropanones) and OTFP on the Hydrolysis of *p*-nitrophenyl Acetate (*p*-NpAc) and Methyl β(1-penthylthio) Propiothioate (MBPTP) by Solubilized Mouse Liver Microsomal Carboxylesterases

Compound No.	Inhibitors	IC_{50}[a] (M) *p*-NpAc	IC_{50}[a] (M) MBPTP
	$CF_3C(O)CH_2S\text{-}(CH_2)_nSCH_2C(O)CF_3$		
I	n: 3	3.29×10^{-8}	2.61×10^{-8}
II	4	3.00×10^{-8}	2.59×10^{-8}
III	5	2.66×10^{-8}	1.74×10^{-8}
IV	6	1.21×10^{-8}	1.09×10^{-8}
V	7	1.42×10^{-7}	6.49×10^{-8}
VI	8	2.77×10^{-7}	1.09×10^{-7}
VII	9	1.62×10^{-7}	7.39×10^{-8}
VIII	10	8.59×10^{-8}	8.18×10^{-8}
IX	11	2.57×10^{-6}	2.03×10^{-6}
X	12	3.17×10^{-7}	1.52×10^{-7}
	$CF_3C(O)CH_2S\text{-}R\text{-}SCH_2C(O)CF_3$		
XI	R: $-(CH_2)_2\text{-}O\text{-}(CH_2)_2-$	5.42×10^{-8}	6.00×10^{-8}
XII	$-(CH_2)_2\text{-}S\text{-}(CH_2)_2-$	3.35×10^{-8}	2.80×10^{-8}
XIII	$-CH(CH_2)_2-$ | CH_3	4.35×10^{-7}	3.55×10^{-7}
XIV	$-CH(CH_2)_3-$ | CH_3	3.08×10^{-7}	2.54×10^{-7}
XV	OTFP[b]	4.36×10^{-9}	3.45×10^{-9}

[a] IC_{50} values were obtained as the means of three determinations. The concentrations of *p*-NpAc and MBPTP were 0.5 mM and 0.2 mM, respectively. Protein concentrations were 75 ug/ml for *p*-NpAc and MBPTP.

[b] OTFP was used as a reference.

with these substrates (Table II) displayed typical Michaelis-Menten kinetics. Substrates containing the heteroatom (S or O) in the γ position are more resistant to base hydrolysis (26 vs. 28), and this is probably due to less inductive effect of the heteroatom in the γ position.

The alkylamino (13) was inactive, as a substrate, and the phenyl carbonate and thiocarbonates (17, 23, and 24) were very poor substrates for the enzymes tested (Table I).

pH Optimum of Porcine Liver Carboxylesterase

Results with several methyl thioesters, e.g. 2, 10, and 15 showed maximal esterase activity from pH 7.4 to pH 9.0. The pH optimum with *p*-NpAc is at pH 8.0–8.5 which is in good agreement with the pH optima of this substrate reported for other carboxylesterases (4).

Inhibition of Solubilized Human and Mouse Liver Microsomal Carboxylesterases (Tables IV and V)

The dramatic effect on enzyme activity caused by the presence of heteroatoms in the β or γ position of the acyl group in some of these subtrates (15, 18, 21, 26 and 28) were also observed with a series of trifluoromethyl ketones (TFK). Placing a sulfur in the β position of the acyl group in the TFKs resulted in increased inhibitory activity with several serine hydrolases (9–11). The TFKs are putative "transition state" esterase inhibitors which are thought to act by forming a tetrahedral covalent hydrate with a catalytically active serine of carboxylesterases. An X-ray crystal structure of a hydrated TFK showed intramolecular hydrogen bonding interaction between the sulfur atom and the hydroxyl group on the carbonyl of the hydrate (14). This suggest that the S atom might play a role in the stabilization of the tetrahedral structure of the hydrate in the enzyme-inhibitor complex.

The structure-activity relationships of a series of α,α'-bis(2-oxo-3,3,3-trifluoropropylthio)alkanes were investigated as potent inhibitors of human and mouse liver microsomal carboxylesterases. These compounds were compared with a reference compound, OTFP, which is a potent transition state inhibitor (8–12). In this study, the bis-TFKs as well as OTFP, are more selective for inhibition of human liver carboxylesterases hydrolyzing *p*-NpAc and MBPTP (15) than malathion (Table IV). Thus, these compounds may be useful tools for distinguishing among esterase isozymes acting on different substrates. The structure-activity relationships for the TFKs appears to be similar for the human and mouse liver enzymes using *p*-NpAc and MBPTP (15) as substrates (Tables IV and V). Two peaks of inhibition were observed for both human and mouse liver carboxylesterases: a higher optimum at C_6 (IV) and a lower optimum at C_{10} (VIII). This suggests that the compounds, after reaching a particular size, might be interacting with two catalytic sites on two different isozymes. Another possible explanation for the presence of a second inhibition maximum is that the second polarized carbonyl group of the inhibitor might be interacting on another highly hydrophilic region of the enzyme, causing a loss of enzyme activity. It is hoped that a library of surrogate substrates and inhibitors will aid in the characterization and affinity purification (8) of mammalian esterases. The very low background hydrolysis but rapid metabolism of compounds such as 2 or 15 may also be useful for diagnostic applications (7).

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