ELSEVIER

Biochimica et Biophysica Acta 1250 (1995) 171-182

BBA Biochimica et Biophysica Acta

Development of surrogate substrates for neuropathy target esterase

Babak Borhan [a,b], Ying Ko [b], Chris Mackay [c], Barry W. Wilson [c], Mark J. Kurth [b], Bruce D. Hammock [a,*]

[a] Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616, USA
[b] Department of Chemistry, University of California, Davis, CA 95616, USA
[c] Department of Avian Sciences, University of California, Davis, CA 95616, USA

Received 21 November 1994; revised 17 February 1995; accepted 21 March 1995

Abstract

Seventeen substrates were synthesized and their activities as surrogate substrates for Neuropathy Target Esterase were tested. Substrates investigated are carbon analogs of phenylvalerate (**1**) with oxygen and sulfur substituted at the α, β and γ positions. Phenol and thiophenol esters of these analogs constitute two series of compounds tested. The ratio of catalytic hydrolysis to background hydrolysis increased at lower pH values with all substrates tested including phenylvalerate (**1**). There was more than a 2.5-fold increase in specific activity with phenylthiopropylethanoate (**6**) at pH of 6.75 compared to phenylvalerate (**1**). Furthermore, a 19-fold decrease in K_m is reported with compound **6**. This and related compounds can be used as the basis of more sensitive assays for neuropathy target esterase. Thiophenyl esters in this series are sufficiently good substrates to hold promise in continuous assays.

Keywords: Neuropathy target esterase; Esterase; Surrogate substrate; Colorimetric substrate

1. Introduction

Insecticidal organophosphate compounds (OPs) cause inhibition of acetylcholinesterase which leads to a build up of acetylcholine and an over-stimulation of cholinergic systems. Intake of certain organophosphorus esters, however, can cause a variety of delayed neurological symptoms where the actual poisoning is preceded by approximately a two-week delay period. Organophosphate-induced delayed neuropathy (OPIDN) is a rare toxic syndrome associated with the irreversible inhibition of Neuropathy Target Esterase (NTE). NTE is an integral membrane protein localized in central and peripheral nerve tissue [1–4]. The physiological substrate for NTE is not known, and its catalytic activity does not seem to be vital to the short term function of neurons. Yet, modification of NTE by OPIDN-causing OPs through covalent binding is associated with an irreversible polyneuropathy [5–9].

Since NTE is not yet available in pure form, activity studies of NTE in vitro presently are accomplished through differential assays developed by Johnson [10,11]. NTE, in crude preparations, also suffers from a low rate of catalysis which further complicates its analysis. We set out to improve the NTE assay by synthesizing analogs of phenylvalerate (the most common surrogate substrate for NTE assay). In our laboratories, we have observed a common theme among substrates for different esterases, ranging from insect juvenile hormone esterase (JHE) to mammalian liver carboxyl esterases. The specific activity of these esterases on substrates in some cases dramatically increased by substituting heteroatoms in the place of carbon in the β or γ positions of the alkyl group attached to the ester (McCutchen et al. [12], Tien L. Huang and Takahiro Shiotsuki, unpublished data). Here in, we report the synthesis of several such analogs of phenylvalerate and their specific activities.

2. Materials and methods

2.1. Chemicals

5,5′-Dithionitrobenzoic acid (DTNB), porcine liver carboxylesterase (Type I, 19 mg protein/ml, 200 U/mg protein), bovine serum albumin (Type V), butyrylcholinesterase (from horse serum, 1000 U/mg protein),

* Fax: +1 (916) 7521537.

0167-4838/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0167-4838(95)00058-5

p-Nitrophenylacetate and 1-naphthylacetate were purchased from Sigma (St. Louis, MO). BCA reagent for protein determination was obtained from Pierce (Rockford, IL). All other chemicals were purchased from Aldrich (Milwaukee, WI). Mipafox was synthesized as previously described by Ishikawa et al. [13]. Solvents used were reagent grade which were freshly distilled before use.

2.2. Analytical procedures

^{1}H-NMR spectra were obtained on a QE-300 MHz spectrometer (General Electric) with deuterated chloroform ($CDCl_3$) as solvent and tetramethylsilane as the internal standard. The purity of compounds were demonstrated by thin-layer chromatography, ^{1}H-NMR, and capillary GC.

2.3. Synthesis of substrates

Substrates **2–17** were synthesized for use in this study. Butyl carbonates of phenol and thiophenol (**4,11**) were prepared by acylation with butylchloroformate according to published procedures [14–16]. Thiobutylphenylcarbonate (**7**) was synthesized by the acylation of butanethiol with phenylchloroformate. Thiophenylvalerate (S-(phenyl) thiopentanoate, **8**) was prepared with the reaction of thiophenol with valeryl chloride in pyridine with catalytic 4-(N,N-dimethylamino)pyridine (DMAP) [17]. Phenylcaproate (phenylhexanoate, **15**) was prepared similarly with phenol and hexanoyl chloride.

2.4. Phenyl-3-ethoxypropanoate (2) and S-(phenyl) 3-ethoxythiopropanoate (9)

3-Ethoxy-1-propanol (2.26 g, 16.7 mmol) was dissolved in acetone (35 ml) and freshly prepared Jones reagent [18] (35 ml) was added to the solution. The reaction was stirred at room temperature for 6 h at which time the solution was extracted with ether (3×25 ml), and the combined organic fraction was washed with 2% H_2SO_4 (3×25 ml) followed by saturated NaCl (acidified) and dried over anhydrous $MgSO_4$. Solvent was removed under reduced pressure yielding 3-ethoxypropanoic acid in sufficiently pure state for further synthesis (1.95 g, 76.2% yield).

2.5. Preparation of phenol ester (2)

3-Ethoxypropanoic acid (900 mg, 7.6 mmol) prepared by the latter procedure was dissolved in CH_2Cl_2 (20 ml). DMAP (186 mg, 1.5 mmol), phenol (717 mg, 7.6 mmol) and dicyclohexylcarbodiimide (DCC, 1.73 g, 8.38 mmol) were added to the solution at room temperature. The reaction was stirred for 16 h, after which it was quenched by addition of 2% H_2SO_4. Hexane was added to facilitate the precipitation of the dicyclohexylurea side product. The reaction mixture was filtered through a bed of celite, picked up with hexane (30 ml) and washed with 2% H_2SO_4 (2×20 ml), 10% $NaHCO_3$ (2×20 ml), saturated NaCl and was finally dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure to yield the product as a yellow oil. Product (2) was purified by prep TLC plate which was developed with 20% ethylacetate in hexane (1.28 g, 86.5% yield, $R_f = 0.52$). ^{1}H-NMR ($CDCl_3$): δ 1.23 (3H,t), 2.82 (2H, t), 3.56 (2H, q), 3.81 (2H, t), 7.09 (2H, d), 7.22 (1H, t), 7.37 (2H, t).

2.6. Preparation of thiophenol ester (9)

3-Ethoxypropanoic acid (700 mg, 5.9 mmol) was dissolved in CH_2Cl_2 (20 ml). DMAP (145 mg, 1.2 mmol), thiophenol (653 mg, 5.9 mmol) and DCC (1.35 g, 6.52 mmol) were added to the solution at room temperature. The reaction was stirred for 16 h, after which it was quenched by addition of 2% H_2SO_4. Hexane was added to facilitate the precipitation of the dicyclohexylurea side product. The reaction mixture was filtered through a bed of celite, picked up with hexane (30 ml) and washed with 2% H_2SO_4 (2×20 ml), 10% $NaHCO_3$ (2×20 ml), saturated NaCl and was finally dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure to yield the product as a yellow oil. Product (**9**) was purified by prep TLC plate which was developed with 5% acetone in hexane (0.98 g, 78.6% yield, $R_f = 0.27$). ^{1}H-NMR ($CDCl_3$): δ 1.22 (3H, t), 2.95 (2H, t), 3.53 (2H, q), 3.78 (2H, t), 7.41 (5H, s).

2.7. Phenyl-3-thioethylpropanoate (5) and S-(phenyl) 3-thioethylthiopropanoate (12)

NaH (60%, 4.54 g, 114 mmol) was washed with hexane (3×10 ml) and suspended in DMF. 3-Mercaptopropanoic acid (5.48 g, 51.6 mmol) was added to the latter solution (dropwise) and stirred for 30 minutes until H_2 evolution had ceased. Bromoethane was then dropwise added to the suspension and additional DMF was introduced to help in the mixing of the solution. Reaction was quenched after 20 h with the addition of H_2O. The solution was acidified to pH of 1 with 12 M HCl and then was extracted with ether (3×50 ml). The combined organic was washed with 2% H_2SO_4 (3×30 ml), acidified saturated NaCl (50 ml) and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure to yield a yellow oil (3-thioethylpropanoic acid) which was sufficiently pure for further reactions (6.80 g, 93.8% yield).

2.8. Preparation of phenol ester (5)

The phenolic ester of 3-thioethylpropanoic acid was synthesized as previously described. The resulting product (**5**) was purified by prep TLC, which was developed with 3% ethylacetate in CH_2Cl_2 (85% yield, $R_f = 0.67$). ^{1}H-NMR ($CDCl_3$): δ 1.28 (3H, t), 2.59 (2H, q), 2.87 (4H, m), 7.10 (2H, d), 7.21 (1H, t), 7.38 (2H, t).

*2.9. Preparation of thiophenol ester (**12**)*

The thiophenolic ester of 3-thioethylpropanoic acid was synthesized as previously described. The resulting product (**12**) was purified by prep TLC which was developed with 4% acetone in hexane (71% yield, $R_f = 0.23$). ^{1}H NMR ($CDCl_3$): δ 1.13 (3H, t), 2.58 (2H, q), 2.89 (4H, m), 7.41 (5H, s).

*2.10. Phenylpropoxyethanoate (**3**) and S-(phenyl) propoxythioethanoate (**10**)*

NaH (60%, 5.28 g, 132 mmol) was washed with hexane (3 × 10 ml) and was suspended in DMF (40 ml). 1-Propanol (3.61 g, 60 mmol) was added to the suspension, dropwise and stirred at room temperature for 20 min. Iodoacetic acid (11.71 g, 63 mmol) dissolved in THF (20 ml) was slowly introduced to the reaction mixture. Reaction was quenched after 20 h by addition of H_2O, acidified to pH of 1 with 12 M HCl and extracted with ether (3 × 50 ml). The combined organic were washed with 2% H_2SO_4 (3 × 30 ml), acidified saturated NaCl (50 ml) and dried over anhydrous Na_2SO_4. The resulting yellow oil (propoxyethanoic acid) was sufficiently pure for subsequent reactions (4.53 g, 64% yield).

*2.11. Preparation of phenol ester (**3**)*

The phenolic ester of propoxyethanoic acid was synthesized as previously described. The resulting product (**3**) was purified by prep TLC which was developed with 20% ethylacetate in hexane (79% yield, $R_f = 0.45$). ^{1}H-NMR ($CDCl_3$): δ 0.97 (3H, t), 1.68 (2H, sx), 3.59 (2H, t), 4.31 (2H, s), 7.11 (2H, d), 7.22 (1H, t), 7.37 (2H, t).

*2.12. Preparation of thiophenol ester (**10**)*

The thiophenolic ester of propoxyethanoic acid was synthesized as previously described. The resulting product (**10**) was purified by prep TLC which was developed with 5% acetone in hexane (87% yield, $R_f = 0.27$). ^{1}H-NMR ($CDCl_3$): δ 1.05 (3H, t), 1.75 (2H, sx), 3.62 (2H, t), 4.24, (2H, s), 7.41 (5H, s).

*2.13. Phenylthiopropylethanoate (**6**) and S-(phenyl) thiopropylthioethanoate (**13**)*

Thiopropylethanoic acid was synthesized as previously discussed for the preparation of propoxyethanoic acid which was used in the synthesis of **3** and **10** (thioether synthesis with 1-propanethiol and iodoacetic acid). 1-Thiopropylethanoic acid was isolated as a dark yellow oil sufficiently pure for further synthesis (70% yield).

*2.14. Preparation of phenol ester (**6**)*

The phenolic ester of thiopropylethanoic acid was synthesized as previously described. The resulting product (**6**) was purified by prep TLC which was developed with 3% ethylacetate in CH_2Cl_2 (89% yield, $R_f = 0.67$). ^{1}H-NMR ($CDCl_3$): δ 1.03 (3H, t), 1.69 (2H, sx), 2.72 (2H, t), 3.41 (2H, s), 7.10 (2H, d), 7.21 (2H, t), 7.38 (2H, t).

*2.15. Preparation of thiophenol ester (**13**)*

The thiophenolic ester of thiopropylethanoic acid was synthesized as previously described. The resulting product (**13**) was purified by prep TLC which was developed with 4% acetone in hexane (90% yield, $R_f = 0.31$). ^{1}H-NMR ($CDCl_3$): δ 10.3 (3H, t), 1.67 (2H, sx), 2.66 (2H, t), 3.49 (2H, s), 7.41 (5H, s).

*2.16. Phenylthioethylethanoate (**14**)*

Phenylthioethylethanoate was synthesized and purified in the exact manner by which phenylthiopropylethanoate (**6**) was prepared. The only difference was that ethanethiol was used with iodoacetic acid to synthesize thioethylethanoic acid. Overall yield of the reaction was 16% (possibly due to the higher water solubility of the intermediate carboxylic acid leading to 14). ^{1}H-NMR ($CDCl_3$): δ 1.33 (3H, t), 2.76 (2H, q), 3.44 (2H, s), 7.11 (2H, d), 7.24 (1H, t), 7.38 (2H, t).

*2.17. Phenyl-3-hydroxythiopropylethanoate (**16**)*

Preparation of **16** involved a multistep synthesis. Ethylmercaptoethanoate (**16a**) derived from mercaptoethanoic acid was converted to methyl-3-hydroxythiopropylethanoate (**16b**) which upon saponification to 3-hydroxythiopropylethanoic acid (**16c**) was protected with *t*-butyldimethylsilyl chloride (TBDMSCl) to yield 3-*t*-butyldimethylsilyloxythiopropylethanoic acid (**16d**). **16d** was subsequently esterified with phenol to give phenyl-3-*t*-butyldimethylsilyloxythiopropylethanoate (**16e**) and after deprotection of the hydroxyl yielded the final product, **16**.

*2.18. Ethylmercaptoethanoate (**16a**)*

Concentrated H_2SO_4 (catalytic, 5 drops) was added to a stirred solution of mercaptoethanoic acid (16.3 g, 178 mmol) in ethanol (300 ml, anhydrous). The solution was refluxed overnight and was neutralized with saturated $NaHCO_3$. Most of the ethanol was removed under reduced pressure. The residue was extracted with ether (3 × 50 ml). The combined ether extracts were washed with saturated NaCl and dried over anhydrous Na_2SO_4. Evaporation of solvent under reduced pressure yielded 6.30 g (30%) of **16a** as an oily liquid. ^{1}H-NMR ($CDCl_3$): δ 1.26 (3H, t), 2.02 (1H, t), 3.26 (2H, d), 4.20 (2H, q).

*2.19. Methyl-3-hydroxythiopropylethanoate (**16b**)*

To a stirred solution of **16a** (6.30 g, 52.5 mmol) in methanol (200 ml), $NaOCH_3$ (25% solution in methanol,

52.5 mmol, 12 ml) was slowly added at room temperature. After 20 minutes, 3-bromopropanol (6.57 g, 47.3 mmol) was added. The solution was stirred for 2 days at room temperature. The reaction mixture changed from pale pink to murky yellowish-brown color. Most of the methanol was evaporated under reduced pressure. Saturated NaCl (50 ml) was then added to the residue before extracting it with ether (3 × 50 ml). The combined extracts were washed with saturated NaCl and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 5.04 g (59%) **16b** as a pale yellow oily liquid. ^{1}H-NMR ($CDCl_3$): δ 1.93 (2H, p), 2.76 (2H, t), 3.26 (2H, s), 3.73 (3H, s), 3.75 (2H, t).

2.20. *3-Hydroxythiopropylethanoic acid (**16c**)*

Compound **16b** (4.16 g, 30.7 mmol) was refluxed in a solution of 1:1 THF:1% H_2SO_4 (250 ml) overnight. THF was removed from the solution under reduced pressure. The residue was extracted with ether (3 × 50 ml). The combined extracts was washed with saturated NaCl and dried over anhydrous Na_2SO_4. Evaporation of solvent under reduced pressure yielded 1.29 g (34%) of **16c** as a thick yellowish oily liquid. ^{1}H-NMR ($CDCl_3$): δ 1.86 (2H, p), 2.75 (2H, t), 3.23 (2H, s), 3.76 (2H, t), 5.48 (2H's exchangeable).

2.21. *3-t-Butyldimethylsilyloxythiopropylethanoic acid (**16d**)*

To a stirred solution of **16c** (1.24 g, 8.27 mmol) in pyridine (50 ml), *t*-butyldimethylsilyl chloride (2.75 g, 18.2 mmol) and DMAP (50 mg, 0.413 mmol) were added. The solution was stirred overnight at room temperature. Reaction was quenched with H_2O. The solution was extracted with CH_2Cl_2 (3 × 50 ml) and the combined organic were washed with 1% H_2SO_4 (3 × 30 ml), 4% $NaHCO_3$ (50 ml), saturated NaCl and was dried over anhydrous Na_2SO_4. Evaporation of solvent under reduced pressure yielded 1.31 g (60%) of **16d** as a yellowish oily liquid. ^{1}H-NMR ($CDCl_3$): δ 0.05 (6H, s), 0.88 (9H, s), 1.81 (2H, p), 2.74 (2H, t), 3.25 (2H, s), 3.69 (2H, t), 5.05 (1H, s).

2.22. *Phenyl-3-t-butyldimethylsilyloxythiopropylethanoate (**16e**)*

To a stirred solution of **16d** (628 mg, 2.38 mmol) in dry CH_2Cl_2 (25 ml), DMAP (59 mg, 0.476 mmol), phenol (224 mg, 2.38 mmol) and DCC (550 mg, 2.62 mmol) were added. The solution was stirred at room temperature for 16 h. Reaction was quenched with 1% H_2SO_4 and hexane. The solution was filtered through a bed of celite and the organic portion of the filtrate was washed with 1% H_2SO_4 (30 ml), 4% $NaHCO_3$ (30 ml), saturated NaCl and was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure. Residue obtained was redissolved in hexane in order to precipitate all of the dicyclohexylurea side product and was again filtered with celite. Evaporation under reduced pressure yielded 0.428 g (53%) of **16e** as a yellowish oily liquid. ^{1}H-NMR ($CDCl_3$); δ 0.05 (6H, s), 0.88 (9H, s), 1.86 (2H, p), 2.82 (2H, t), 3.43 (2H, s), 3.72 (2H, t), 7.11 (2H, d), 7.24 (1H, t), 7.38 (2H, t).

2.23. *Phenyl-3-hydroxythiopropylethanoate (**16**)*

To a stirred solution of **16e** (315 mg, 0.0926 mmol) in CH_3CN (25 ml), 40 drops of HF/pyridine was added. The solution was stirred for 20 h at room temperature at which time H_2O was added to the reaction mixture. After extraction with ether (3 × 20 ml), the combined extracts was washed with 1% H_2SO_4 (20 ml), 4% $NaHCO_3$ (20 ml), saturated NaCl and was dried over anhydrous Na_2SO_4. Evaporation of solvent under reduced pressure yielded **16** as a yellowish oily liquid. The product was purified by prep TLC which was developed with 20% ethylacetate in hexane (0.242 g, 91.0% yield). ^{1}H-NMR ($CDCl_3$): δ 1.63 (1H, s), 1.92 (2H, p), 2.86 (2H,t), 3.45 (2H, s), 3.76 (2H, t), 7.11 (2H, d), 7.24 (1H, t), 7.38 (2H, t).

2.24. *p-Nitrophenylthiopropylethanoate (**17**)*

Procedures were practically identical to synthesis of any of the phenolic esters. Thiopropylethanoic acid (refer to synthesis of **6**, 126 mg, 0.939 mmol), DMAP (24 mg, 0.188 mmol), DCC (217 mg, 1.033 mmol), and *p*-nitrophenol (131 mg, 0.939 mmol) were used. Compound **17** (249 mg, 100% yield) was isolated as a yellowish oily liquid. ^{1}H-NMR ($CDCl_3$): δ 1.03 (3H, t), 1.70 (2H, sx), 2.72 (2H, t), 3.45 (2H, s), 7.32 (2H, d), 8.25 (2H, d).

2.25. *Enzyme preparation*

The procedure used for the isolation of chicken embryo NTE was previously described [19]. Embryos from 18–19 day old chicken eggs were removed and the brain cut from the back of the cranium. Tissue was homogenized with 5 ml/g of isolation buffer (50 mM Tris, 0.5 M NaCl, 2 mM EDTA, 0.5 mM DTT, pH 7.2) on ice with Polytron setting at 7 for 20 s. The homogenate was centrifuged at 11 000 × *g* for 20 min (4° C), supernatant removed and centrifuged at 110 000 × *g* for 60 min (4° C). The pellet contained most of the NTE activity, which was resuspended in appropriate buffer. Enzyme preparation used for determination of NTE specific activity with different substrates and determination of K_m was kept on ice at all times and was used before freezing and subsequent thawing.

2.26. *NTE Enzyme assays*

Enzymatic assays were performed as described previously [11,20,21]. Typically, 10 μl samples of appropriate

dilution of enzyme (ranging from to 2 up to 24-fold dilution based on activity of sample and sensitivity of substrate) were incubated with addition of inhibitors in a volume of buffer (50 mM Tris, 0.2 mM EDTA, pH 6.75 or pH 8.00) at 37° C for 20 min to yield a final volume of 735 μl. The inhibitor solutions were as follows: (a) 100 μl of the latter buffer (non-inhibited sample); (b) 50 μl of 0.6 mM paraoxon in 50 mM Tris-citrate buffer (pH 6.0); (c) 50 μl of 0.6 mM paraoxon in 50 mM Tris-citrate buffer (pH 6.0) and 50 μl of 0.75 mM mipafox in 50 mM Tris-citrate buffer (pH 6.0). A fourth sample which did not contain enzyme was also prepared with both inhibitors present as a blank for non-enzymatic chemical hydrolysis measurement of substrates. Ten microliters of a 66.7 mM solution of substrates in DMF was added and the tubes were incubated for an additional 20 minutes at 37° C. Enzymatic hydrolysis was quenched by addition of 500 μl of 1% (w/v) SDS, 0.025% (w/v) aminoantipyrine, 50 mM Tris-HCl, 0.2 mM EDTA, pH 8.0 (stop solution). The color was developed by addition of 250 μl 0.4% $K_3Fe(CN)_6$ (w/v) in water to each test tube. The absorbance of each sample was determined in triplicate by placing 300 μl sample of each test tube in a well of a 96-well microtiter plate. Absorbences were measured with a V_{max} ™ plate reader (Dynatech, Virginia, VA) at 490 nm. Phenolic concentration was determined based on a previously determined extinction coefficient of 16812 $M^{-1}cm^{-1}$ [19]. Enzymatic assays involving the release of thiophenol were essentially identical in procedure. The only difference was in using 250 μl solution of DTNB (0.06%, w/v, in water) instead of $K_3Fe(CN)_6$ for colorimetric development [22]. Standard curve of a wide range of thiophenol concentration in final enzymatic assay mixture provided the extinction coefficient to be 12400 $M^{-1}cm^{-1}$. For each enzyme concentration an incubation of the enzyme with $K_3Fe(CN)_6$ or DTNB and other reagents but without substrate was used to insure that significant absorbance did not occur from reaction of phenols and thiols in the enzyme solution.

At least three replicates of each assay were run each day. For the comparison of relative rates of enzymatic hydrolysis, the rate of hydrolysis of each compound was compared to that of phenylvalerate run at the same time and on the same preparation. Also, the rate of each compound was compared to others hydrolyzed at a similar rate run on the same day with the same enzyme preparation. The relative rates are based on at least four separate runs with at least four separate enzyme preparations. The comparison of phenylvalerate (phenylpentanoate, **1**) and phenylthiopropylethanoate (**6**) was run with at least four separate preparations. For each assay used to support the substrate comparison in Table 3, the rates of hydrolysis were shown to be linear with time and protein concentration.

The NTE enzyme preparation was made with a widely accepted protocol and the phenylvalerate difference assay is the most widely used method for determining NTE. However, as this preparation is used to compare rate of enzymatic hydrolysis with different substrates, it must be remembered that a given substrate could be hydrolyzed by a different population of esterases in the NTE preparation even though this population would escape inhibition by paraoxon and be inhibited by mipafox.

2.27. *pH optimization of NTE with phenylvalerate (1)*

NTE enzymatic assays were performed as usual with varying assay buffer pH values ranging from 6.00 to 8.50. In order to obtain full color development, the pH of the stop solution was changed to 9.00 for samples with assay buffer pH values below 6.5.

2.28. *Substrate activity with other esterases*

Porcine liver carboxylesterase (PlC), mouse liver microsomal carboxylesterase (MLC), human liver microsomal carboxylesterase (HLC) and butyrylcholinesterase (BuChE) were diluted in phosphate buffer (100 mM, pH 7.4) to 0.475 μg/ml, 0.71 μg/ml, 37.8 μg/ml and 0.9 μg/ml, respectively. The activity of phenolics esters were measured as was done with NTE, with the exception of the 20 minute inhibitor incubation and reducing the enzyme/substrate incubation period to five minutes. The final substrate concentration was 0.2 mM. For the thiophenol series, the phosphate buffer was doped with DTNB (0.075% w/v). In a typical assay, 298 μl of enzyme/reagent solution were added to wells of a 96 well ELISA plate. Enzyme assays were initiated by the addition of 2 μl of substrate to achieve a final concentration of 0.2 mM. Hydrolytic rates were monitored at 23° C for at least 2 min. Final concentration of *p*-nitrophenylacetate and 1-naphthylacetate were 0.5 mM. The rates of hydrolysis in Table 4 were shown to be linear with time and protein concentration.

2.29. *K_m determination of **1** and **6***

K_m for compounds **1** and **6** were obtained under steady state conditions using six substrate concentrations ($1.0 \cdot 10^{-5}$ to $5 \cdot 10^{-3}$ M). Assay conditions for kinetic measurements were identical to ones described for NTE specific activity assay. Substrate incubation times were changed to 10 min for samples with concentration below $5 \cdot 10^{-5}$ M.

3. Results and discussion

Heteroatoms such as nitrogen, oxygen and sulfur, substituted in the β and γ position of alkyl groups attached to esters, can greatly influence hydrolytic activity. We examined the effect of such substitutions on the activity of NTE by synthesizing heteroatom substituted analogs of phenyl-

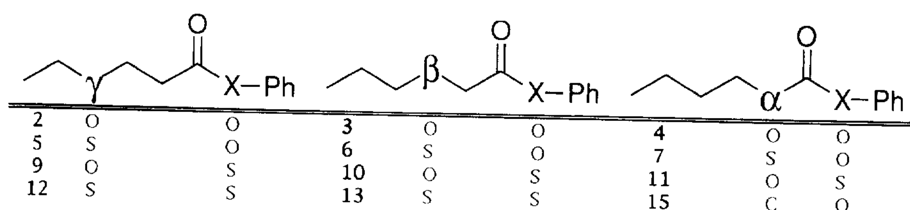

Fig. 1. Substrates synthesized for this study consist of all sulfur and oxygen permutations possible in the α, β, γ and X positions (except for α = S, X = S).

valerate (**1**). Initially, the number of carbon atoms in the alkyl chain was considered. Carbon analogs of phenylvalerate (**1**) are defined as substrates synthesized with the same number of carbon atoms with the same carbon skeleton as **1**. Atom length analogs of phenylvalerate (**1**) are defined as substrates with identical number of atoms as phenylvalerate (**1**). Table 1 clearly demonstrate the relationship between carbon analogs and length analogs by comparison of their alkyl chain length. Carbon analogs of phenylvalerate (**1**), such as **6**, would be one atom longer than phenylvalerate. Atom length analogs of phenylvalerate (**1**), such as **14**, are one carbon shorter, however, with the addition of the heteroatom within the alkyl chain they will have the same number of atoms as phenylvalerate (**1**) and phenylcaproate (**15**). Considering the latter results and the fact that the chemical yield of synthesizing the shorter analog was lower, the carbon analogs of phenylvalerate (**1**) were synthesized. Table 1 illustrates the approximate length of the alkyl chain measured from the carbonyl carbon to the end of the chain by MM2 calculation for a selected set of substrates.

The choice of which series to synthesize (carbon analogs versus length analogs) was determined by comparing specific activities of **1**, **6**, **15** and **16**. As Table 2 shows substrate **6**, the carbon analog of **1**, proved to be a better substrate than **15**, the length analog. There was not a large difference in specific activity between phenylvalerate (**1**) were synthesized.

Fig. 1 depicts the arrangement of heteroatoms within the substrates prepared for this study. Compounds **2–7** were synthesized to investigate the effect of heteroatom substitution within the alkyl chain. Substrates **8–13** were also synthesized with the same idea in mind, however, they differ only in the leaving group. The thiophenol leaving group was incorporated for three reasons. Firstly, thiol leaving groups can react with DTNB, a spectrochemical indicator, which allows for real time kinetic measurement of hydrolytic activity. Even though phenyl ester hydrolysis can be followed continuously at 270 nm, usually the high background and low sensitivity makes it impractical. Also, the use of DTNB allows for visible spectrophotometric measurements with 96 well plate ELISA readers. Secondly, there is no need for stop buffers and colorizing solutions to be added as done presently to perform NTE assays with phenylvalerate (**1**). Thirdly, thioesters could be used for histochemical staining of biological tissues for NTE localization studies.

Preliminary data on NTE hydrolysis of **1–16**, utilizing Johnson's standard NTE assay protocol was not satisfactory due to the high non-enzymatic hydrolysis of some of

Table 1
Interatomic distance of selected NTE substrates from the carbonyl carbon to the end of the chain (Å)

Substrate	Carbonyl carbon → end of chain
$CH_3(CH_2)_3C(O)OPh$ (**1**)	4.97 Å
$CH_3(CH_2)_4C(O)OPh$ (**15**)	6.27 Å
$CH_3(CH_2)_2OCH_2C(O)OPh$ (**3**)	6.06 Å
$CH_3(CH_2)_2SCH_2C(O)OPh$ (**6**)	6.55 Å
$CH_3CH_2SCH_2C(O)OPh$ (**14**)	5.30 Å

Table 2
Specific activity comparison of carbon analogs of **1** vs. length analogs of **1**

Substrate	Relative specific activity (%)	Yield in chemical synthesis (%)
$CH_3(CH_2)_3C(O)OPh$ (**1**)	100	—
$CH_3(CH_2)_4C(O)OPh$ (**15**)	77	89
$CH_3(CH_2)_2SCH_2C(O)OPh$ (**6**)	343	65
$CH_3CH_2SCH_2C(O)OPh$ (**14**)	220	16

Assay conditions are described under materials and methods. Activity of each substrate was determined using a final substrate concentration of 0.895 mM. Percent relative specific activity refers to the activity of each substrate compared to phenylvalerate (**1**). Data were determined from at least four independent replicates from at least two enzyme preparations.

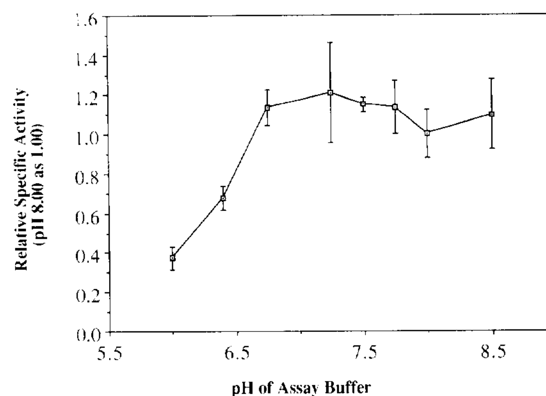

Fig. 3. pH profile of NTE assayed with phenylvalerate (**1**). NTE activity was monitored using a pH range of 6.00 to 8.50.

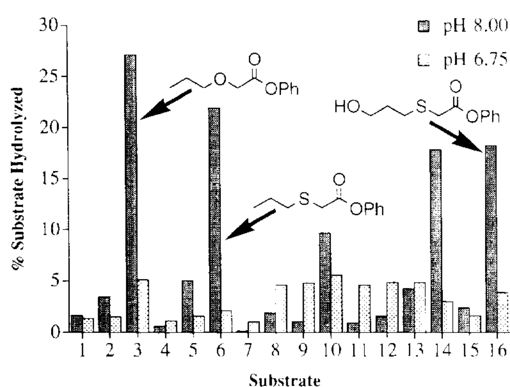

Fig. 2. Non-enzymatic hydrolysis of phenyl and thiophenol esters at a pH of 8.00 and 6.75. The non-enzymatic hydrolysis of β-substituted esters were greatly reduced at pH of 6.75. Hydrolysis assays were performed without enzyme, but otherwise exactly as enzyme assays described in (see section 2). Percent hydrolysis refers to the actual amount of substrate hydrolyzed with in the 20 minute incubation of substrate in assay buffer. The hydrolytic rate of **17** was very high and the data were not reproducible.

the new substrates at pH of 8.00 (Fig. 2). The β-substituted heteroatom substrates were most sensitive to pH of 8.00. For instance with in the 20 minute incubation period of substrates in the enzyme assay buffer close to 27% of substrate **3** and 22% of substrate **6** were hydrolyzed non-enzymatically. This hydrolysis did not preclude collection of meaningful data at pH of 8.00, but the high back ground hydrolysis reduced the possible advantages offered by improved substrates. At pH of 6.75, the non-enzymatic hydrolysis of the same substrates was reduced to below 5%. This led us to look into the possibility of lowering the non-enzymatic hydrolysis by lowering the pH of the assay buffer.

A pH profile study of NTE, utilizing phenylvalerate (**1**) as the diagnostic substrate, was performed to determine the lowest possible pH at which no significant NTE activity was lost. Result of the pH study with phenylvalerate (**1**) are shown in Fig. 3. Activity of NTE seems to vary little with in the pH range of 6.75 to 8.00. Our results agree well

Table 3
Specific activity (μmol/min per mg protein)

No.	Substrate	Total enzyme (non-inhibited)	Paraoxon inhibited	Paraoxon and Mipafox inhibited	NTE Activity	Selectivity (%)
1	$CH_3(CH_2)_3C(O)OPh$	0.165 ± 0.007	0.145 ± 0.003	0.090 ± 0.004	0.055 ± 0.005	37.9
2	$CH_3CH_2O(CH_2)_2C(O)OPh$	0.21 ± 0.002	0.016 ± 0.001	0.010 ± 0.001	0.006 ± 0.002	37.5
3	$CH_3(CH_2)_2OCH_2C(O)OPh$	0.134 ± 0.020	0.112 ± 0.006	0.085 ± 0.009	0.027 ± 0.011	24.1
4	$CH_3(CH_2)_3OC(O)OPh$	0.050 ± 0.007	0.049 ± 0.001	0.034 ± 0.009	0.015 ± 0.009	30.6
5	$CH_3CH_2S(CH_2)_2C(O)OPh$	0.078 ± 0.002	0.076 ± 0.004	0.053 ± 0.002	0.023 ± 0.005	30.3
6	$CH_3(CH_2)_2SCH_2C(O)OPh$	0.474 ± 0.020	0.466 ± 0.012	0.309 ± 0.016	0.157 ± 0.020	33.7
7	$CH_3(CH_2)_3SC(O)OPh$	0.006 ± 0.001	0.006 ± 0.001	0.005 ± 0.004	0.001 ± 0.004	16.7
8	$CH_3(CH_2)_3C(O)SPh$	0.020 ± 0.002	0.022 ± 0.001	0.019 ± 0.001	0.003 ± 0.002	13.6
9	$CH_3CH_2O(CH_2)_2C(O)SPh$	0.018 ± 0.001	0.020 ± 0.002	0.015 ± 0.001	0.005 ± 0.002	25.0
10	$CH_3(CH_2)_2OCH_2C(O)SPh$	0.104 ± 0.004	0.102 ± 0.004	0.079 ± 0.007	0.023 ± 0.008	22.5
11	$CH_3(CH_2)_3OC(O)SPh$	0.003 ± 0.001	0.004 ± 0.002	0.003 ± 0.003	0.001 ± 0.004	25.0
12	$CH_3CH_2S(CH_2)_2C(O)SPh$	0.045 ± 0.002	0.043 ± 0.001	0.034 ± 0.002	0.009 ± 0.002	20.9
13	$CH_3(CH_2)_2SCH_2C(O)SPh$	0.105 ± 0.002	0.099 ± 0.001	0.076 ± 0.002	0.023 ± 0.002	23.2

Assay conditions are described in (see section 2). Activity of each substrate was determined using a final substrate concentration of 0.895 mM. Final concentration of paraoxon and mipafox were 40 μM and 50 μM, respectively. Data were determined from at least four independent replicates from at least three enzyme preparations. % Selectivity refers to the ratio of NTE activity to the paraoxon inhibited activity.

with Johnson's pH profile study [4]. The only concern with lowering the pH of the assay buffer was whether or not the color development at the end of the assay would be effected. A solution of phenol (40 μM final concentration) at various assay buffer pH's were developed spectroscopically, and it was found that as long as the final pH of the solution (after addition of stop solution and colorizing reagent) was above 7.50 that no significant color loss due to pH was observed. It is also possible to increase the pH of the stop solution in order to compensate for the lowering of assay buffer pH to achieve the full color development. Thus, even with the standard phenylvalerate (**1**) based NTE assay the ratio of the enzymatic to back ground hydrolysis can be increased with no apparent loss of specificity by reducing the pH of the assay.

Table 3 lists data accumulated from NTE hydrolysis of substrates **1–13** at pH of 6.75. Paraoxon is considered to inhibit most of the non-NTE esteratic activity. As can be seen in the table, little of the total enzyme activity (non-inhibited) is inhibited by paraoxon, indicating small general esterase contamination in the enzymatic preparations used in this study. It is generally accepted that NTE activity is defined as the mipafox sensitive esteratic portion of the total enzyme activity. Specific activity of each substrate for NTE was calculated as the difference in activity of paraoxon and the paraoxon/mipafox inhibited enzyme fractions. Fig. 4 represents NTE specific activity of substrates **1–16** with respect to phenylvalerate (**1**) at pH of 6.75. The activity of substrates are represented as percent specific activity with respect to the specific activity of phenylvalerate (taken to be 100%). This method was adopted, since different batches of NTE exhibited slightly different specific activities. All of the experiments with different substrates were performed along with phenylvalerate (**1**) in order to normalize the data.

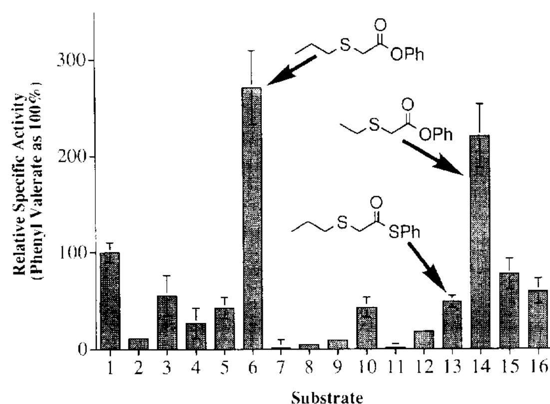

Fig. 4. Catalytic activity on substrates **2–16** expressed as percent specific activity with respect to specific activity of phenylvalerate (taken to be 100%). For these studies the specific activity of NTE with phenylvalerate (**1**) was 0.055 ± 0.005. Enzymatic assays were performed at pH 6.75 and the activities are the difference in activity of paraoxon/mipafox inhibited and paraoxon inhibited fractions.

Fig. 4 clearly demonstrates that phenylthiopropylethanoate (**6**), which has a sulfur substituted in the β-position of the alkyl group, has a much higher specific activity compared to the rest of the substrates. Substrates **2** and **5** with the γ-substituted heteroatom exhibit reduced total enzyme activity and small NTE specific activity. Generally, within other groups of esterases, such a large differ-

Table 4
Specific activities for porcine liver carboxylesterase, solubilized mouse and human liver microsomal carboxylesterases and butyrylcholinesterase with phenylvalerate analogs

No.	Substrate	Specific activity (μmol/min per mg protein)			
		porcine	mouse	human	BuChE
1	$CH_3(CH_2)_3C(O)OPh$	209 ± 4.3	7.88 ± 0.36	10.5 ± 0.03	82.3 ± 5.3
2	$CH_3CH_2O(CH_2)_2C(O)OPh$	127 ± 5.1	2.20 ± 0.07	5.05 ± 0.16	8.9 ± 0.5
3	$CH_3(CH_2)_2OCH_2C(O)OPh$	171 ± 5.6	9.21 ± 0.07	8.10 ± 0.51	52.6 ± 2.0
4	$CH_3(CH_2)_3OC(O)OPh$	14.8 ± 3.4	1.02 ± 0.01	1.12 ± 0.04	9.9 ± 0.1
5	$CH_3CH_2S(CH_2)_2C(O)OPh$	286 ± 2.8	7.88 ± 0.17	12.3 ± 0.31	N.D. †
6	$CH_3(CH_2)_2SCH_2C(O)OPh$	299 ± 3.0	10.1 ± 0.3	10.2 ± 0.24	88.3 ± 5.9
7	$CH_3(CH_2)_3SC(O)OPh$	5.08 ± 1.3	N.H. ‡	0.23 ± 0.04	1.0 ± 0.1
8	$CH_3(CH_2)_3C(O)SPh$	121 ± 17	1.27 ± 0.11	6.82 ± 0.29	22.5 ± 2.6
9	$CH_3CH_2O(CH_2)_2C(O)SPh$	118 ± 4.6	0.36 ± 0.02	1.96 ± 0.15	3.7 ± 0.3
10	$CH_3(CH_2)_2OCH_2C(O)SPh$	369 ± 12	1.75 ± 0.09	9.81 ± 0.57	75.2 ± 4.3
11	$CH_3(CH_2)_3OC(O)SPh$	N.H. ‡	N.H. ‡	N.H. ‡	N.H. ‡
12	$CH_3CH_2S(CH_2)_2C(O)SPh$	366 ± 20	1.68 ± 0.15	10.3 ± 0.43	23.3 ± 1.5
13	$CH_3(CH_2)_2SCH_2C(O)SPh$	201 ± 15	1.35 ± 0.16	8.48 ± 0.16	57.0 ± 5.9
	p-Nitrophenylacetate	119 ± 7.5	2.25 ± 0.10	0.85 ± 0.04	12.4 ± 0.3
	1-Naphthylacetate	150 ± 1.3	3.56 ± 0.03	3.89 ± 0.2	120.9 ± 0.6

Assay conditions are described in (see section 2). Each value represents the mean ± S.D. of four independent trials using at least 3 independent enzyme preparations. Protein concentrations for porcine, mouse, and human carboxylesterases and butyrylcholinesterase were 0.475 μg/ml, 71 μg/ml, 37.8 mg/ml and 0.9 μg/ml, respectively. The final substrate concentrations were 0.5 mM for *p*-nitrophenylacetate and 1-naphthylacetate and 0.2 mM for the other substrates.

† N.D. indicates not determined.

‡ N.H. indicates no hydrolysis or less than 0.10 μmol/min per mg protein.

ence between the β-and γ-substituted compounds is not observed (Table 4). Possibly one could use a ratio of the hydrolytic activity of an enzyme preparation on compound **6** versus compound **5** as an effective indicator of the presence of NTE. For instance this ratio is 1.04 for porcine carboxylesterase but 6.80 for NTE.

Carbonate substrates are very slowly turned over by most esterase families. In most cases, this can be associated to their inhibitory action due to the slow rate of deacylation of the acylated enzyme. Carbonates were also slowly turned over with NTE as can be seen with the activity of **4** and **7**. Substrate **3**, the oxy analog of **6**, exhibits a larger non-enzymatic hydrolysis compared to **6** (Fig. 2), however, **6** is a much better enzymatic substrate. The latter observation suggests that the inductive or other chemical properties of the β-oxy analog makes it much more susceptible to chemical hydrolysis. However, this is not the driving force for hydrolysis within the enzyme, since the enzymatic hydrolysis of the β-thio analog is more than 5-fold higher. Considering that there are not any resonance contribution considerations from the β-position, the large difference between the β-thio and the β-oxy analogs seem to suggest that the electronic effects of the β-thio analog are not totally responsible for its increased activity. Chemically, the difference between oxygen and sulfur stems from the larger atomic size of sulfur. This size leads to a higher degree of polarizability, but it also means that sulfur is more hydrophobic than oxygen. Sulfur, therefore, can provide electron density with a small charge density. If strong polarity can not be tolerated within the alkyl chain binding site, but there are sites for electron stacking interactions, such as π-electron interaction of the substrate with aromatics, then one can speculate that oxygen is too polar and lacks the ability of lending its non-bonding electrons for stacking type interaction, but sulfur is not very polar and yet it could interact as a pseudo π-electron donor.

An interesting observation with substrate **6** is the high level of enzymatic hydrolysis it detects within the paraoxon/mipafox inhibited preparation. Even though the sample is severely inhibited with OPs, the level of its detection of the non-inhibited esterase(s) is more than 3-fold higher than phenylvalerate. Using Johnson's definition of NTE, compound **6** should offer improved detection over the more commonly used phenylvalerate (**1**). However, as shown in Table 3, there is no improvement in the NTE selectivity of substrate **6** or any other substrates as compared to phenylvalerate (% selectivity is defined as the ratio of NTE specific activity to the paraoxon inhibited specific activity [4]).

In comparison with the phenol esters (**1–7**), the thiophenol esters (**8–13**) are turned over more slowly with NTE. Thiophenylvalerate (**8**), the sulfur analog of phenylvalerate (**1**), exhibits a substantially reduced activity. Within the thiophenyl esters series, both the β-oxy and β-thio analogs **10** and **13** are improved substrates. The β-heteroatoms do increase the activity by **8**-fold. The difference between sulfur and oxygen in the β position is not evident in the thioester series. Once again one can speculate that thioesters are turned over more slowly than the oxygen esters and the increased affinity of each substrate for NTE is overshadowed by the decrease in the rate of hydrolysis. It should be noted that even though thioesters are chemically more labile (thiophenyl is a better leaving group compared to phenoxide), enzymatically this is not true. Compounds **9** and **12** (γ-substituted heteroatoms) are intermediate in activity, but are higher than thiophenylvalerate (**8**). This observation is unlike the ester series where phenylvalerate is considerably higher in activity compared to its γ-oxygen and γ-sulfur-substituted analogs. Thiocarbonate **11** is almost inactive, even with the non-inhibited enzyme mixture. In comparing compound **1** (phenylvalerate) versus its thioester analog compound **8**, one sees an unacceptable 18-fold decrease in rate of hydrolysis by NTE coupled with an increase in non-enzymatic hydrolysis. However, by inserting a heteroatom β to the carbonyl in compounds **10** and **13**, the rates increase almost **8**-fold to almost half of the rate observed with phenylvalerate (**1**). The ease of using DTNB based detection system and a continuous enzyme assay as commonly done with acetylthiocholine [23] or the ability to monitor NTE directly in whole cells or thin sections may make these compounds very useful NTE substrates in certain situations [24]. Interestingly in the thiophenyl ester series both the β-thio and β-oxy ethers are good substrates. The differences between the ester and thioester series might suggest two possible hydrolytic pathways for each series.

Two additional derivatives of **6** were synthesized. With the introduction of sulfur, the solubility of **6** in aqueous media decreased. A terminal hydroxyl analog of **6**, phenyl-3-hydroxythiopropylethanoate (**16**), was synthesized in order to increase its solubility in the assay buffer and also study the effect of increased polarity on the alkyl side chain of a good substrate. However, as Fig. 4 illustrates, by the introduction of the hydroxyl the activity of the substrate decreased to about 60% of phenylvalerate (**1**). The decrease in activity may be due to several factors. If one assumes that the NTE side-chain binding pocket to contain hydrophobic amino-acid residues (since most NTE substrates have hydrophobic tails) then by placing the hydroxyl group at the end of the chain, we have created a hydrophilic tail that may disrupt the binding of the substrate to NTE. Also, it is possible that the hydroxyl group on **16** is hydrated by water molecules in aqueous medium and severely distorts its shape compared to that of **6** or simply is too large for the catalytic site. However, the hydroxyl group provides a site for the attachment of a variety of groups such as photoaffinity labels which could be useful for the study of NTE and which at the same time could block the polarity.

It is interesting to see, however, that all the increase in activity gained by positioning the sulfur in the β-position

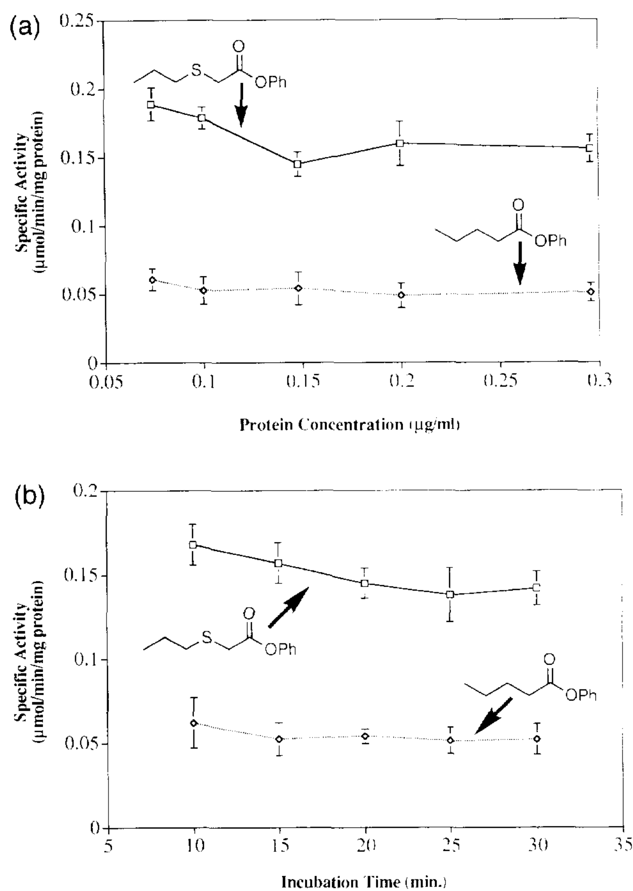

Fig. 5. (a) Specific activity of **1** (◇) and **6** (□) determined at various concentrations of the NTE preparation. (b) Specific activity of **1** (◇) and **6** (□) determined at various substrate incubation periods. All enzymatic assays were performed as described in Section 2.

is lost by increasing the polarity of the same side chain. Our speculation for substrate **3**'s lack of activity (the β-oxy analog of phenylvalerate), the possibility that its polarity is disruptive to the function of the enzyme, seems even more plausible.

p-Nitrophenylthiopropylethanoate (**17**), was the second derivative synthesized. Since the *p*-nitrophenolate anion generated in the hydrolysis of **17** is a spectrophotometric reagent by itself, **17** could be a useful substrate for studying kinetics in real time. It could be detected without the aid of stop solution and colorizing agents. Unfortunately, **17** had such a high tendency for non-enzymatic hydrolysis that reliable and reproducible data could not be obtained from the assays. Possibly more stable esters such as the ethyl ester would yield continuous assays with out a high non-enzymatic hydrolysis rate. Yet this substrate is structurally diverse enough from phenylvalerate (**1**) that we felt it could be misleading to use in crude preparations.

Substrate **6** proved to have the highest rate with NTE of all those synthesized. As Fig. 5 illustrates, the specific activity of **1** and **6** were essentially constant over a range of protein concentrations (0.074 μg/ml → 0.296 μg/ml final concentration) and times (10 min → 30 min). To further compare phenylthiopropylethanoate (**6**) with phenylvalerate (**1**) as substrate for NTE, the apparent K_m for each were determined. Fig. 6 illustrates the Lineweaver–Burk plots for **1** and **6**. K_m of **6** was determined to be 0.26 mM and the K_m of **1** was found to be 4.90 mM. Johnson reports the K_m for phenylvalerate (**1**) as 10 mM, about 2-times higher than our value. Although K_m is an intrinsic value for an enzyme and therefore should remain constant, it is important to remember that NTE is not isolated in pure form. Thus the discrepancy may be caused by purity differences in various NTE preparations. Comparisons of our experimental K_m's for **1** and **6** illustrates a 19-fold decrease. For esterases K_m is a complex term, but within one series of compounds the K_m is probably related to affinity of the enzyme for the substrate and/or reaction intermediates and transition states.

Substrates **1–13** were subjected to analysis with a variety of other esterases (Table 4). At first glance one can see that both the ester and thioester series are turned over quickly. The β-oxy and β-thio analogs have high specific activities. The carboxyl esterases, however, exhibit a high specific activity for the γ-substituted substrates. As observed before, the thio analogs are more active than their oxy counterparts for the β-and γ-substituted compounds, with the only exception being the high activity of compound **10** (β-oxythioester). However, there are some differences between each type of carboxyl esterase. Compound **10** (β-oxy thioester) and **12** (γ-thio thioester) exhibit the highest activity with porcine liver carboxyl esterase. Compound **3** and **6** (β-oxy and β-thio esters) are the most active substrates with mouse liver microsomal carboxyl esterase, while human liver microsomal carboxyl

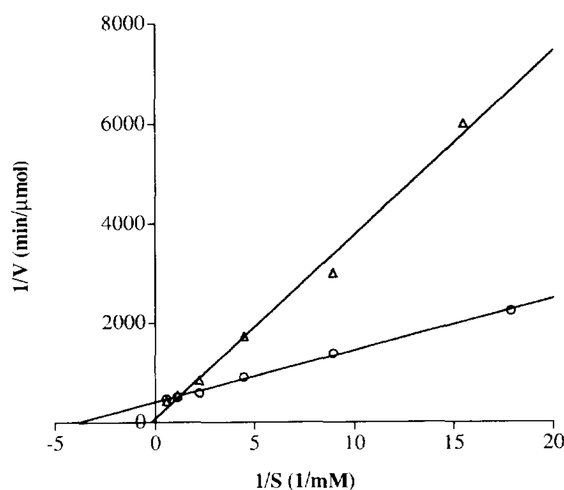

Fig. 6. Lineweaver–Burk plot of **1** △ and **6** ○ K_m of **6** (0.26 mM) was found to be 19-fold less than **1** (4.90 mM). The latter data was obtained by linear fits of the Lineweaver–Burk plots which compares well with the K_m obtained by fitting the data to the Michaelis–Menten equation by non-linear least-squares procedure (K_m of **6**: 0.25 ± 0.03 mM and K_m of **1**: 4.12 ± 0.21 mM).

esterase prefers compound **5** (γ-thio ester). *p*-Nitrophenylacetate and 1-naphthylacetate are the surrogate substrates most commonly used for carboxyl esterase assays. The specific activity of some the phenylvalerate analogs synthesized for this study are up to twofold higher than the latter diagnostic substrates and may be useful in differential detection of hepatic enzymes.

Since pure preparations are not available for NTE. we are defining the activity as originally described by Johnson [25] based on difference assays. Thus the quality of a substrate is based on a variety of factors. The key factor is the total activity detected and the size of the window between activity detected in the presence of paraoxon and in the presence of mipafox which currently in the field is defined as NTE activity. Phenylthiopropylethanoate (**6**) compares favorably to phenylvalerate (**1**) in having a higher total rate and a larger window of NTE activity. A second parameter is background hydrolysis. The background with phenylthiopropylethanoate (**6**) at a pH of 8.0 is higher than that of phenylvalerate (**1**). However, Johnson pointed out that the NTE assay can be run at a variety of pHs. With phenylvalerate (**1**) the activity is slightly greater at a pH of 6.75 to 7.5 than it is at 8.0. At a pH of 6.75, under the conditions described in this paper the signal to noise of the assay (the ratio of enzymatic hydrolysis to non-enzymatic hydrolysis) based on phenylthiopropylethanoate (**6**) is greater than that seen with phenylvalerate (**1**) at pH of 6.75 or 8.00. The signal-to-noise ratio with phenylthiopropylethanoate (**6**) can be improved further by using shorter incubation times and higher protein concentrations. An argument against using phenylthiopropylethanoate (**6**) to replace phenylvalerate (**1**) is that both substrates are surrogate substrates for an unknown endogenous substrate. NTE hydrolysis is simply associated with anatomical lesions and toxicology. The large amount of literature based on phenylvalerate (**1**) argues for its continued use for screening studies, even though phenylthiopropylethanoate (**6**) appears to be a better reagent based on Johnson's original definitions. A possible compromise is that phenylvalerate (**1**) could be used for screening and for monitoring initial solubilization in conjunction with phenylthiopropylethanoate (**6**) and then more sensitive phenylthiopropylethanoate (**6**) based assays could be used for more detailed monitoring of the enzyme activity during purification or physiological studies. A second caution is that conclusions from data generated with preparations from an avian species may not be applicable to NTE preparations from other organisms.

Another limitation of phenylvalerate (**1**) is the low sensitivity and labor involved in the development of the color from the release of phenol. *S*-(Phenyl)thiopropylthioethanoate (**13**) offers an advantage over phenylvalerate (**1**) regarding color development as do a variety of other substrates. Once one moves beyond the first few purification steps with NTE when the selectivity factor become less important, then it may be possible to use a variety of substrates offering less selectivity but far greater sensitivity and ease of use than either phenylvalerate (**1**) or phenylthiopropylethanoate (**6**) to follow purification of NTE. *S*-(Phenyl)thiopropylthioethanoate (**13**) was determined to be the best thiophenol substrate of this series. It is promising for both biochemical assays and histochemical staining.

In summary, phenylthiopropylethanoate (**6**), is found to be a more sensitive substrate for the NTE differential assay at pH of 6.75. Compared to phenylvalerate (**1**), **6** is more than 3-fold more sensitive and it exhibits a much reduced K_m value (19-fold).

Acknowledgements

This work was supported by NIEHS grant 2R01 ES02710-14. Y.K. is the recipient of the Donald Crosby summer research internship for undergraduates, awarded for research on NTE. M.J.K. is an NIH RCDA recipient (1989–1994; EC00182). U.C. Davis is an E.P.A. Center for Ecological Health Research (CR 819658-010) and NIEHS Health Sciences Center (P30 ES05707).

References

[1] Richardson, R.J., Davis, C.S., Johnson, M.K. (1979) J. Neurochem. 32, 607–615.
[2] Dudek, B.R. and Richardson, R.J. (1982) Biochem. Pharmacol. 31, 1117–1121.
[3] Davis, C.S. and Richardson, R.J. (1987) Biochem. Pharmacol. 36, 1393–1399.
[4] Johnson, M.K. (1982) Rev. Biochem. Toxicol. 4, 141–212.
[5] Bidstrup, P.L., Bonnell, J.A., Beckett, G. (1953) Br. Med. J. 1068–1072.
[6] Lotti, M., Becker, C.E., Aminoff, M.J. (1984) Neurology 34, 658–662.
[7] Capodicasa, E., Scapellato, M.L., Moretto, A., Caroldi, S., Lotti, M. (1991) Arch. Toxicol. 65, 150–155.
[8] Lotti, M., Caroldi, S., Capodicasa, E., Moretto, A. (1991) Tox. App. Pharm. 108, 234–241.
[9] Lotti, M. (1992) Toxicology 21, 465–488.
[10] Johnson, M.K. (1969) Biochem. J. 114, 711–717.
[11] Johnson, M.K. (1977) Arch. Toxicol. 37, 113–115.
[12] McCutchen, B.F., Uematsu, T., Szekacs, A., Huang, T.L., Shiotsuki, T., Lucas, A., Hammock, B.D. (1993) Arch. Biochem. Biophys. 307, 231–241.
[13] Ishikawa, Y., Chow, E., McNamee, M.G., McChesney, M., Wilson, B.W. (1983) Toxicol. Lett. 17, 315–320.
[14] Copeland, C. and Stick, R.V. (1984) Aust. J. Chem. 37, 1483–1487.
[15] Ashour, M.A., Harshman, L.G., Hammock, B.D. (1987) Pestic. Biochem. Physiol. 29, 97–111.
[16] Shobana, N., Amirthavalli, M., Deepa, V., Shanmugam, P. (1988) Ind. J. Chem. 27B, 965–966.
[17] Smith, G.G., Jones, D.A.K., Taylor, R. (1963) J. Org. Chem. 28, 3547–3550.
[18] Jones, E.R.H. (1953) J. Chem. Soc. 457–464.
[19] Thomas, T.C., Szekacsm, A., Rojas, S., Hammock, B.D., Wilson, B.W., McNamee, M.G. (1990) Biochem. Pharmcol. 40, 2587–2596.

[20] Soliman, S.A. and Curley, A. (1981) J. Anal. Toxicol. 5, 183–186.
[21] Thomas, T.C., Ishikawa, Y., McNamee, M.G., Wilson, B.W. (1989) Biochem. J. 257, 109–116.
[22] Riddles, P.W., Blakeley, R.L., Zerner, B. (1979) Anal. Biochem. 94, 75–81.
[23] Ellman, G.L., Courtney, K.D., Andres, V.J., Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95.
[24] Karnovsky, M.J. and Roots, L. (1964) J. Histochem. Cytochem. 12, 219–221.
[25] Johnson, M.K. (1975) Biochem. Pharmacol. 24, 797–805.