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CHARACTERIZATION OF NEUROPATHY TARGET ESTERASE USING TRIFLUOROMETHYL KETONES

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Abstract—Neuropathy target esterase (NTE) is a membrane-bound carboxylesterase activity which is proposed as the target site in nerve tissue for initiation of organophosphate-induced delayed neuropathy. This activity is identified as phenyl valerate hydrolysis which is resistant to treatment with paraoxon and sensitive to co-incubation with paraoxon and mipafox. NTE preparations were obtained, which did not contain paraoxon-sensitive or mipafox-resistant hydrolases, by selective reconstitution of detergent-solubilized NTE from chick embryo brain into asolectin vesicles during gel filtration. The topography of the catalytic site of NTE was then examined by investigating the inhibition of NTE by a series of 3-alkylthio- and 3-arylthio-1,1,1-trifluoro-propan-2-ones. These trifluoromethyl ketones were found to be rapidly reversible, competitive inhibitors of NTE with I_{50} values from 1.3×10^{-4} M to 4.9×10^{-8} M. Correlation of I_{50} values with octanol/water partition coefficients *(P)*, in the range of $\log P = 1.5$ to 5.9, indicated that the optimal lipophilicity for NTE substrates and inhibitors is in the range of $\log P = 3.0$ to 3.4. Electrophilic substitution at the *meta* position of aromatic rings increased the inhibitory capacity of these inhibitors, whereas substitution at the *ortho* position reduced inhibitory capacity. These results indicate both that a large hydrophobic pocket is closely associated with the catalytic residue of NTE, and that affinity for the active site is affected by steric and electronic parameters.

Neuropathy target esterase (NTE,** also known as neurotoxic esterase) is an integral membrane protein found predominantly in central and peripheral nerve tissue [1–3]. NTE is proposed as the target site at which certain organophosphates (OPs) such as diisopropyl phosphorofluoridate (DFP) and diisopropylphosphorodiamidic fluoride (mipafox) act to initiate organophosphate-induced delayed neuropathy (OPIDN) [4–7]. The symptoms of this potentially irreversible neuropathy appear 2–3 weeks after a single acute exposure to OPs and are accompanied by a distal degeneration of the long, large diameter peripheral nerves and the distal ends of the ascending and descending tracts of the spinal cord [8–10]. Determination of the role of NTE in the proper function of the nerve axon will require both the purification of NTE and the characterization of its physical and catalytic properties.

The relationship between the structures and inhibitory potencies of a wide range of NTE inhibitors has been examined in two previous studies [11, 12]. In excess of 150 different phosphates, phosphonates, phosphinates, carbamates, and sulfates were examined. Analysis of the results involved the examination of a homologous series of compounds, but attempts were not made to quantitatively relate observed trends to the physicochemical parameters of the compounds. Although a strong correlation between structure and activity was not identified, the results indicated that both hydrophobic and steric parameters were important.

It has been reported previously that trifluoromethyl ketones are potent inhibitors of a large number of serine active site hydrolases [13–15], including human brain NTE (Talcott, Ketterman and Lotti, personal communication to B.D.H.). The substitution of the hydrogen atoms α to the carbonyl group with fluorines enhances the reactivity of the carbonyl with the serine in the catalytic site [13], and it is postulated that these compounds behave as transition state analogs [16]. In the present study we investigated the interaction of NTE with a series of twenty-two aliphatic and aromatic trifluoromethyl ketone sulfides. The I_{50} values for inhibition of NTE by each of these compounds were determined, and an analysis of quantitative structure–activity relationships (QSAR) was performed based on the physicochemical properties of each inhibitor. The purpose of this analysis was 3-fold: (1) to characterize the active site of NTE, (2) to provide information for the synthesis of more specific substrates and inhibitors, and (3) to identify appropriate ligands for affinity chromatography of NTE.

In addition, we report a method for obtaining an

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** Abbreviations: NTE, neuropathy target esterase; OPIDN, organophosphate-induced delayed neuropathy; OTFP, 3-octylthio-1,1,1-trifluoropropan-2-one; TFK, trifluoromethyl ketone; mipafox, *N,N'*-diisopropylphosphorodiamidic fluoride; DFP, diisopropyl phosphorofluoridate; paraoxon, phosphoric acid diethyl 4-nitrophenyl ester; *P*, octanol/water partition coefficient; and EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate.

NTE preparation which is free from contamination by non-NTE-type phenyl valerate hydrolases. Operationally, NTE is identified as that portion of total phenyl valerate hydrolyzing activity which is resistant to pretreatment with non-neuropathic paraoxon (40 μM), but sensitive to pretreatment with paraoxon and neuropathic mipafox (50 μM) [17]. Therefore, to determine NTE activity, pairs of differentially inhibited samples must be assayed. Protocols to characterize the interaction of NTE with other inhibitory compounds must confront two potential problems. First, the catalytic sites of NTE may be less than 50% saturated with substrate during assay [18, 19], in which case, nonbound paraoxon and mipafox may compete with the substrate or the inhibitor of interest for interaction with hydrolytic sites. Second, if nonbound paraoxon and mipafox are removed prior to addition of test compounds and substrate, then reversibly inhibited hydrolases may be reactivated. Evidence identifying the existence of such a hydrolase in chick embryo brain membrane fractions is presented. The problems associated with these methods were avoided in the present study by screening inhibitors against an NTE-type phenyl valerate hydrolase preparation from which all paraoxon-sensitive and mipafox-resistant activities had been removed.

MATERIALS AND METHODS

Chemicals. Aliphatic and aromatic 3-substituted thio-1,1,1-trifluoropropanones (TFKs) used in this study were synthesized previously by the reaction of the appropriate thiol with 3-bromo-1,1,1-trifluoropropan-2-one, purified, and analyzed [15, 20]. Mipafox (*N*,*N'*-diisopropylphosphorodiamidic fluoride) and phenyl valerate were synthesized as previously described [21] according to the methods of Johnson [17]. The purity of mipafox was monitored by determination of its melting point (60.5°, white crystals), and the structures of mipafox and phenyl valerate were analyzed by NMR and i.r. Paraoxon (phosphoric acid diethyl 4-nitrophenyl ester, Aldrich Chemical Co., Milwaukee, WI) was analyzed for interfering contaminants by thin-layer chromatography and gas phase chromatography, and by determination of its I_{50} to NTE (600 μM) as recommended by Johnson [22]. Stock solutions of mipafox (10^{-1} M) in 50 mM Tris-citrate, pH 6.0 and paraoxon (10^{-1} M in acetone) were prepared and stored in a desiccator at −25°. Triton X-100 (Surfact-Amps X-100) was obtained from Pierce (Rockford, IL). Asolectin was obtained from Associated Concentrates (Woodside, NY).

Animal. Fertilized White Leghorn chicken eggs were obtained from De Kalb West (Turlock, CA) and incubated by the Department of Avian Sciences, University of California, Davis. White Leghorn laying hens were obtained from flocks maintained by the Department of Avian Sciences.

Assay for phenyl valerate hydrolysis. Phenyl valerate hydrolysis was assayed colorimetrically by the method of Johnson [17] with modifications described previously [23, 24]. In addition, this method has been scaled down 4-fold to yield the following protocol. Samples (10–50 μL) were incubated for 20 min at 37° in an appropriate volume of 50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0, and an inhibitor solution such that the final volume was 0.74 mL. The inhibitor solution was one of the following: (a) 50 μL of above buffer; (b) 50 μL of 0.6 mM paraoxon in 50 mM Tris-citrate, pH 6.0; (c) 50 μL of 0.75 mM mipafox in 50 mM Tris-citrate, pH 6.0; or (d) 50 μL each of the above paraoxon and mipafox solutions. Ten microliters of phenyl valerate (12.5 mg PV/mL in dimethylformamide) was added and tubes were incubated for 30 min at 37°. Enzymatic hydrolysis was stopped by adding 0.5 mL of 1% (w/v) SDS, 0.25% (w/v) 4-aminoantipyrine, 50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0. Then the colored complex was developed by adding 0.25 mL of 0.4% (w/v) $K_3Fe(CN)_6$. The absorbance of each assay tube was determined in triplicate using a Bio-Tek 96-well plate reader (Winooski, VT) by addition of 300 μL from each tube to each of three wells of a Falcon 96-well plate (Becton-Dickinson, Oxnard, CA). Absorbance was determined at 490 nm and the concentration of phenol was calculated using an absorption coefficient equal to 16,812 M^{-1} cm^{-1}. Units of activity are reported as micromoles of phenol produced per minute (I.U.).

Protein determinations. Protein was quantitated using the method of Lowry *et al.* [25] with bovine albumin (Fraction V, Sigma, St. Louis, MO) as a standard. To avoid the interference caused by precipitation of Triton X-100, 0.1 mL of 24% (w/v) sodium dodecyl sulfate (SDS) was added to samples containing Triton X-100 (final assay vol. 1.3 mL) [26].

Preparation of microsomal membranes from chicken embryo brains. Brains from day 19 chicken embryos were homogenized for 25 sec in 10 mL of ice-cold Buffer A (50 mM Tris–HCl, 0.5 M NaCl, 2 mM EDTA, 2 mM EGTA, pH 7.2, at 21°) per g of tissue with a Polytron homogenizer on setting 7 (Brinkmann Instruments, Westbury, NY). Homogenates were centrifuged in an SS34 rotor (Sorvall, Wilmington, DE) for 10 min at 4° and 1100 *g* (r_{av} 8.26 cm). The upper layer of foam was aspirated and the supernatant was isolated. The low speed supernatant was centrifuged in a Type 60 Ti rotor (Beckman, Palo Alto, CA) for 20 min at 4° and 100,000 *g* (r_{av} 6.15 cm) to obtain a crude microsomal membrane fraction. Membrane pellets were resuspended in Buffer B (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0, at 21°) and stored in liquid nitrogen for future use.

Inhibition of membranes with paraoxon. Membrane suspensions were treated with 100 μM paraoxon for 20 min at 37°. Inhibition was stopped by 10-fold dilution of suspensions into ice-cold Buffer B. Samples were centrifuged in a Type 60 Ti rotor at 100,000 *g* for 60 min at 4°. Pellets were resuspended in the same volume of ice-cold buffer and centrifuged as before. These pellets were resuspended at *ca.* 20 mg protein/mL of buffer and either used immediately or stored in liquid nitrogen.

Detergent solubilization. Two types of samples were prepared for gel filtration. In the first case, control membranes (no pretreatment with paraoxon)

were extracted at 1 mg protein/mL in ice-cold 0.2% (w/v) Triton X-100, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Tris–HCl (pH 7.2 at 21°) for 60 min on ice with occasional mixing. The sample was centrifuged in a Type 60 Ti rotor at 100,000 *g* for 1 hr at 4°. The supernatant was concentrated overnight at 4° against 5 L of 0.2% Triton X-100 (w/v), 1 mM EDTA, 1 mM EGTA, 0.5 M NaCl, 20 mM Tris–HCl (pH 7.2 at 21°) in a Micro-ProDiCon apparatus with PA-30 dialysis membranes (30,000 M_r cut-off; Bio-Molecular Dynamics, Beaverton, OR). This resulted in a 40-fold decrease in volume.

In the second case, paraoxon-treated membranes were extracted at 5 mg protein/mL in ice-cold 0.3% (w/v) Triton X-100, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Tris–HCl (pH 7.2 at 21°) for 30 min on ice with occasional mixing. The sample was centrifuged in a Type 75 Ti (Beckman) rotor at 100,000 *g* for 1 hr at 4°. The supernatant was recovered and used without further concentration.

Gel filtration. A Superose 12 gel filtration column (HR 10/30, Pharmacia) was equilibrated at room temperature in 0.02% (w/v) Triton X-100, 0.02% (w/v) asolectin, 0.5 M NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM Tris–HCl, pH 7.2. Samples (500 μL) were loaded and eluted at 0.25 mL/min using an FPLC system (Pharmacia). Elution was monitored at 280 nm, and 0.5-mL fractions were collected. Fractions were placed on ice as they were collected. When paraoxon-treated membranes were detergent solubilized and fractionated by gel filtration, NTE-containing fractions were combined (see Fig. 2 for range) and stored in liquid nitrogen.

Determination of I_{50} values and partition coefficients. All I_{50} determinations with the 3-substituted thio-1,1,1-trifluoropropan-2-ones were performed with gel filtration purified NTE from paraoxon-treated membranes. Rates of phenyl valerate hydrolysis were determined as described above, except that samples were not preincubated for 20 min at 37° with mipafox and paraoxon. Phenyl valerate and NTE were added simultaneously to tubes containing appropriate concentrations of inhibitor. Assay tubes were incubated at 37° for 30 min prior to addition of SDS to stop substrate hydrolysis.

Inhibitors were added in a volume of 10 μL of ethanol. Ethanol controls were included in each assay. To determine I_{50} values, a broad titration was first performed with inhibitor concentrations in the range of 10^{-4}–10^{-9} M. This was followed by a more complete titration curve in the linear region of a percent activity vs −log [inhibitor] plot. All inhibitor concentrations were assayed in triplicate. I_{50} values were determined by linear regression analysis of narrow titrations in which a minimum of two points were above 50% inhibition and two points were below 50% inhibition. Reported I_{50} values are the average of two such narrow titrations.

Octanol/water partition coefficients were calculated by the FRAGMENT method of Hansch and Leo [27, 28]. The partition coefficient for OTFP was measured [29, 30] at room temperature in cyclohexane, using an HP-5890A gas chromatograph with a DB-17 megabore column (F & W Scientific, Folsom, CA) and an electron capturing detector. Log *P* value for the octanol/water solvent system was then calculated using a solvent regression equation from the literature [30, 31].

RESULTS

Gel filtration purification of soluble NTE. Crude microsomal membrane fractions were solubilized with Triton X-100 and fractionated on a Superose 12 gel filtration column. The column had been equilibrated in a buffer containing 0.5 M NaCl, 0.02% Triton X-100 and 0.02% asolectin. Aliquots were removed from fractions and analyzed for total, paraoxon-resistant, mipafox-resistant and paraoxon + mipafox-resistant phenyl valerate hydrolase activity (Fig. 1A). Results indicate that these activities were resolved into three peaks which differed in their organophosphate sensitivities. Peak I (fractions 15–22) contained the paraoxon-resistant, but paraoxon + mipafox-sensitive activity known as neuropathy target esterase (NTE) (Fig. 1B). Peak II (fractions 21–25) contained both a mipafox-resistant, paraoxon-sensitive activity and a paraoxon + mipafox-resistant activity. Peak III (fractions 27–31) contained a paraoxon-sensitive and mipafox-sensitive activity.

The NTE peak (Peak I) eluted in the excluded volume of the column, indicating an apparent molecular weight of greater than 1,500,000. Fractions 15–20 were combined and determined to have a specific activity of 0.212 I.U. of NTE/mg protein. This is a 2.7-fold purification over the starting fraction, and a 71% recovery of NTE activity. Similar results were obtained with a Sephacryl S300 column (1.5 × 100 cm, 10 mL/hr, 4°, sample vol. 1.8 mL). When an identical sample was eluted in a buffer which did not contain asolectin, NTE eluted as a symmetrical peak with an apparent molecular weight of 850,000 (results not shown).

In an attempt to eliminate the paraoxon-sensitive activity (Peak II) which overlaps with NTE, membrane fractions were pretreated with paraoxon. These membranes were then solubilized with Triton X-100 and fractionated on a Superose 12 column (Fig. 2). This process of treating the membranes with paraoxon successfully removed the paraoxon-sensitive activity in Peak II. Unexpectedly, the paraoxon-sensitive and mipafox-sensitive activity in Peak III was not eliminated by this treatment. After washing paraoxon-treated membranes to remove nonbound paraoxon, this peak of paraoxon-sensitive activity was still present in the activity profile. When this fractionation was performed using membranes treated simultaneously with both paraoxon and mipafox, Peak III was still present (data not shown). Preliminary experiments, however, appear to indicate that the degree of reactivation was not equivalent to that seen when membranes were treated with paraoxon alone.

Fractions 16–21 containing the NTE activity were combined and assayed. In the three preparations used in the TKF inhibition experiments, paraoxon + mipafox-resistant activity represented an average of 4.5% (SD_{n-1} = 1.3%) of the total activity. This compares to 17% paraoxon + mipafox-resistant activity in brain homogenates. These combined fractions were stored as 1-mL aliquots in

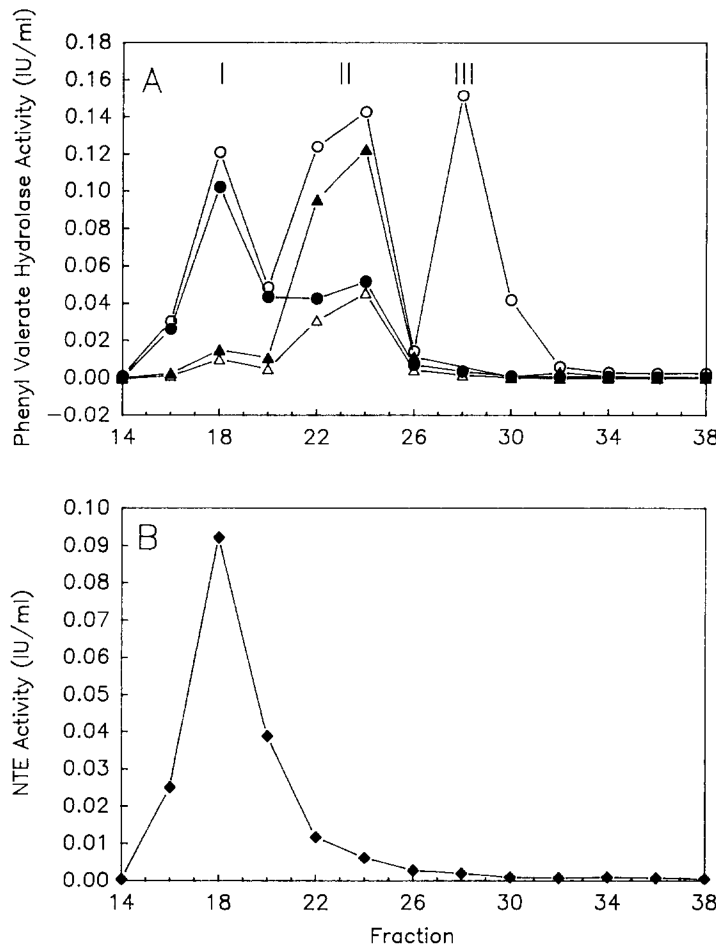


Fig. 1. Gel filtration of Triton X-100/NaCl solubilized phenyl valerate hydrolases from a microsomal preparation of chick embryo brain. Proteins were solubilized and fractionated on a Superose 12 column using a Pharmacia FPLC system as described in Materials and Methods. (A) Individual aliquots from each fraction were incubated with either buffer (○), paraoxon (●), mipafox (▲), or paraoxon + mipafox (△) prior to addition of substrate. Peaks have been labeled I, II and III to correspond with text. (B) NTE activity is plotted as the difference between paraoxon-resistant and paraoxon + mipafox-resistant activities.

liquid nitrogen. No significant loss of activity was observed in samples stored up to 13 months.

Velocity vs substrate concentration. The relationship of velocity to substrate concentration was analyzed using fractions 15–20 from the gel filtration fractionation profiled in Fig. 1. The maximum velocity obtained using substrate concentrations up to 10 mM was 1.1×10^{-3} I.U. V_{max} appeared to be reached at substrate concentrations between 1 and 1.5 mM which is in the region of maximum substrate solubility. A Lineweaver–Burk reciprocal plot of eight substrate concentrations from 0.2 to 0.9 mM indicated that the K_m of phenyl valerate for NTE was 5.33 mM ($r = 0.999$) and the V_{max} was 5.1×10^{-3} I.U.

Time-dependent incubation with trifluoroketones. Superose 12 fractions 16–21 obtained from paraoxon-pretreated membranes were incubated with 7×10^{-8} M 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) for 0, 5, 10 and 20 min prior to addition of substrate, and hydrolysis was stopped after 30 min. The activity of samples was inhibited an average of 44% at this inhibitor concentration regardless of the length of incubation prior to addition of substrate.

In a related experiment, a sample was incubated with 2.5×10^{-7} M OTPF at 37° for 6 min (sample D in Table 1). This sample was then diluted 25-fold and assayed for phenyl valerate hydrolysis at a final inhibitor concentration of 1×10^{-8} M OTFP. Control samples, which did not contain inhibitor, were incubated and then diluted as above. These were assayed in the presence of either 2.5×10^{-7} M OTFP (sample B) or 1×10^{-8} OTFP (sample C). Upon 25-fold dilution in assay buffer, sample D, which had been incubated at 2.5×10^{-7} M OTFP, behaved as if it were being inhibited at the post-dilution concentration of 1×10^{-8} M (Table 1). The same results were achieved when samples were incubated at 4° for 20 hr. In an additional experiment, it was observed that the inhibition of samples with 1×10^{-3} M OTFP could be reversed rapidly and completely by gel filtration on a desalting column.

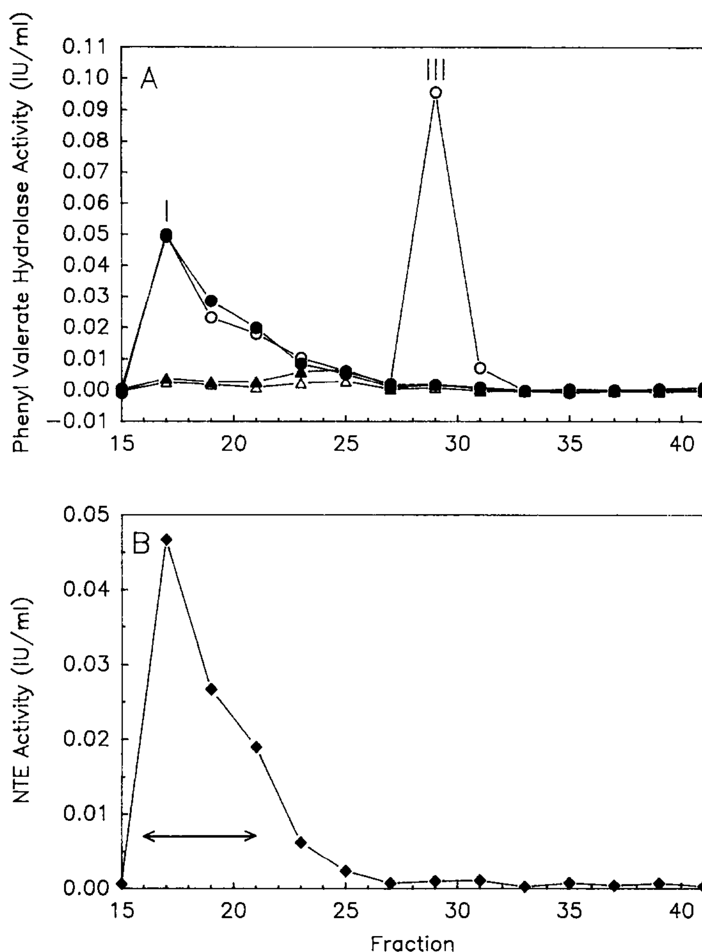


Fig. 2. Gel filtration of Triton X-100/NaCl solubilized phenyl valerate hydrolases from a paraoxon-treated membrane fraction. Membrane fraction from chick embryo brains was treated with paraoxon prior to treatment with Triton/NaCl extraction buffer. Methods and symbols are as described in Fig. 1. The arrowed line in panel B indicates the region of the NTE peak which was combined and used to screen inhibitors.

Table 1. Reversible inhibition of neuropathy target esterase by 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP)

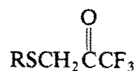
	OTFP concn (M)		NTE activity (%)	
Sample	Incubation	Assay	6 min	20 hr
A	0	0	100	100
B	0	2.5×10^{-7}	16.7	19.8
C	0	1.0×10^{-8}	72.2	73.6
D	2.5×10^{-7}	1.0×10^{-8}	85.0	83.6

Identical samples of gel filtration purified NTE from paraoxon-treated membranes were incubated for either 6 min at 37° or 20 hr at 4°. In each case, sample D contained 2.5×10^{-7} M OTFP. Samples were then diluted 25-fold into assay buffer, and OTFP was added to samples B and C. Samples were incubated at 37° for 7 min prior to addition of substrate. One hundred percent NTE activity at 6 min was 0.027 I.U./mL and at 20 hr was 0.026 I.U./mL. The volume of sample used in the assay was 30 μL.

Determination of I_{50} values. The inhibitory capacities of twenty-two 3-substituted thio-1,1,1-trifluoropropan-2-ones against NTE were determined (Table 2). These compounds fall into two groups containing either an *n*-alkylthio group (N = 4–12) or a substituted arylthio group. Titrations were performed by simultaneous addition of both sample and substrate to assay tubes containing appropriate concentrations of inhibitor. Assay tubes were incubated immediately for 30 min at 37°, followed by determination of released phenol. A broad titration curve was obtained for each inhibitor, followed by two narrow titrations in the linear region of the curve (Fig. 3). Reported I_{50} values are the average of the two I_{50} values obtained by linear regression of each narrow titration (Table 2).

Octanol/water partition coefficient. The octanol/water partition coefficients for the carbonyl form of each TFK were calculated and are presented as log *P* (Table 2). In addition log *P* was calculated for several carboxylesterase substrates including phenyl valerate

Table 2. Logarithmic octanol/water partition coefficients and inhibitory potencies for 3-substituted thio-1,1,1-trifluoropropan-2-ones



Inhibitor No.	Compound (R-)	log P	I_{50} (M) (slope)*
1	butyl-	1.54	7.42×10^{-6} (47)
2	hexyl-	2.62	2.87×10^{-7} (50)
3	heptyl-	3.16	9.50×10^{-8} (53)
4	octyl-	3.70	5.88×10^{-8} (56)
5	decyl-	4.78	7.15×10^{-8} (52)
6	undecyl-	5.32	1.09×10^{-7} (45)
7	dodecyl-	5.86	9.64×10^{-8} (51)
8	cyclohexyl-	2.31	1.59×10^{-6} (56)
9	benzyl-	1.59	3.72×10^{-6} (51)
10	phenyl-	1.49	2.37×10^{-6} (55)
11	2-chlorophenyl-	2.20	2.96×10^{-6} (57)
12	3-chlorophenyl-	2.20	1.62×10^{-7} (49)
13	4-chlorophenyl-	2.20	2.01×10^{-6} (53)
14	3,4-dichlorophenyl-	2.91	1.76×10^{-7} (55)
15	2,6-dichlorophenyl-	2.91	1.26×10^{-4} (63)
16	2,5-dichlorophenyl-	2.91	6.80×10^{-7} (52)
17	2-methoxyphenyl-	1.47	4.30×10^{-5} (50)
18	3-methoxyphenyl-	1.47	8.28×10^{-7} (54)
19	2-bromophenyl-	2.35	1.25×10^{-5} (61)
20	3-methyl-4-bromophenyl-	3.01	1.97×10^{-7} (57)
21	3-trifluoromethylphenyl-	2.37	2.90×10^{-7} (51)
22	4-*tert*-butylphenyl-	3.38	4.93×10^{-8} (53)
	phenyl valerate	3.11	
	phenyl 2-(octylthio)acetate	4.07	

* I_{50} values were determined by linear regression of titration curves generated as described in the legend of Fig. 3. Reported values are the average of two determinations.

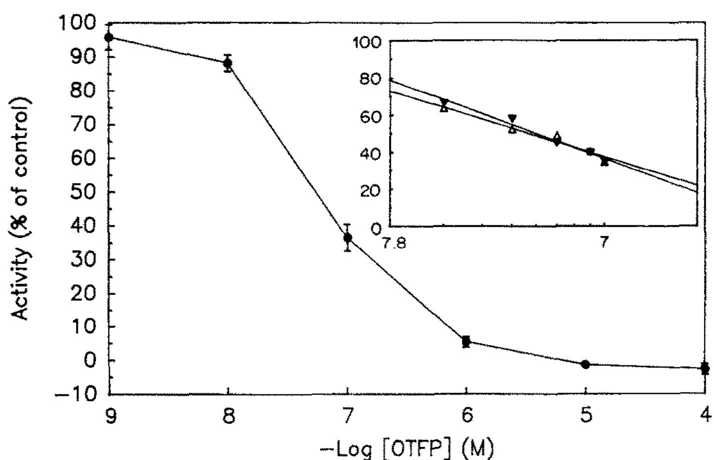


Fig. 3. Titration of paraoxon-pretreated, Superose 12 purified NTE with OTFP. A broad titration was performed (full figure) to determine the linear range of the curve. One hundred percent activity is 0.029 I.U./mL. Two separate titrations were performed in this range (inset; 100% activity is 0.032 I.U./mL). A separate regression line is plotted for each titration (inset). Error bars on the broad titration represent ± one standard deviation (N-1). Full figure labels also apply to inset figure.

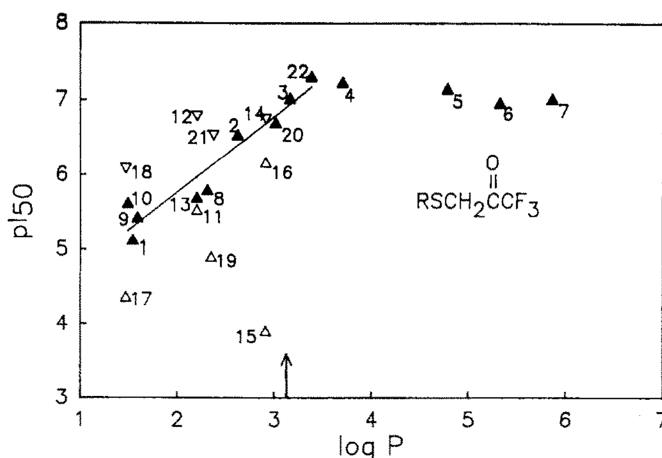


Fig. 4. Relationship of inhibitory potency to lipophilicity (pI_{50} vs log *P*). The pI_{50} and log *P* values of twenty-two 3-substituted thio-1,1,1-trifluoropropan-2-ones were determined as described in Materials and Methods. Substituents were either *n*-alkyl or substituted phenyl groups. Compounds containing a phenyl ring mono- or di-substituted in the *ortho* position have been plotted with open triangles (△), while those substituted with an electron withdrawing group in the *meta* position have been plotted with open inverted triangles (▽). Those compounds which are not included in the above two groups were plotted as filled triangles (▲). A regression line has been plotted for the members of this last group found in the range of log *P* between 1.5 and 3.4. An arrow indicates the log *P* value of phenyl valerate. The number next to each symbol corresponds to the Inhibitor No. in Table 2.

and phenyl octylthioacetate (Table 2). The relationship between lipophilicity and inhibitory potency is presented as pI_{50} vs log *P* (Fig. 4).

The partition coefficient for the distribution between an organic and an aqueous phase was measured for OTFP. The octanol/water partition coefficient could not be measured directly, due to the formation of a hemiacetal with octanol. Therefore, the partition coefficient was measured in cyclohexane and was calculated for octanol, using the solvent regression equation [30, 31]

$$\log K_{o/w} = 0.941 \log K_{c/w} + 0.69$$

where partition coefficients are $K_{o/w}$ for the octanol/water and $K_{c/w}$ for the cyclohexane/water systems. Log $K_{c/w}$ was measured to be 2.21 ± 0.11, and log $K_{o/w}$ was calculated to be 2.77 ± 0.09. This value is 0.93 less than the log *P* value calculated by the FRAGMENT method of Hansch and Leo (Table 2) [27, 28].

DISCUSSION

The relationship between the structural and physicochemical characteristics of twenty-two aliphatic and aromatic 3-substituted-1,1,1-trifluoropropan-2-ones and their inhibitory potencies toward NTE was investigated. These compounds, which resemble the transition state intermediate of many inhibitors of serine active site hydrolases, were shown to be very effective inhibitors of NTE. The two best inhibitors were identified as 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) and 3-[4-*tert*-butyl] - phenylthio - 1,1,1 - trifluoropropan - 2 - one, which have I_{50} values of 5.88×10^{-8} M and 4.93×10^{-8} M respectively. By comparison, the I_{50} values for inhibition of juvenile hormone esterase (*Trichoplusia ni*), acetyl cholinesterase (electric eel), trypsin (bovine) and chymotrypsin (bovine) by OTFP are 2.3×10^{-9} M, 2.3×10^{-6} M, and $>1 \times 10^{-5}$ M, and $>1 \times 10^{-4}$ M [15]. Johnson reported that the organophosphate sensitivity of NTE was very similar to that of α-chymotrypsin and trypsin, but dissimilar to that of acetyl cholinesterase [11]. In contrast, these results indicate that trifluoromethyl ketones are very good inhibitors of esterases such as NTE, juvenile hormone esterase and acetyl cholinesterase, but are poor inhibitors of peptidases.

The rapidly reversible inhibition of NTE by these TFKs indicates that they are concentration-dependent, competitive inhibitors of NTE. This is similar to the kinetics of inhibition of acetyl cholinesterase by these compounds but contrasts with the kinetics of juvenile hormone esterase inhibition which show slow tight binding [32, 33]. Juvenile hormone esterase requires a period of incubation with inhibitor prior to addition of substrate. No time-dependent inhibition of NTE by these trifluoroketones was observed, so I_{50} titrations were performed by simultaneous addition of sample and substrate to assay tubes containing appropriate concentrations of inhibitor. The failure to observe time-dependent inhibition of NTE may be partially due to the low occupancy of catalytic sites by phenyl valerate. The high K_m (1–10 mM) and low solubility (1.5 mM) of phenyl valerate may result in less than 50% saturation of NTE catalytic sites [18, 19].

To characterize more fully the interaction of NTE with the TFKs, the inhibitory potency of each TFK against NTE was correlated to several hydrophobic parameters, including the octanol/water partition coefficient of the molecules, molar refractivity, and π lipophilicity substituent constants of the alkyl or

the aryl substituent group on the sulfur. Since these parameters are highly colinear by nature (cross correlation = 0.87), the one with the highest correlation to the pI_{50} values, in this case the octanol/water partition coefficient (P), is discussed.

Figure 4 presents the relationship between the pI_{50} and the $\log P$ values of each TFK. In the range of lower lipophilicities ($\log P = 1.5$ to 3.5), the inhibition of NTE appears to be a linearly increasing function of lipophilicity on the double-logarithmic plot. A regression line for appropriate compounds within this range is plotted and defined by the equation $pI_{50} = 1.014 \log P + 3.74$ ($r = 0.958$). Compounds which appeared to have steric or electronic contributions (discussed later) to their inhibitory capacity were not included in this regression analysis. In the higher $\log P$ zone ($\log P = 3.5$ to 5.5), inhibition does not increase further with $\log P$. This saturation at higher lipophilicity is also observed with inhibition of juvenile hormone esterase by TFKs [15, 20].

The above results should provide useful information for the synthesis of more specific and sensitive substrates and inhibitors for NTE. Analysis of the relationship between pI_{50} and $\log P$ indicates that the optimal compromise between lipophilicity and solubility of NTE substrates and inhibitors is likely to be found in the range of $\log P$ between 3.0 and 3.4. This is consistent with the fact that phenyl valerate, which is more rapidly hydrolyzed and more selective for NTE than phenyl butyrate and phenyl caproate, has a calculated $\log P$ of 3.11. Phenyl butyrate and phenyl caproate, which differ from phenyl valerate by only one methylene group, have $\log P$ values outside this range (2.45 and 3.79 respectively). When phenyl 2-(octylthio)acetate, which has a $\log P$ value of 4.07, was tested as a hydrolase substrate, it too was found to be a sensitive and selective substrate of NTE [12]. Our results indicate that phenyl 2-(heptylthio)acetate, which has a $\log P$ value of 3.41, may be a more selective substrate of NTE than phenyl 2-(octylthio)acetate.

Two additional trends appear upon analysis of these TFKs. First, substitution of the phenyl group at the *ortho* position reduced the inhibitory potency of the compound. This was probably due to steric hindrance. Second, substitution of the phenyl ring at the *meta* position with an electron withdrawing group enhanced inhibitory potency compared to substitution at the *para* position. This may indicate an electronic interaction between the enzyme and substituents in the *meta* position. Unfortunately, the number of different compounds included in these groups was insufficient to determine a quantitative structure–activity relationship for steric and electronic parameters. With further study, some of the trifluoromethyl ketones may be of value as diagnostic inhibitors for NTE activity. They offer the advantages of high inhibitory activity and ease of synthesis. In addition, studies in both mice [15] and chickens (unpublished) indicate that these compounds have very low toxicity and are non-neuropathic.

It has been demonstrated [34] that fluoromethyl ketones predominantly exist in their hydrated form, as geminal diols. However, it was only possible to use $\log P$ fragment constants to calculate the $\log P$ values for the carbonyl forms. As such, the calculated $\log P$ value for OTFP (3.70) and the measured $\log P$ value for OTFP (2.77) differed by almost one order of magnitude. Since the hydration/dehydration equilibrium is unlikely to be the rate-limiting step in the enzyme inhibition [35], the form which is actually involved in the inhibition of the enzyme should be used to calculate $\log P$. The transition state mimic theory [36, 37] assumes that the geminal diol form, which is tetrahedral in geometry, is responsible for inhibition. In this case the difference between the calculated and measured $\log P$ values of OTFP (0.93) can be used as a general correction factor for all TFKs. This correction would result in the structure–activity relationship curve (Fig. 4) being shifted down by about one unit along the $\log P$ axis. It should be noted, however, that due to the electronic parameters of substituents on the sulfur, the equilibrium between the carbonyl and geminal diol forms varies among the different compounds [38]. In contrast to the transition state mimic theory, it has been suggested that in the lipophilic microenvironment of the enzyme surface, the geminal diol form may undergo a dehydration [32]. Inhibition would then occur in an addition reaction between the enzyme and the carbonyl form of the inhibitor. This is consistent with recent evidence indicating that acetylcholinesterase is inhibited by the carbonyl form of trifluoromethyl ketones [39].

NTE is one of four classes of phenyl valerate hydrolases found in brain tissue. These classes are distinguished by their differential sensitivities to the organophosphates mipafox and paraoxon. NTE is identified as the difference in activity between paired samples, one of which was incubated with paraoxon while the other was incubated with paraoxon and mipafox. However, the presence of paraoxon and mipafox in this differential assay can interfere with the determination of the sensitivity of NTE to other inhibitors. There are now several lines of evidence that paraoxon can competitively decrease the rate of phosphorylation of NTE and other proteins by mipafox [24, 40], our unpublished results). It is reasonable to assume that paraoxon can have this effect on other inhibitors, and should be removed prior to inhibitor titration of NTE. However, Figs. 1 and 2 clearly show that when nonbound paraoxon was removed prior to gel filtration, the paraoxon- or mipafox-sensitive activity in Peak III was reactivated. Experiments with membranes that were treated simultaneously with paraoxon and mipafox indicate that when nonbound paraoxon and mipafox are removed, this esterase may not be fully reactivated. If there is a differential reactivation of the activity in Peak III, then the difference between titrations of paraoxon and paraoxon + mipafox-treated membranes does not yield a titration curve for NTE solely.

We have not provided evidence that the presence or removal of paraoxon and mipafox prior to titration of NTE yields substantially different I_{50} values. However, the potential drawbacks to these methods bring into question their suitability for the type of characterization pursued in this study. To avoid this issue, a method for isolating an essentially pure NTE-type phenyl valerate hydrolase fraction was sought.

Previous chromatographic fractionation of detergent solubilized fractions has indicated that the different types of phenyl valerate hydrolases are separable [21, 41, 42]. Sucrose gradient centrifugation succeeded in separating NTE from the majority of other phenyl valerate hydrolases [21, 24], but yields of active enzyme were very poor and there was a loss of specific activity. Gel filtration chromatography has resulted in better yields and increases in specific activity, but the phenyl valerate hydrolase activities are poorly resolved ([41], our unpublished results). In the present study, we have improved the ability of gel filtration to completely resolve NTE by selectively reconstituting it into vesicles during fractionation. This was achieved by including asolectin in the elution buffer and by decreasing the concentration of detergent in the buffer. Without asolectin present, NTE migrated as a single peak with an apparent molecular weight of 850,000. This is consistent with the previously reported value of 880,000 for 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) solubilized NTE [41]. Upon inclusion of asolectin, NTE migrated in the void volume of the column ($M_r > 1.5 \times 10^6$). This significantly reduced the overlap between Peak I, containing NTE, and Peak II, containing paraoxon +mipafox-resistant activity (Fig. 1).

A final consideration regarding the use of this gel filtration purified NTE fraction is the possibility that detergent solubilization may have altered the characteristics of the enzyme [43]. Davis and Richardson [3] have reported that solubilization of NTE with Triton X-100 does not alter the sensitivity of NTE for mipafox. In addition, we have determined that the K_m of this NTE preparation for phenyl valerate is 5.3 mM. Previous investigations using intact membranes have reported K_m values of 1 and 10 mM [18, 19]. These results confirm that Triton X-100 solubilized NTE has the same characteristics as membrane associated NTE.

A method has been described for the rapid, high yield preparation of an NTE fraction which is suitable for study without the use of differential inhibition protocols. This eliminates the drawbacks inherent in previously used methods and results in considerable savings of time and sample. In addition, this fraction can be stably stored in liquid nitrogen, so that numerous inhibitors can be tested against the same preparation.

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