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# SUBSTITUTED TRIFLUOROKETONES AS POTENT, SELECTIVE INHIBITORS OF MAMMALIAN CARBOXYLESTERASES

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**Abstract**—A series of substituted trifluoroketones were tested as inhibitors of mammalian liver microsomal carboxylesterase(s) hydrolyzing a variety of substrates including malathion, diethylsuccinate (DES) and *p*-nitrophenyl acetate (*p*-NpAc). The trifluoroketones used were very potent "transition state" inhibitors of crude mouse and human liver microsomal carboxylesterases as well as commercial porcine liver carboxylesterase (Sigma EC 3.1.1.1 Type I). These enzymes were found to differ in their sensitivity to the inhibitors employed, and some compounds caused dramatic activation of the hydrolysis of DES. In some but not all cases, a thioether beta to the carbonyl increased the inhibitory potency of the compound. Structure–activity relationships also were evaluated among aliphatic versus substituted and unsubstituted aromatic trifluoroketones. Kinetic parameters [i.e. $K_m$, $V_{max}$ and $(T_{1/2})_e$] for the mouse liver microsomes and the porcine carboxylesterase hydrolyzing DES were determined. Apparent high- and low-affinity forms were observed with each preparation. 3-Nonylthio-1,1,1-trifluoropropan-2-one was synthesized by the reaction of the corresponding thiol with 3-bromo-1,1,1-trifluoroacetone, and apparent synergism was observed when it was coadministered i.p. with malathion to mice.

Carboxylesterases (EC 3.1.1.1) are highly efficient catalysts for the hydrolysis of a wide range of aliphatic and aromatic esters, as well as amides and thioesters, and are important in several different processes including chemotaxis and the metabolism of drugs and pesticides [1–5]. However, in many cases their function remains unknown. In mammalian liver there are multiple forms of carboxylesterases with most catalytic activity associated with membrane fractions such as the microsomes [6–16]. Studies on esterase specificities have shown that changes in the acyl and alcohol moieties of their substrates affect reactivity [17–24].

Three esterase substrates were selected for this study. *p*-Nitrophenyl acetate (*p*-NpAc‡) is metabolized rapidly by carboxylesterases from a variety of sources, and this reactivity is useful for distinguishing between carboxylesterases and proteases [16, 25]. Malathion is an insecticide which exhibits a broad spectrum of effectiveness against a wide variety of insects, and its selectivity, low mammalian toxicity and development of resistance are related to its metabolism by carboxylesterases [26–33]. It is widely used in situations where direct exposure of humans and domestic animals may occur. In addition, Talcott *et al.* [34] suggested that hepatocellular damage and malathion carboxylesterase solubilization are related. They discussed the assay of serum ethylesterases as a prospective liver function test, and they explored the use of diethylsuccinate as an alternative to malathion for this test.

To further characterize these important hydrolytic enzymes, three research directions clearly are important. First, one needs tools for distinguishing groups of carboxylesterases in a crude state. Diagnostic substrates and inhibitors have been used for this in the past [35–37], and additional diagnostic compounds would be useful. Second, one needs selective inhibitors to evaluate the *in vivo* role of these enzymes. *In vivo* inhibition is of practical concern in some cases. For example, inhibition of hepatic microsomal carboxylesterases was found to be a primary factor in the potentiation of acute malathion toxicity [30, 38]. Finally, simple high yield methods of purification are needed for these enzymes.

Trifluoromethylketones were found to be extraordinarily selective and potent inhibitors of cholinesterase [39, 40], juvenile hormone esterase (JHE) [41–46], and meperidine carboxylesterases from mouse and human livers [47]. Thus, a series of these compounds first were examined for their abilities to inhibit the carboxylesterase activity from several sources acting on three substrates as an indication of potential selectivity. Second, one of the best compounds in the series was synthesized in large-scale, and its *in vivo* action on malathion toxicity to mice was examined. Since Abdel-Aal and Hammock [46] have demonstrated that trifluoroketones can be used in the high yield affinity purification of some insect enzymes, this study also provides the basis for the

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‡ Abbreviations: *p*-NpAc, *p*-nitrophenyl acetate; DES, diethylsuccinate; BTFA, 3-bromo-1,1,1-trifluoroacetone; INT, *p*-iodonitrophenyltetrazolium violet; and DFP, diisopropylphosphofluoridate.

rational design of affinity purification systems for mammalian hepatic carboxylesterases.

## MATERIALS AND METHODS

*Chemicals.* Malathion (99.2%), *O,O*-dimethyl-*S*-[1,2-di(ethoxycarbonyl)ethyl] phosphorodithioate, was provided by Dr. M. Mallipudi, American Cyantbamid Corp. (Princeton, NJ). Diethylsuccinate (DES),*p*-nitrophenyl acetate (*p*-NpAc), and *n*-nonyl mercaptan were purchased from the Aldrich Chemical Co. (Milwaukee, WI). 3-Bromo-1,1,1-trifluoroacetone (BTFA) was purchased from PCR Research Chemicals Inc. (Gainesville, FL). *p*-Iodonitrophenyltetrazolium violet (INT), alcohol dehydrogenase, NAD and NAD diaphorase were purchased from the Sigma Chemical Co. (St. Louis, MO). 3-Nonylthio-1,1,1-trifluoropropan-2-one was synthesized, and the rest of the substituted trifluoromethylketones used were available from previous syntheses [41–43].

*Synthesis.* 3-Nonylthio-1,1,1-trifluoropropan-2-one was synthesized by the reaction of *n*-nonyl mercaptan with 3-bromo-1,1,1-trifluoroacetone (Fig. 1). *n*-Nonyl mercaptan (75 mmol) was transferred via syringe to a dry, round-bottom, flask containing 10 ml carbon tetrachloride and sealed with rubber septum. BTFA (75 mmol) was added dropwise via syringe while the reaction was stirred magnetically. The reaction was allowed to proceed under a gentle stream of nitrogen, and the evolving hydrogen bromide gas was trapped with sodium hydroxide pellets. The mildly exothermic reaction, accompanied by a gradual change from a light tan slurry to a red-brown solution was monitored by gas liquid chromatography (GLC) using a Varian Series 1400 equipped with a flame ionization detector and a glass column (2 m × 2 mm i.d.) packed with 5% SE-30 on 80–100 Gas Chrom Q (air, 300 ml/min; $H_2$, 20 ml/min; $N_2$, 15 ml/min). The temperature was 145° and 180° for column and detector respectively. After 36 hr, 20 ml of aqueous sodium bicarbonate (5%, w/v) was added to the reaction mixture and then extracted with chloroform (50 ml × 2). The combined chloroform layers were dried over anhydrous sodium sulfate, and the solvent was evaporated to give a faintly orange oil in 90% isolated yield which by GLC showed only a trace of the starting material with 87% purity. The oil was dissolved in an equal volume of pentane and kept at −20° until cyrstallization and then filtered to give colorless waxy crystals with an m.p. around room temperature. The structural assignment was supported by infrared (i.r.); nuclear magnetic resonance (NMR) [42]; and mass spectrum, *m/z* (rel. intensity) 288 $M^+$ (30), 269 (20), 199 (15), 173 (100), 159 (100), 142 (15), 129 (14), 115 (14), 101 (17), 87 (34), 75 (12); using V.G.ZAB-2F with a VG11-250 data system (direct probe); $NH_3$ chemical ionization ($pNH_3 = 2 \times 10^{-5}$ millibar measured near the source); source temperature, 180°; emission current, 0.5 mA; and electron energy, 100 cV. The product yielded one spot on thin-layer chromatography (TLC) developed in hexane–ether, 5:1 [42], and one peak on GLC with 98.5% apparent purity.

$$C_9H_{19}SH + BrCH_2C(=O)CF_3 \longrightarrow C_9H_{19}SCH_2C(=O)CF_3 + HBr$$

Fig. 1. Synthesis of 3-nonylthio-1,1,1-trifluoropropan-2-one. *n*-Nonyl mercaptan is reacted with 3-bromo-1,1,1-trifluoroacetone in carbon tetrachloride with the evolution of HBr.

*Enzyme preparation.* Male Swiss–Webster mice (Bantin–Kingman, Fremont, CA, 25–30 g) were housed in steel cages with kiln-dried pine shavings as bedding in an environmentally controlled room (12 hr light cycle, 22.5–24.0°, constant humidity). Food (Purina rodent chow) and tap water were provided *ad lib*. Mice were killed by cervical dislocation, and livers were removed, perfused with cold 1.15% KCl, placed into 76 mM sodium phosphate buffer (pH 7.4), and weighed. Homogenates (20-sec burst with Polytron on setting 6) from individual livers were then used to prepare cell fractions. Conventional crude microsomal fractions were prepared from 10% (of original liver weight, w/v) homogenates in the buffer as described earlier [48]. The homogenates were centrifuged at 10,000 *g* for 10 min, and the supernatant fractions were decanted and centrifuged at 100,000 *g* for 1 hr to obtain the microsomal pellet, which was washed once and resuspended in 0.1 M sodium phosphate buffer, pH 7.4, and stored at −70°. The human liver microsomal fraction was prepared as described above using an apparently normal section of liver from a 53-year-old male. Commercial, partially purified porcine liver carboxylesterase type I (160 units/mg) was purchased from the Sigma Chemical Co. and stored at 4°. Stock solutions and subsequent dilutions for each enzyme were prepared daily using the appropriate buffer.

*Protein assay.* Protein was measured by a dye binding method [49] as modified by Bio-Rad, using bovine serum albumin (fraction V, Sigma) as standard protein. To a cuvette, 100 $\mu$l of protein was added, followed by the addition of 400 $\mu$l of 5× diluted Bio-Rad reagent (20% in distilled water), and the mixture was incubated for 10 min at room temperature. Absorbance at 620 nm was then measured using a Gilford EIA manual ELISA reader interfaced with an Atari 400 microcomputer (programmed by R. Wixtrom, this laboratory).

*Enzyme assays.* The spectrophotometric method of Talcott [50] was used to assay malathion and diethylsuccinate carboxylesterase activities in crude mouse liver microsomes and commercial porcine liver carboxylesterase. The hydrolysis of malathion and DES was coupled to the reduction of INT with alcohol dehydrogenase and NADH diaphorase. The enzyme activity, which is equivalent to the reduction rate of the tetrazolium dye, was calculated from the linear portion of the recording (change in absorption at 500 nm with time) using an extinction coefficient of 13.8 $mM^{-1}$ $cm^{-1}$. All assays were monitored for 5 min at 37° in cuvettes containing 1.0 ml of incubation mixture using a Varian–Cary 219 UV/visible spectrophotometer equipped with time drive, temperature-controlled sample compartment, and inter-

faced with an Apple II computer. The incubation mixture contained 0.5 ml of enzyme solution and 0.5 ml of reagent mixture in 0.1 M Tris–HCl buffer, pH 7.5. Reagent blank was used in the reference cuvette. Protein concentration was 200 $\mu$g/ml for mouse liver microsomes. In the case of porcine carboxylesterase, 1.6 and 0.16 $\mu$g protein/ml were used for malathion and DES respectively. The reaction was started by injecting 1.0 $\mu$l of an acetone solution of substrate into the cuvette and shaking. Thus, the final concentrations of malathion and DES were $3 \times 10^{-4}$ and $5 \times 10^{-4}$ M respectively. These concentrations were sufficient to saturate the carboxylesterases assayed in this study. *p*-Nitrophenyl acetate esterase(s) activity was assayed spectrophotometrically [10]. The rate of *p*-NpAc hydrolysis was monitored at 37° for 5 min in the Varian–Cary 219 spectrophotometer, and the liberation of *p*-nitrophenol was monitored at 400 nm. The molar absorption coefficient for *p*-nitrophenol at pH 7.8 was 17 $mM^{-1} cm^{-1}$. Preliminary work was carried out to select substrate and protein concentrations that give linear hydrolysis rates with time. The sample cuvette contained 4.0 $\mu$g protein (mouse) and 80 ng protein (porcine) in 1.0 ml Tris–HCl buffer, pH 7.8, and 1.0 $\mu$l of an ethanol solution of *p*-NpAc was added to the cuvette, yielding a 0.15 mM final substrate concentration. Except for the enzyme, the reference and sample cuvettes contained the same components.

*Determination of apparent* $K_m$ *and* $V_{max}$. The computational method of Wilkinson [51] was used to obtain the apparent $K_m$ and $V_{max}$ for diethylsuccinate carboxylesterase(s). Eleven substrate concentrations ($5 \times 10^{-4}$–$4.9 \times 10^{-7}$ M) were used with both crude mouse liver microsomes and the commercial porcine enzyme. Assay conditions were as mentioned before.

*Inhibition studies*. For malathion and diethylsuccinate carboxylesterases, the inhibitor was added in 10 $\mu$l acetone to a cuvette containing 0.5 ml enzyme solution, the cuvette was shaken and incubated for 10 min at 37°, then 0.5 ml of the reagent mixture was added followed by injection of 1.0 $\mu$l of substrate. Acetone controls were done and the enzyme assay was performed as mentioned before. In the case of *p*-NpAc esterase(s), the inhibitor was added in 10 $\mu$l ethanol 10 min prior to the substrate and preincubated at 37°. Ethanol controls were always run, and the enzyme assays were carried out as mentioned before.

At first, all the inhibitors were tested at $1.0 \times 10^{-4}$ M final concentration; then a series of concentrations were tested for those compounds that showed some inhibition. At least three replicates of each inhibitor concentration were used. A minimum of two inhibitor concentrations showing greater than, and two showing less than, 50% inhibition on the linear region of the curve were used to determine the $I_{50}$ values from semilog plots. Only those points on the linear portion of the curve were used to calculate the $I_{50}$ and slope values by least-squares regression of the lines.

In vivo *studies*. The acute toxicity of 3-nonylthio-1,1,1-trifluoropropan-2-one was evaluated by diluting it appropriately in corn oil and injecting a 50-$\mu$l aliquot of the resulting solution interperitoneally (i.p.) into male Swiss–Webster mice to give single, 125, 250, 500 and 750 mg/kg doses. Five groups of six mice for each treatment were used. The control mice received corn oil only. The mice were observed continually for the first 6 hr; then the symptoms and mortality were recorded at 6, 12, 24, 48 and 72 hr. The surviving mice were kept under observation for 3 additional months. Dose–response studies were carried out to determine the $LD_{50}$ for malathion with and without the inhibitor. A dose of 250 mg/kg body wt in 50 $\mu$l corn oil, which caused no mortality, was injected i.p. into mouse just prior to the i.p. treatment of malathion. Six mice were used for each dose. An additional six mice were treated with corn oil only to serve as a control group. The mice were observed 6, 12 and 24 hr after treatment. The $LD_{50}$ values were based on 24-hr mortality and were determined by probit analysis.

## RESULTS

*Specific activities of crude mouse and commercial porcine liver microsomal carboxylesterases*. The rates of hydrolysis of malathion, DES, and *p*-NpAc by intact mouse and partially purified porcine liver microsomal carboxylesterases are shown in Table 1. The three substrates were hydrolyzed by partially purified porcine carboxylesterase(s) at a much faster rate than by crude mouse liver microsomes. *p*-NpAc was hydrolyzed 21 (mouse) and 1.9 (porcine) times and 78.1 (mouse) and 15.2 (porcine) times more rapidly than DES and malathion respectively. Moreover, porcine and mouse carboxylesterases hydrolyzed DES, respectively, 8.0 and 3.7 times faster than malathion.

*Kinetic parameters for DES*. The data for the double-reciprocal plots (Figs. 2 and 3) were generated from eleven concentrations of DES with the activity being measured using a dilution of 200 and 0.16 $\mu$g protein/ml for crude mouse liver microsomes and commercial, partially purified porcine liver carboxylesterase, respectively. Although the two figures represent a typical plot for two different enzyme preparations from two species, each figure shows a straight line at the lowest four concentrations and then a downward curvature making a steeper line at the highest seven concentrations. It is interesting that the data in Table 1 indicate that the low-affinity forms from both crude mouse and partially purified porcine preparations have identical $K_m$ values ($55.6 \times 10^{-6}$ M), whereas the high-affinity forms have different $K_m$ values (4.2 and $1.7 \times 10^{-6}$ M respectively). The velocity of hydrolysis of DES by the two enzyme systems was greater at high substrate concentrations than low concentrations. For crude mouse preparations, the $V_{max}$ was 44.8 and 4.6 nmol/min/mg protein, while in porcine preparations it was 14.9 and 1.2 $\mu$mol/min/mg protein for the high- and low-affinity forms respectively. The more rapid hydrolysis of DES by the partially purified porcine carboxylesterase(s) resulted from increased maximum velocity in this system compared to the crude mouse liver preparation. The enzymatic half-lives $(T_{1/2})_e$ of DES in each enzyme system were calculated using mean kinetic parameter values (Table 1). These values describe relative enzyme activity

Table 1. Malathion, diethylsuccinate and *p*-nitrophenyl acetate esterase activities in crude mouse liver microsomes and commercial porcine carboxylesterase*

| Enzyme source | Specific activity† | | | Kinetic parameters for DES† | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $K_m$‡ | | $V_{max}$ | | $(T_{1/2})_e$§ | |
| | Malathion | *p*-NpAc | DES | I | II | I | II | I | II |
| Mouse liver microsomes | 11.2 ± 0.6‖ | 875 ± 10‖ | 41.7 ± 11.6‖ | 55.6 ± 3.2 | 4.2 ± 0.2 | 44.8 ± 5.7‖ | 4.6 ± 0.3‖ | 0.86 | 0.759 |
| Porcine liver | 1.9 ± 0.3¶ | 28.1 ± 6.4¶ | 14.8 ± 4.9¶ | 55.6 ± 4.5 | 1.7 ± 0.1 | 14.9 ± 3.1¶ | 1.2 ± 0.1¶ | 0.003 | 0.001 |

* Sigma EC 3.1.1.1 Type I.
† Values are means ± SD for three determinations of each eleven substrate concentrations.
‡ M × $10^{-6}$; (I) the highest seven DES concentrations, (II) the lowest four concentrations.
§ Min (mg protein)/ml; $(T_{1/2})_e = 0.693\ K_m/V_{max}$.
‖ Expressed in nmol/min/mg protein.
¶ Expressed in µmol/min/mg protein.

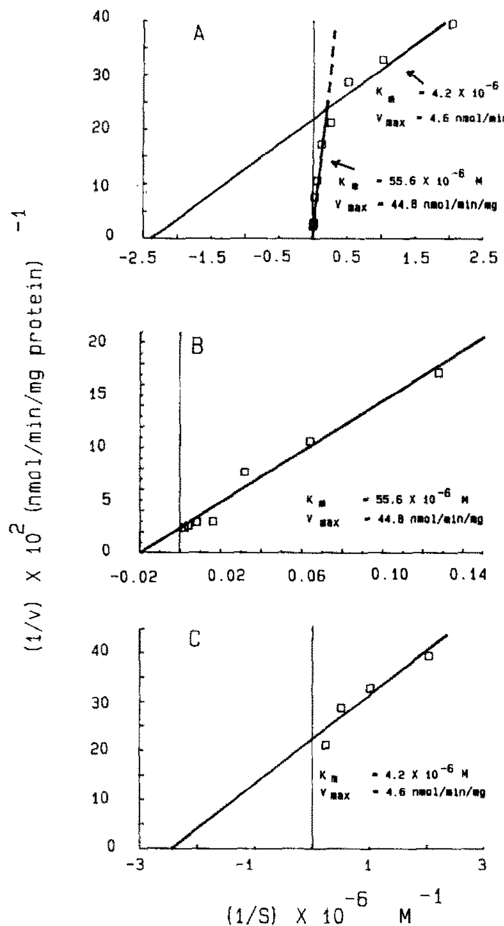


Fig. 2. Double-reciprocal plots showing the hydrolysis of DES by crude mouse liver microsomes using (A) eleven substrate concentrations, (B) the highest seven concentrations, and (C) the lowest four concentrations.

independent of substrate concentration [27]. On the basis of $(T_{1/2})_e$ values, the commercial porcine liver carboxylesterase(s) was 287 (low-affinity) and 759 (high-affinity) times more active than the crude mouse liver microsomal carboxylesterase(s).

*Inhibition of mouse liver microsomal carboxylesterase(s).* The $I_{50}$ values presented in Table 2 and the example inhibition curves (Fig. 4) clearly indicate the inhibitory potency of the 3-substituted thio-1,1,1-trifluoropropan-2-ones tested on the hydrolysis of malathion by crude mouse liver microsomes. Under these assay conditions, the nonyl and decyl sulfides were the most potent inhibitors. The cyclohexyl sulfide was biologically more active than the phenyl sulfides. On the other hand, substitution on the phenyl ring clearly increased the inhibitory activity. The inhibitory potency of the tested substituted phenyl sulfides could be arranged in decreasing order as follows: *o,o*-dichloro; *m*-chloro; *m,p*-dichloro; *p*-*t*-$C_4H_9$ and *p*-chloro.

Unlike malathion, the esterases hydrolyzing DES were activated by low-inhibitor concentrations whereas high concentrations were strongly inhibitory (Fig. 5). Data in Table 2 also show that the non-specific esterases hydrolyzing *p*-NpAc were inhibited

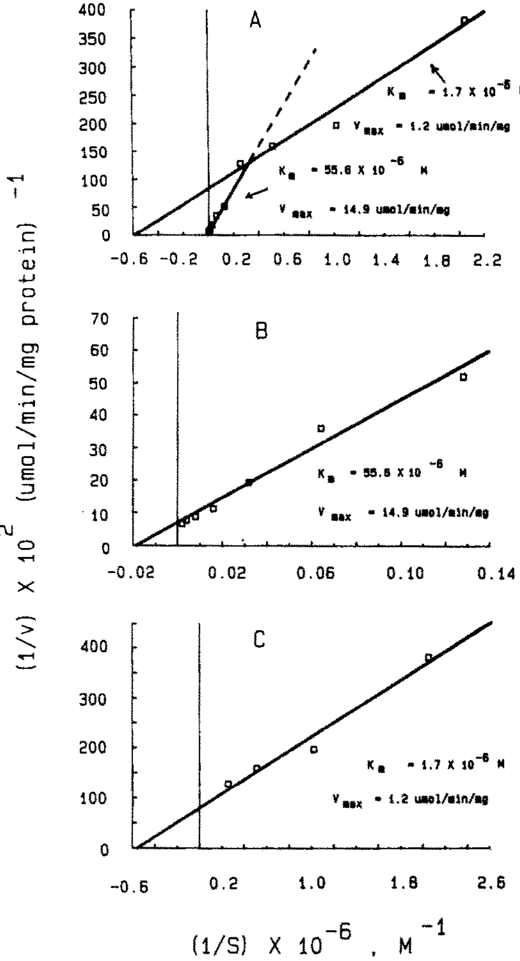


Fig. 3. Double-reciprocal plots showing the hydrolysis of DES by commercial porcine liver carboxylesterase using (A) eleven substrate concentrations, (B) the highest seven concentrations, and (C) the lowest four concentrations.

by the nonyl and phenyl sulfides. The two inhibitors were more selective for inhibition of *p*-NpAc esterases compared to malathion and DES carboxylesterases.

Table 3 and Fig. 6 show the inhibitory activities of substituted trifluoroketones lacking the sulfide bond on mouse liver microsomal carboxylesterases hydrolyzing malathion, DES and *p*-NpAc. Inhibition of malathion hydrolysis by the compounds used was increased as the carbon chain and distance from the phenyl ring to the carbonyl increased in the aliphatic and aromatic compounds respectively. Comparison of the inhibitory potency of the nonyl and phenyl sulfides (Table 2) and the corresponding compounds lacking the sulfide bond (Table 3) indicated that the former were more active as they gave lower $I_{50}$ values and steeper slopes. 1,1,1-Trifluorooctan-2-one (compound **14**) was the most potent compound tested with DES carboxylesterase(s) followed by **17**, **15**, and **16**. At inhibitor concentrations below those showing any inhibition of DES carboxylesterases there was an activation (Fig. 6). It is interesting to notice that **14** was more selective for DES carboxylesterase(s) and **15**, **16**, and **17** were more selective for *p*-NpAc esterases.

*Inhibition of porcine liver carboxylesterase(s).* The inhibitory potencies of some 3-substituted thio-1,1,1-trifluoropropan-2-ones on the commercial porcine liver carboxylesterase hydrolyzing *p*-NpAc, malathion and DES were examined (Table 4). Under these conditions, the aliphatic series showed high inhibitory potency. The phenyl compound (**7**) had the highest $I_{50}$ value ($6.5 \times 10^{-7}$ M) compared with the rest of the inhibitors tested; however, substitution on the phenyl increased the inhibitory activity. The *p*-methyl (**19**) was the most potent substituent (with an $I_{50}$ value about one order of magnitude lower than the phenyl) followed by the *p*-fluoro, *p*-methoxy, and *p*-chloro (**21**, **20**, **9**). Comparison of the inhibitory potencies of the undecyl (**4**) and the phenyl (**7**) compounds on the hydrolysis of

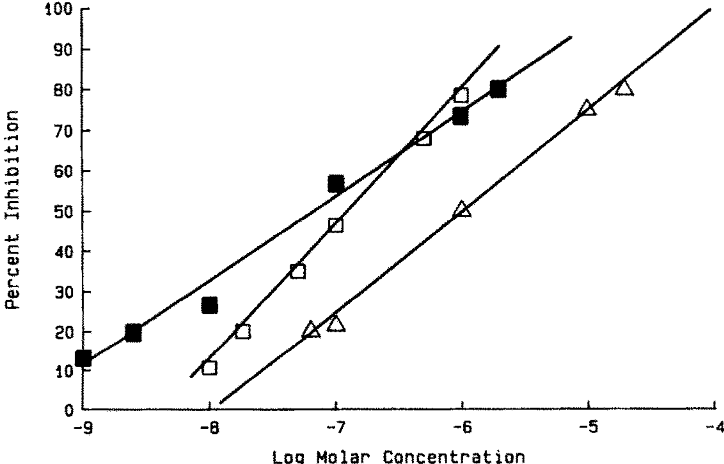


Fig. 4. Plot of percentage inhibition of mouse liver microsomal carboxylesterase(s) hydrolyzing malathion against log molar concentrations of 3-nonylthio-1,1,1-trifluoropropan-2-one (■), 3-phenylthio-1,1,1-trifluoropropan-2-one (△), and DFP (□).

Table 2. Inhibitory potencies of 3-substituted thio-1,1,1-trifluoropropan-2-ones

$$(\mathrm{R{-}S{-}CH_2{-}\overset{O}{\overset{\|}{C}}CF_3})$$

on the hydrolysis of malathion, diethylsuccinate and *p*-nitrophenyl acetate by mouse liver microsomes

| | | $I_{50}$* (M) (slope) for the hydrolysis of | | |
|---|---|---|---|---|
| Number | R | Malathion | DES† | *p*-NpAc |
| 1 | $C_8H_{17}$— | $2.5 \times 10^{-6}$ (15) | $4.0 \times 10^{-7}$ | —‡ |
| 2 | $C_9H_{19}$— | $7.2 \times 10^{-8}$ (21) | $3.4 \times 10^{-7}$ | $8.9 \times 10^{-9}$ (18) |
| 3 | $C_{10}H_{21}$— | $9.7 \times 10^{-8}$ (22) | — | — |
| 4 | $C_{11}H_{23}$— | $3.8 \times 10^{-7}$ (31) | — | — |
| 5 | $C_{12}H_{25}$— | $2.7 \times 10^{-7}$ (21) | — | — |
| 6 | | $2.4 \times 10^{-7}$ (14) | $9.5 \times 10^{-7}$ | — |
| 7 | | $1.0 \times 10^{-6}$ (24) | $4.2 \times 10^{-6}$ | $2.6 \times 10^{-7}$ (31) |
| 8 | *t*—$C_4H_9$— | $6.1 \times 10^{-7}$ (26) | $4.0 \times 10^{-6}$ | — |
| 9 | Cl— | $7.4 \times 10^{-7}$ (19) | — | — |
| 10 | Cl | $2.9 \times 10^{-7}$ (34) | — | — |
| 11 | Cl, Cl— | $4.6 \times 10^{-7}$ (23) | — | — |
| 12 | Cl, Cl | $2.2 \times 10^{-7}$ (23) | — | — |
| 13 | DFP§ | $1.4 \times 10^{-7}$ (34) | — | — |

* $I_{50}$ values were obtained from at least three separate determinations of two assays per treatment. The substrate concentrations were 0.3, 0.5, and 0.15 mM respectively. Protein concentrations were 200 μg/ml for malathion and DES and 4.0 μg/ml for *p*-NpAc.

† At inhibitor concentrations just below those causing inhibition, activation was observed thus making calculation of slope difficult.

‡ $I_{50}$ was not determined.

§ DFP was used as a reference.

*p*-NpAc, malathion and DES indicated that these compounds were more selective for inhibition of *p*-NpAc esterases and more potent on DES esterase(s) as compared to malathion esterase(s).

*Species specificity*. Data in Table 5 show the inhibitory activities of the nonyl, cyclohexyl, and phenyl sulfides (**2**, **6**, **7**) on the hydrolysis of malathion by crude human and mouse liver microsomes as well as

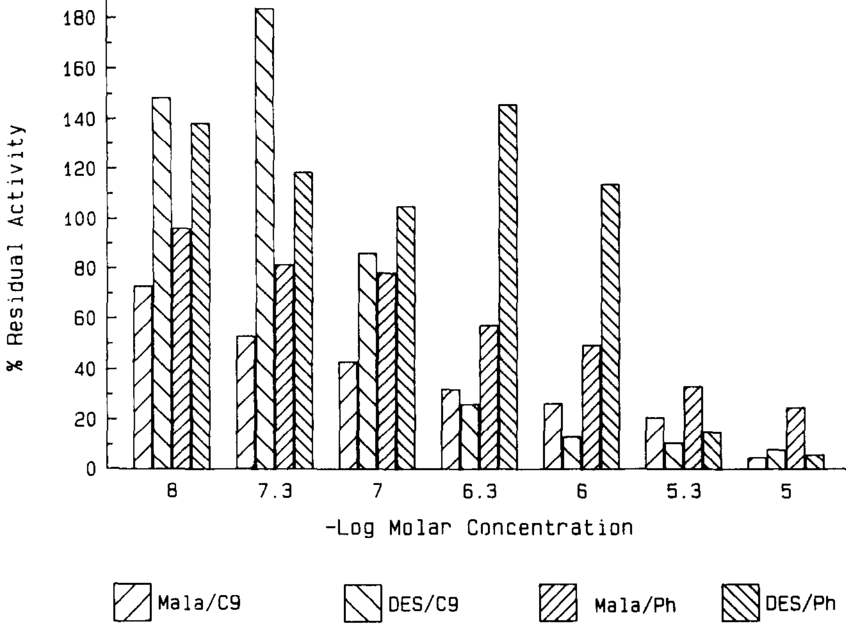


Fig. 5. Plot of percentage residual activity of mouse liver microsomal carboxylesterase(s) hydrolyzing malathion and diethylsuccinate against log molar concentrations of 3-nonylthio-1,1,1-trifluoropropan-2-one (C9) and 3-phenylthio-1,1,1-trifluoropropan-2-one (Ph). Activation of carboxyesterase(s) using malathion as a substrate was not observed as low as $10^{-10}$ M with either inhibitor.

commercial porcine liver carboxylesterase. On the basis of $I_{50}$ and slope values, it is interesting to notice a species specificity of malathion hydrolysis in the tested systems. Although the commercial porcine liver carboxylesterase is partially purified, the enzyme hydrolyzing malathion was less sensitive to the inhibitors tested compared to the crude preparations of human and mouse liver microsomes. The three compounds used were more selective for inhibition of human carboxylesterase(s) hydrolyzing malathion, and the nonyl sulfide was the most potent followed by the cyclohexyl and phenyl sulfides.

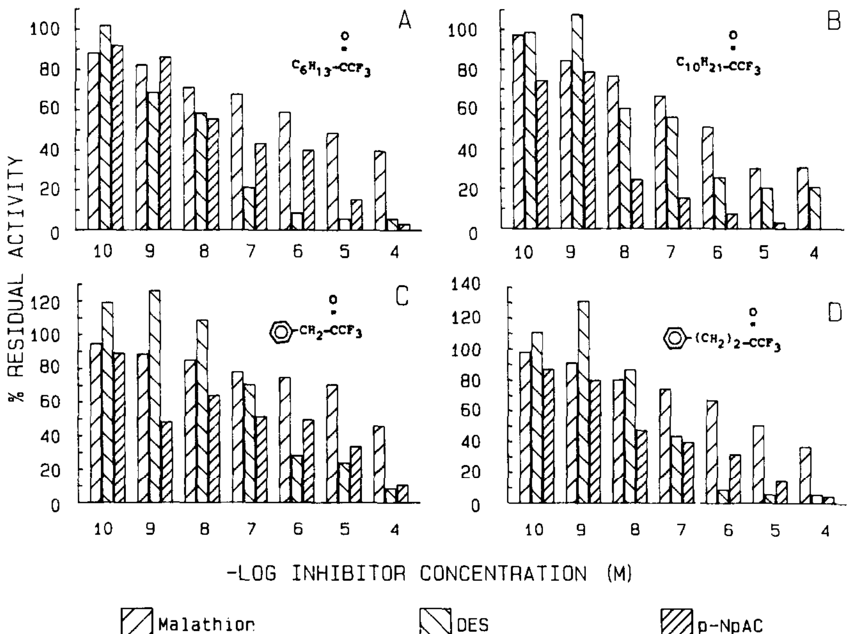


Fig. 6. Plot of percentage residual activity of crude mouse liver microsomal carboxylesterase(s) hydrolyzing malathion, DES and *p*-NpAc against log molar concentrations of (A) 1,1,1-trifluorooctan-2-one, (B) 1,1,1-trifluorododecan-2-one, (C) 3-phenyl-1,1,1-trifluoropropan-2-one, and (D) 4-phenyl-1,1,1-trifluorobutan-2-one.

Table 3. Inhibitory potencies of some substituted trifluoroketones ($R—\overset{O}{\overset{\|}{C}}CF_3$) on the hydrolysis of malathion, diethylsuccinate and *p*-nitrophenyl acetate by mouse liver microsomes

| | | $I_{50}$* (M) (slope) for the hydrolysis of | | |
|---|---|---|---|---|
| Number | R | Malathion | DES† | *p*-NpAc |
| 14 | $C_6H_{13}$— | $8.15 \times 10^{-6}$ (8) | $1.03 \times 10^{-8}$ (22) | $7.35 \times 10^{-8}$ (15) |
| 15 | $C_{10}H_{21}$— | $8.19 \times 10^{-7}$ (13) | $8.05 \times 10^{-8}$ (15) | $4.82 \times 10^{-9}$ (32) |
| 16 | $C_6H_5$—$CH_2$— | $8.1 \times 10^{-4}$ (9) | $2.46 \times 10^{-6}$ (49) | $2.55 \times 10^{-7}$ (13) |
| 17 | $C_6H_5$—$(CH_2)_2$— | $1.26 \times 10^{-5}$ (11) | $8.19 \times 10^{-8}$ (39) | $3.34 \times 10^{-8}$ (15) |

* $I_{50}$ values were obtained from at least three separate determinations of two assays per treatment. The substrate concentrations were 0.3, 0.5, and 0.15 mM respectively. Protein concentrations were 200 μg/ml for malathion and DES and 4.0 μg/ml for *p*-NpAc.

† At inhibitor concentrations just below those causing inhibition, activation was observed. The slope was calculated from the clear linear portion of the curve.

Table 4. Inhibitory potencies of 3-substituted thio-1,1,1-trifluoropropan-2-ones

$$(R—S—CH_2—\overset{O}{\overset{\|}{C}}CF_3)$$

on the hydrolysis of *p*-nitrophenyl acetate, malathion, and diethylsuccinate by commercial porcine liver carboxylesterase*

| | | $I_{50}$† (M) (slope) for the hydrolysis of | | |
|---|---|---|---|---|
| Number | R | *p*-NpAc | Malathion | DES |
| 2 | $C_9H_{19}$— | $4.2 \times 10^{-10}$ (25) | —‡ | — |
| 3 | $C_{10}H_{21}$— | $2.6 \times 10^{-10}$ (23) | — | — |
| 4 | $C_{11}H_{23}$— | $3.0 \times 10^{-11}$ (19) | $7.0 \times 10^{-8}$ (65) | $1.8 \times 10^{-8}$ (34) |
| 5 | $C_{12}H_{25}$— | $2.7 \times 10^{-10}$ (26) | — | — |
| 18 | —$CH_2COOH$ | $6.1 \times 10^{-7}$ (42) | — | — |
| 7 | $C_6H_5$— | $6.5 \times 10^{-7}$ (34) | $7.8 \times 10^{-6}$ (20) | $1.7 \times 10^{-6}$ (17) |
| 19 | $CH_3$—$C_6H_4$— | $4.3 \times 10^{-8}$ (23) | — | — |
| 20 | $CH_3O$—$C_6H_4$— | $1.5 \times 10^{-7}$ (18) | — | — |
| 21 | F—$C_6H_4$— | $1.1 \times 10^{-7}$ (21) | — | — |
| 9 | Cl—$C_6H_4$— | $2.4 \times 10^{-7}$ (79) | — | — |

* Sigma EC 3.1.1.1 Type I.

† $I_{50}$ values were obtained from at least three separate determinations of two assays per treatment. The substrate concentrations were 0.15, 0.3, and 0.5 mM respectively. Protein concentrations were 80 ng, 1.6 μg, and 160 ng for *p*-NpAc, malathion and DES respectively.

‡ $I_{50}$ was not determined.

Table 5. Comparative inhibitory potencies of three selected 3-substituted thio-1,1,1-trifluoropropan-2-ones

$$(R{-}SCH_2{-}\overset{O}{\overset{\|}{C}}CF_3)$$

on human, mouse, and porcine liver malathion carboxylesterase(s)

| Number | R | $I_{50}$* (M) (slope) Human | Mouse | Porcine |
|---|---|---|---|---|
| 2 | $C_9H_{19}$— | $5.3 \times 10^{-8}$ (44) | $7.2 \times 10^{-8}$ (21) | $7.9 \times 10^{-8}$ (66) |
| 6 | cyclohexyl— | $1.0 \times 10^{-7}$ (24) | $2.4 \times 10^{-7}$ (14) | $1.5 \times 10^{-6}$ (23) |
| 7 | phenyl— | $3.4 \times 10^{-7}$ (32) | $1.0 \times 10^{-6}$ (24) | $7.8 \times 10^{-6}$ (20) |

* $I_{50}$ values were obtained from at least three separate determinations of two assays per treatment. The substrate concentration was $3 \times 10^{-4}$ M. Protein concentrations were 200 $\mu$g/ml for human and mouse and 1.6 $\mu$g/ml for porcine.

In vivo *effects*. When i.p. doses of 125 and 250 mg/kg of 3-nonylthio-1,1,1-trifluoropropan-2-one (**2**) were given to male Swiss–Webster mice, a hyperactivity was noted for a few minutes. Partial loss of facial and dorsal hair was observed with both of these doses after several weeks. No mortality or other symptoms were recorded over a period of 3 months. Higher doses of the compound caused 100% mortality after 24–48 hr with initial hyperactivity the only clear symptom observed (Table 6). The lack of mortality at 250 mg/kg enabled us to study the effect of the nonyl sulfide (**2**) on malathion toxicity to mice (Table 7). An i.p. dose of 250 mg/kg of the inhibitor was given to mice just prior to i.p. doses of malathion. This treatment resulted in lower $LD_{50}$, $LD_{99}$ and slope values compared with the corresponding values for malathion alone. Like the effect of the high doses of malathion, a rapid death of mice (6 hr after treatment) was observed in the inhibitor-malathion treatment. Thus, 3-nonylthio-1,1,1-trifluoropropan-2-one enhanced the toxicity of malathion to mice 2.3 times.

## DISCUSSION

Several studies have characterized carboxylesterases as a heterogeneous group of enzymes with broad and overlapping substrate specificities, different specificities toward inhibitors, as well as different molecular properties [11, 29, 36].

Table 6. Toxicity of different doses of 3-nonylthio-1,1,1-trifluoropropan-2-one administered i.p. to Swiss–Webster mice

| Dose (mg/kg body wt*) | % Mortality 6 | 12 | 24 | 48 | 72 hr | → | 3 months |
|---|---|---|---|---|---|---|---|
| 125 | 0 | 0 | 0 | 0 | 0 | | 0 |
| 250 | 0 | 0 | 0 | 0 | 0 | | 0 |
| 500 | 0 | 0 | 0 | 100 | | | |
| 750 | 0 | 0 | 100 | | | | |

* Body weights of male mice were from 25 to 30 g.

Table 7. Effect of 3-nonylthio-1,1,1-trifluoropropan-2-one on the toxicity of malathion to Swiss–Webster mice

| Treatment* | $LD_{50}$ (g/kg body wt) | $LD_{99}$ (g/kg body wt) | Slope† |
|---|---|---|---|
| Malathion | 1.6 | 2.75 | 5.7 |
| Malathion + 250 mg C9/kg body wt | 0.71 | 2.19 | 3.0 |

* The inhibitor was administered i.p. just prior to malathion.

† 95% Confidence interval was 1.7–1.5 and 0.79–0.64 for malathion alone and malathion + the inhibitor respectively.

Our results demonstrate that carboxylesterases from mouse and porcine livers have considerable specificity toward the hydrolysis of *p*-NpAc, DES and malathion, as has been demonstrated previously for other model substrates [3, 13, 16, 37, 52–54].

Double-reciprocal plots (Figs. 2 and 3) for the activities of mouse and porcine liver carboxylesterases hydrolyzing DES show that data points fit two lines for each enzyme. Such data could be explained by multiple catalytic sites on the same enzyme molecule or more likely by two families of enzymes showing, on the average, different kinetic values. It is rather interesting that only the carboxylesterases hydrolyzing DES are activated by very low concentrations of the substituted trifluoroketones tested as enzyme inhibitors, whereas higher trifluoroketone concentrations are potent inhibitors. As an explanation we propose that the isozymes and/or the active sites differ in their sensitivities toward inhibition; however, more kinetic studies are needed. The dimeric structure of native pig liver esterase was shown to contain two active sites per molecule [55]. Levy and Ocken [56] have demonstrated that the velocity of hydrolysis of ethylbutyrate by pig liver esterase is greater at high substrate concentrations than is demanded by extrapolation of low substrate data. They proposed a single esterolytic site with a control site. Our study indicates that, regardless of the nature of this second control site, it can be affected by exceptionally low concentrations of trifluoroketones. This potency indicates that the control site has at least some resemblance to the catalytic site.

The present study shows that substituted trifluoroketones are powerful, selective inhibitors of hepatic carboxylesterases hydrolyzing malathion, DES and *p*-NpAc. They also have species specificity as they are more selective for inhibition of human hepatic malathion carboxylesterase(s) when compared with porcine and mouse hepatic malathion esterases. Thus, these compounds are promising for distinguishing among esterase families either in different species or acting on different substrates. Trifluoromethylketones are probably potent inhibitors due to their resemblance to a tetrahedral transition state or transient intermediate on the reaction coordinate to the acylated enzyme [41, 56–61]. In addition, fluorines possess strong electron withdrawing properties so that in aqueous solutions fluoroketones are largely in the hydrate state [62]. Gelb *et al.* [40] have demonstrated that trifluoroketones inhibit acetylcholinesterase by formation of a stable hemiketal with the active-site serine residues. It has been reported [63] that a reversible inhibition of chicken liver carboxylesterase by benzils occurs through formation of a tetrahedral adduct at the active site of the enzyme. In this regard, our data indicate that both the electrophilicity of the carbonyl group and the stereochemical requirements make trifluoromethylketones potent, selective inhibitors of carboxylesterases, and although the trifluoromethyl group plays the major part in polarizing the carbonyl group, the apolar end of the molecule seems to be important in stabilizing the interaction with the enzyme. So it is worth arguing that the potency and selectivity can be further increased by the synthesis of more exact mimics of good carboxylesterase substrates as well as by modifications affecting the polarity of the carbonyl moiety. Previous studies have reported the inhibitory potency of trifluoromethylketones on juvenile hormone, α-naphthyl acetate (α-NA), and meperidine esterases, as well as acetylcholine esterase and bovine trypsin [39–47]. Some of these workers reported that the sulfide bond *beta* to the carbonyl greatly increased inhibitory potency. However, data from this study indicate that the role of the thioether in enhancing inhibitor potency is by no means universal.

The synergistic effect of the nontoxic dose of 3-nonylthio-1,1,1-trifluoropropan-2-one (**2**) is somewhat surprising since trifluoromethylketones are found to be reversible inhibitors, and such inhibitors commonly have weaker *in vivo* effects than irreversible inhibitors [41, 44]. The high inhibitory potency *in vitro* of the nonylsulfide on mouse hepatic malathion carboxylesterase(s) (this study) supports the argument that malathion synergism occurs because of the inhibition of the majority of carboxylesterases which either degrade malathion or act as alternate targets for the oxon. This inhibition would allow malathion to reach the target site at a high enough concentration to cause mortality. Since some trifluoroketones were found to be cholinesterase inhibitors [39, 40, 63], it also can be argued that the low dose of the nonyl sulfide, when used alone, was not enough to cause effective cholinesterase inhibition; however, in the presence of malathion the enzyme was highly inhibited and thus the toxicity of malathion was potentiated. High doses of the inhibitor are toxic to mice by an unknown mechanism. Low acute toxicity of other substituted trifluoromethylketones to mice and their *in vivo* effects on insects have been reported earlier [41, 44].

In conclusion, this study proves that substituted trifluoromethylketones are very potent "transition state" inhibitors of hepatic carboxylesterases, and their selective inhibitor activity can be used to distinguish among hepatic esterases from different species. In addition, they are active *in vivo* so, from a practical point of view, it may be possible to use such compounds as drug or pesticide synergists or potentiators. Thus, trifluoroketones offer a tremendous potential as probes for the investigation of a wide variety of enzymes. This study may also lay the groundwork for the use of such compounds in the affinity purification of solubilized, hepatic carboxylesterases. Certainly such purification is a necessary step in the definitive characterization of these enzymes, and simple, high yield purification systems may facilitate their use in therapy.

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