Synthesis and Study of Thiocarbonate Derivatives of Choline as Potential Inhibitors of Acetylcholinesterase

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Fourteen alkyl and aryl thiocarbonate derivatives of choline were synthesized and studied as potential inhibitors of acetylcholinesterase (AChE). Twelve of the compounds inhibited AChEs derived from calf forebrain, human red blood cells, and octopus brain ranging from low to moderately high inhibition potency. The concentration of each inhibitory compound giving 50% inhibition of enzyme activity (IC_{50} values, which ranged from 1×10^{-2} to 8×10^{-7} M) was determined and is reported; inhibitor constants (K_i values) for the most inhibitory compounds, (1-pentylthiocarbonyl)choline chloride and (1-heptylthiocarbonyl)choline chloride, were calculated from kinetic data and are also reported. The inhibitors are competitive with substrate, and they are not hydrolyzed by the AChE activities. Certain of these new compounds may provide direction for the development of new drugs that have anticholinesterase activity and may be used for the treatment of Alzheimer's disease.

Introduction

The involvement of cholinesterase in various pathological conditions of the nervous system, such as Alzheimer's disease (AD), has led to clinical trials of cholinomimetics, including cholinesterase inhibitors. AD is a neurodegenerative disorder characterized by a progressive deterioration of memory and learning; its invariant pathological feature is the increased number of neurite plaques and neurofibrillary tangles in the cerebral cortex, hippocampus, and amygdala.[1] The molecular mechanisms that underlie these phenomena are still obscure. Among the numerous neurochemical abnormalities described in AD, the decrease in the activity levels of choline acetyltransferase, the enzyme catalyzing the synthesis of acetylcholine, is the most prominent.[2] This reduction is commensurate with the severity of the disease and suggests the existence of selective cholinergic lesions.[3] Moreover, different groups of neurons, in which lesions occur in AD, were shown to share a common feature in the presence of acetylcholinesterase (AChE).[4−8] Thus, the cholinergic hypothesis of age-related dementia arose,[9] and it is consistent with a vast animal literature that supports a critical involvement of the brain cholinergic system in cognitive activity.[10]

Studies of anticholinesterase drugs in the treatment of central cholinergic disorders such as AD have been carried out using physostigmine (eserine) and tetrahydroaminoacridine (THA).[11,12] Although both compounds give some beneficial results, their relatively high toxicity and chemical instability have prompted searches for effective AChE inhibitors having lower toxicity, longer duration of action, and more specific binding with the brain AChE active site. Recently, new and potent bifunctional AChE inhibitors, alkylene-linked bis-THA analogs, have been approved by the U.S. Food and Drug Administration for testing for the palliative treatment of mild and moderate AD.[13] The compounds are proposed to bind with both the catalytic site, at the bottom of the enzymatic binding pocket (gorge), and the so-called peripheral binding site, at the opening of the pocket of AChE.[14]

The purpose of the research reported herein was to synthesize new and specific AChE inhibitors having low mammalian toxicity and high chemical stability, with the aim that these inhibitors might give direction to the synthesis of new and more effective drugs than are currently available for the treatment of AD. It was determined that the compounds to be synthesized would have a functionality similar to the ester group functionality of acetylcholine, that they would contain the choline moiety, and that they would also possess various hydrocarbon groups that could facilitate binding with the hydrophobic amino acid residues in the active site pocket of AChE.[15] Thus, a series of alkyl and aryl thiocarbonate derivatives of choline were synthesized. We found in previous studies that the thiocarbonate functionality, in selected chemical structures, provides effective competitive inhibitors of certain enzymes having hydrolase or thiohydrolase activity.[16−18] Finally, the structures selected, even if they served as weak substrates for AChE activity, should not exhibit any significant mammalian toxicity.

Experimental Section

General. ^{1}H NMR spectra were obtained on a Varian Gemini 200 NMR spectrometer in D_2O or $CDCl_3$, and the chemical shifts are in parts per million using tetramethylsilane as internal standard. Infrared spectra were obtained on a Perkin Elmer 1760X computer-driven FTIR spectrometer in acetonitrile or chloroform solutions. Spectrophotometric enzyme assays were conducted using a Shimadzu UV−vis spectrophotometer, model UV-1201. Thin layer chromatography was conducted using silica gel-precoated plates obtained from Eastman Kodak Co.; the developing solvent was methanol. Choline chloride and the thiol compounds used in this study were obtained from Aldrich Chemical Co. Phosgene (compressed gas, 99%) was obtained from Alphagaz, Edison, NJ. Acetylcholinesterase (AChE) from human red blood cells (RBC), acetylthiocholine chloride (ATC), procainamide, bacitracin, and aprotinin were purchased from Sigma Chemical

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Co. 5,5′-Dithiobis(2-nitrobenzoic acid), DTNB, was obtained from Merck (FRG). Affinity resin for AChE purifications was prepared by coupling procainamide to epoxy-activated Sepharose (Farmacia, Sweden) according to an established procedure.[19] All other reagents were analytical grade products from various sources, and all solutions were made up in twice-distilled water.

Organic Syntheses. Choline Chloride Chloroformate (1). Phosgene gas (fume hood!) was bubbled into 45 mL of cold, dry acetone, under minimal nitrogen flow, until approximately 15 g (0.15 M) of phosgene had been dissolved (about 30 min). The phosgene solution was then added under nitrogen to solid choline chloride (5.0 g, 35.8 mmol); the mixture was stirred in an ice bath for 1 h and then at room temperature for 16 h. Ethyl acetate/hexane (1:9, 50 mL) was added to the reaction mixture with stirring for an additional 10 min. The upper layer was decanted. The material in the lower layer was treated with 50 mL of ethyl acetate, and the precipitate obtained was filtered and dried under high vacuum to give the title compound as a white crystalline, deliquescent solid (7.1 g, 98%): silica gel TLC, single spot (iodine), R_f = 0.05 (chlorine chloride starting material R_f = 0.5); FTIR in acetonitrile, 1784.0 cm^{-1} (C=O strain), no O−H band characteristic of starting material; ^1H NMR in DMSO, 3.12 (9H, s), 3.42 (2H, m), 3.80 (2H, m).

Choline Chloride Thiocarbonates 2−15. To a stirred, cold slurry of sodium hydride (60 mg, 1.48 mmol, 60% oil emersion) in tetrahydrofuran (4 mL, freshly distilled), under nitrogen and in an ice bath, was added dropwise the appropriate thiol (1.48 mmol) via syringe. A reaction occurred instantly with the evolution of hydrogen gas. Stirring was continued for 30 min in an ice bath, and a white emulsion formed. To the latter was added, dropwise via syringe, a suspension of choline chloride chloroformate (0.30 g, 1.48 mmol) in dry acetonitrile (4 mL). Stirring was continued in an ice bath for 1 h and then at room temperature overnight. Chloroform (20 mL) was added, and the mixture stirred for 30 min. The reaction mixture was then filtered under nitrogen pressure. The retentate was washed with chloroform (2 × 5 mL), and the filtrate and washings were concentrated *in vacuo* (fume hood) to give a pale yellow oil. Under nitrogen, the oil was washed with dry hexanes (3 × 10 mL) and then precipitated two times from a chloroform solution (2 mL) using dry hexane (15 mL). The hexane-rich upper layers were removed by pipet. The chloroform-rich lower layer (suspension) was transferred to a vial and concentrated under high vacuum to give the desired product as a pale yellow, deliquescent solid which was stored in a nitrogen atmosphere at −20 °C. The yields ranged from 20% (**4**) to 84% (**2**).

Characterization Data for Choline Chloride Thiocarbonates 2−15. All the thiocarbonates listed below were synthesized according to the general method given above. All gave a single spot using silica gel TLC with R_f values of approximately 0.1 (compared to an R_f of 0.05 for the starting material, choline chloride chloroformate).

(Ethylthiocarbonyl)choline chloride (2): FTIR in chloroform, key peak 1670 cm^{-1} (C=O strain); ^1H NMR in D_2O, 1.30 (3H, t), 2.92 (2H, m), 3.20 (9H, s) 3.76 (2H, m), 4.7 (2H, m).

(1-Propylthiocarbonyl)choline chloride (3): FTIR in chloroform, key peak 1670 cm^{-1} (C=O strain); ^1H NMR in D_2O, 0.95 (3H, t), 1.67 (2H, m), 2.90 (2H, t), 3.20 (9H, s), 3.77 (2H, m), 4.70 (2H, m).

(1-Butylthiocarbonyl)choline chloride (4): FTIR in chloroform, key peak 1709.0 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 0.91 (3H, t), 1.39 (2H, m), 1.60 (2H, m) 2.87 (2H, t), 3.55 (9H, s), 4.20 (2H, m), 4.68 (2H, m).

(1-Pentylthiocarbonyl)choline chloride (5): FTIR in chloroform, key peak 1710.8 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 0.89 (3H, t), 1.34 (4H, m), 1.62 (2H, m), 2.87 (2H, t), 3.54 (9H, s), 4.20 (2H, m), 4.68 (2H, m).

(1-Hexylthiocarbonyl)choline chloride (6): FTIR in chloroform, key peak 1710.9 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 0.88 (3H, t), 1.27 (6H, m), 1.60 (2H, m), 2.88 (2H, t), 3.53 (9H, s), 4.20 (2H, m), 4.70 (2H, m).

(1-Heptylthiocarbonyl)choline chloride (7): FTIR in chloroform, key peak 1711.3 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 0.87 (3H, t), 1.30 (6H, m), 1.60 (2H, m), 2.58 (2H, m), 2.85 (2H, t), 3.53 (9H, s), 4.20 (2H, m), 4.69 (2H, m).

[[1-(3-Methylbutyl)]thiocarbonyl]choline chloride (8): FTIR in chloroform, key peak 1712.7 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 0.82 (6H, d), 1.45 (2H, m), 1.76 (1H, m), 2.79 (2H, t), 3.42 (9H, s), 4.08 (2H, m), 4.62 (2H, m).

[[1-(2-Methylbutyl)]thiocarbonyl]choline chloride (9): FTIR in chloroform, key peak 1712.5 cm^{-1} (C=O strain): ^1H NMR in $CDCL_3$, 0.90 (6H, m), 1.25 (2H, m), 1.40 (1H, m), 2.80 (2H, dd), 3.49 (9H, s), 4.12 (2H, m), 4.49 (2H, m).

(2-Propylthiocarbonyl)choline chloride (10): FTIR in chloroform, key peak 1708.0 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 1.33 (6H, s), 2.12 (1H, s), 3.50 (9H, s), 4.12 (2H, m), 4.62 (2H, m).

[[1-(1,1-Dimethylethyl)]thiocarbonyl]choline chloride (11): FTIR in chloroform, key peak 1712.7 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 1.40 (9H, s), 3.45 (9H, s), 4.10 (2H, m), 4.58 (2H, m).

[[1-(1,1-Dimethylpropyl)]thiocarbonyl]choline chloride (12): FTIR in chloroform, key peak 1712.2 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 0.95 (3H, m), 1.44 (6H, s), 1.72 (2H, m), 3.50 (9H, s), 4.18 (2H, m), 4.65 (2H, m).

(Phenylthiocarbonyl)choline chloride (13): FTIR in chloroform, key peak 1723.6 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 3.44 (9H, s), 4.12 (2H, m), 4.72 (2H, m), 7.30−7.48 (5H, m).

[(4-Ethylphenyl)thiocarbonyl]choline chloride (14): FTIR in chloroform, key peak 1723.0 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 1.22 (3H, t), 2.67 (2H, m), 3.46 (9H, s), 4.19 (2H, m), 4.69 (2H, m), 7.22 (2H, m), 7.41 (2H, m).

[(2-Ethylphenyl)thiocarbonyl]choline chloride (15): FTIR in chlorofrom, key peak 1719.4 cm^{-1} (C=O strain); ^1H NMR in $CDCl_3$, 1.18 (3H, t), 2.78 (2H, m), 3.44 (9H, s), 4.14 (2H, m), 4.67 (2H, m), 7.20−7.54 (4H, m).

Biological Studies. AChE Extraction and Purification from Calf or Octopus Brain. Ten grams of perfused calf forebrain (CFB) or 5 g of octopus brain (OB) was homogenized at 4 °C in 5 vol of high-salt Triton buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 M NaCl, 1% Triton X-100), which also contained bacitracin (0.1 mg/mL) and the trypsin inhibitor aprotinin (0.008 IU/mL), to minimize proteolysis. The 100000*g* supernatant solution gave HST fractions (high-salt Triton soluble). AChEs in the HST extracts were chromatographed (4 °C) on procainamide-Sepharose gel columns (1.5 × 2.0 cm) as previously described.[20] Active fractions were dialyzed against the Triton buffer (above) modified to contain 0.2 M NaCl. This procedure led to the recovery of 40% and 35% of the initial activity of CFB and OB, respectively. Protein concentrations were measured by the Bradford method,[21] using bovine serum albumin as standard. Specific activities of 272 IU (CFB) and 90 IU (OB) per mg of protein were achieved. Purification to homogeneity was shown by SDS−PAGE, giving single protein bands at 68 kDa (CFB) and 66 kDa (OB).

AChE Inhibition Studies. AChE from human RBC and from CFB and OB were employed in the studies. The final concentrations of all enzyme preparations were adjusted to 0.1 IU/mL, where 1 IU is defined as the amount of enzyme which catalyzes the hydrolysis of 1 umol of substrate/min at saturating substrate concentration.

The AChE inhibitory properties of the thiocarbonate compounds reported herein were studied using 1 mM ATC as the substrate. It has been established that thiocholine-containing substrates give comparable rates of hydrolysis by AChE as does acetylcholine.[22] The assays employing the various enzyme preparations were performed at 20 °C by a modification of the method described by Ellman.[23] The rate of production of the hydrolysis product thiocholine was determined spectrophotometrically by measuring the rate of absorbance increase (412 nm) resulting from the reaction of this thiol with DTNB. Concentrations of the test compounds in the range of 10^{-9}−10^{-2} M were used in the assays, and residual AChE activity was determined (two determinations at each compound concentration). Specifically, the reaction was started, after a 1

min preincubation of the enzyme and test compound (thiocarbonate), by addition of the substrate. The concentrations of the test compounds that inhibited the hydrolysis reaction by 50% (IC_{50} values) were determined by interpolation using increasing levels of these substances in the assays. Inhibition constants, K_i values, were calculated from kinetic data.[24] Four concentrations of each inhibitor and five substrate concentrations were used. Duplicate assays were conducted at each of the concentration combinations, and the mean of the enzyme velocity values was used for graphical analysis (individual velocity values were all within ±5% of the mean value). Determinations of possible substrate activity of the thiocarbonates were conducted using assay mixtures (containing DTNB) in which ATC was omitted.

Results and Discussion

All of the thiocarbonates of choline synthesized in this study are very deliquescent, as is the starting material, choline chloride. We found it to be extremely difficult to reproducibly remove all traces of water from the 14 compounds synthesized; consequently, it was difficult to obtain elemental analyses that would provide definitive information relative to the purity of the compounds. Product purity was checked in all instances by TLC (using iodine) and, where applicable, also UV detection; all of the products exhibited a single spot on the chromatograms. The final products were also evaluated using the Ellman test;[23] the results were uniformly negative when conducted at pH 7.0. When the pH was raised above 8.5, all products gave a positive reaction, indicating that hydrolysis of the thiocarbonate had occurred. ^{1}H NMR and FTIR data were utilized to confirm chemical structure and as additional indicators of probable purity.

The synthesis of the thiocarbonates presented some initial difficulties due to the high reactivity and low solubility of choline chloroformate in a number of preferred solvents. It has been reported that alkyl chloroformates undergo a dimethylformamide (DMF)-catalyzed decarboxylation, due to the readiness of DMF to be O-acylated via its polarized carbonyl functionality.[25] We also found that a potentially useful reaction solvent, dimethyl sulfoxide, caused a rapid decarboxylation of the choline chloroformate, perhaps by a process similar to that caused by DMF. It has also been reported that 1-adamantyl chloroformate reacts with hydroxylic solvents to yield carbon dioxide and both solvolysis and decomposition products.[26] This type of process probably accounts for the observation that there is a rapid effervescence when choline chloroformate is added to water or when exposed to moist air. These reactivity and solubility problems associated with choline chloroformate allowed only a very limited choice of solvents. We ultimately found that choline chloroformate was both soluble and stable in acetonitrile; it was the solvent of choice for the formation of thiocarbonates, and the reaction proceeded smoothly via sodium thiolate salts. The sequence of reactions employed for the synthesis of the thiocarbonate derivatives of choline is summarized below:

$$(CH_3)_3N^+CH_2CH_2OH\cdot Cl^- + COCl_2 \rightarrow (CH_3)_3N^+CH_2CH_2O\text{-}CO\text{-}Cl\cdot Cl^-$$

1

$$R\text{-}S^-Na^+ + \mathbf{1} \rightarrow (CH_3)_3N^+CH_2CH_2O\text{-}CO\text{-}S\text{-}R\cdot Cl^-$$

2−**15**

Table 1. Inhibition of AChE Activities by Thiocarbonate Derivatives of Choline[a]

$(CH_3)_3N^+CH_2CH_2O\text{-}CO\text{-}S\text{-}R\cdot Cl^-$

compd	R-group structure or name	AChE IC_{50} (M) human RBC	purified CFB	purified OB
2	CH_3CH_2-	$>1 \times 10^{-2}$	1×10^{-2}	$>1 \times 10^{-2}$
3	$CH_3(CH_2)_2$-	$>5 \times 10^{-3}$	1×10^{-3}	$>1 \times 10^{-3}$
4	$CH_3(CH_2)_3$-	5×10^{-4}	1×10^{-4}	1×10^{-3}
5	$CH_3(CH_2)_4$-	8×10^{-7}	1×10^{-6}	5×10^{-5}
6	$CH_3(CH_2)_5$-	8×10^{-5}	2×10^{-5}	1×10^{-4}
7	$CH_3(CH_2)_6$-	5×10^{-5}	1×10^{-5}	5×10^{-4}
8	$CH_3CH(CH_3)CH_2CH_2$-	5×10^{-5}	1×10^{-5}	5×10^{-5}
9	$CH_3CH_2CH(CH_3)CH_2$-	1×10^{-3}	8×10^{-4}	NI[b]
10	$(CH_3)_2CH$-	NI	NI	NI
11	$(CH_3)_3C$-	NI	NI	NI
12	$CH_3CH_2(CH_3)_2C$-	4×10^{-5}	1×10^{-5}	NI
13	phenyl-	1×10^{-3}	1×10^{-4}	NI
14	4-ethylphenyl-	5×10^{-4}	1×10^{-4}	5×10^{-5}
15	2-ethylphenyl-	5×10^{-5}	1×10^{-5}	NI
	procainamide[c]	1×10^{-6}	5×10^{-7}	1×10^{-6}
	edrophonium[c]	2×10^{-7}	5×10^{-8}	5×10^{-7}

[a] See the Experimental Section for details of bioassay and for purification/preparation of AChEs. Values are expressed as the mean of two independent measurements. [b] NI, not inhibitory. [c] Known AChE competitive inhibitors.

Table 2. Inhibition Constants of AChEs with Selected Thiocarbonate Derivatives of Choline

$(CH_3)_3N^+CH_2CH_2O\text{-}CO\text{-}S\text{-}R\cdot Cl^-$

compd	R-group number or name	AChE K_i (M)[a] human RBC	purified CFB	purified OB	type of inhibition
5	$CH_3(CH_2)_4$-	2×10^{-6}	1×10^{-6}	3×10^{-6}	competitive[b]
7	$CH_3(CH_2)_6$-	2×10^{-5}	1×10^{-5}	4×10^{-5}	competitive
	procainamide	1×10^{-6}	1×10^{-6}	1×10^{-6}	competitive
	edrophonium	2×10^{-7}	2×10^{-7}	4×10^{-7}	competitive

[a] K_i values were calculated from kinetic data (see ref 24); values are expressed as the mean of two independent measurements. [b] See Figure 1 for a Lineweaver−Burk plot of kinetic data obtained from studies with compound **4** and CFB AChE.

Newly synthesized compounds were tested *in vitro* for selectivity and potency as AChE inhibitors. These studies were performed with AChEs from three sources which show comparable catalytic efficiencies and substrate specificities,[15,27] even though belonging to phylogenetically distinct organisms as mammalian (man, calf) and a cephalopod mollusck (octopus). With the aim of performing a rigorous test of the anti-AChE properties of our compounds, the kinetics of enzyme inhibition were studied in purified enzyme preparations. The inhibition data are shown in Table 1. Additionally, known competitive inhibitors of AChE (procainamide and edrophonium) were included in the study for comparative purposes. With the exception of compounds **10** and **11**, all the thiocarbonates exhibited inhibitory activity in human RBC, CFB, and/or OB AChEs. For those compounds exhibiting at least some inhibitory activity, IC_{50} values were determined. For those compounds exhibiting relatively high activity, K_i values were also determined (Table 2). Within the series of thiocarbonate derivatives bearing unbranched alkyl substituents (compounds **2**−**7**), there is a general enhancement in inhibitory potency with increasing chain length, peaking with the pentyl thiocarbonate (K_i values of 2×10^{-6}, 1×10^{-6}, and 3×10^{-6} M for the RBC, CFB, and OB AChEs, respectively) and decreasing

somewhat with the hexyl and heptyl thiocarbonate derivatives.

Among the thiocarbonates bearing branched alkyl substituents (compounds **8**−**12**), there appears to be little correlation of inhibitory effects with the presence of, or position of, the alkyl branch; however, there is a correlation with the longest chain length associated with the alkyl substituent. Thus, compounds **10** and **11**, which have a 2-carbon longest chain length, are, as with compound **2**, essentially noninhibitory to RBC, CFB, and OB AChEs. On the other hand, compounds **8** and **9** (which bear a 4-carbon longest chain) and compound **12** (which bears a 3-carbon longest chain) exhibit significant inhibition of the RBC and CFB AChEs; compound **8** also is inhibitory to OB AChE. The inhibitors of RBC and CFB AChEs by these compounds are reasonably comparable to that given by the thiocarbonate bearing an unbranched 4-carbon chain (compound **4**).

Compounds **13**−**15** are aryl thiocarbonates of choline. While all three of these compounds exhibited some inhibitory activity with the human RBC and CFB AChEs, only one of them (compound **14**, containing the 4-ethylphenyl thiocarbonate substituent) was inhibitory to OB AChE activity. This is especially interesting, in that the 2-ethylphenyl thiocarbonate was the most potent inhibitor of the three aryl thiocarbonates with the RBC and CFB AChEs. This finding, along with some of the inhibitory data obtained for the branched alkyl thiocarbonates, may relate to differences among the substrate-binding pockets of RBC, CFB, and OB AChEs.

Two of the more inhibitory thiocarbonate derivatives of choline (compounds **5** and **7**) were studied further to characterize the nature of the inhibition of the three AChE activities. Table 2, showing the inhibition constants (K_i values) for RBC, CFB, and OB AChEs, summarizes the results of this study. Inhibition data for compound **5**, the pentyl thiocarbonate (at 10^{-5} and 10^{-6} M), with CFB AChE are also presented in a Lineweaver-Burk plot (Figure 1); the graphical presentations for compounds **5** and **7**, with all three AChEs, are similar. The inhibitions were found to be clearly of the competitive type, indicating that binding occurs within the active site pocket of the three AChEs studied. However, none of the thiocarbonate derivatives served as substrates for any of the AChEs studied, when added to assay mixtures in which acetylthiocholine (but not DTNB) had been omitted. This was somewhat unexpected, since thiocarbonate derivatives of glutathione, which are very potent competitive inhibitors of the thiolhydrolase activity of the enzyme glyoxalase II, also serve as weak substrates for that enzyme.[16] Glyoxalase II is not, however, a serinesterase.

The synthesis and study of choline derivatives containing the thiocarbonate functionality were conducted, since previous syntheses in this laboratory had provided thiocarbonate derivatives of glutathione that are very potent and specific competitive inhibitors of the glyoxalase system enzyme glyoxalase II.[16−18,28,29] The reported presence of a hydrophobic region surrounding the anionic moiety of cholinesterases[15,30,31] suggested the synthesis of hydrophobic alkyl thiocarbonates possessing various carbon chain lengths, both branched and unbranched and the synthesis of phenyl thiocarbonates, both alkyl-substituted and unsubstituted, for enzymatic study.

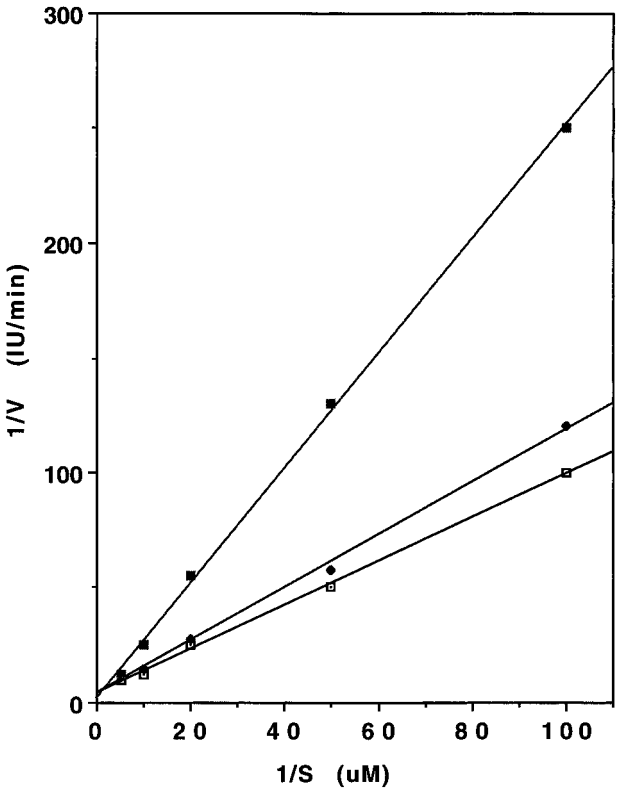

Figure 1. Lineweaver−Burk plot of inhibition data obtained for compound **5** ((1-pentylthiocarbonyl)choline chloride) with AChE purified from calf forebrain (CFB). Concentrations of **5**: (□) 0 M, (◆) 10^{-6} M, and (■) 10^{-5} M.

We can generalize from the findings of our study that increasingly effective competitive inhibitors of AChE are produced with unbranched alkyl groups, plateauing at around 5−7 carbons (compounds **2**−**7**). Branching, at least with methyl groups, does not appear to greatly influence inhibitory activity of the thiocarbonates as long as there is a sufficiently long chain length for the parent alkyl substituent (compare compounds **8**−**12**). The effects of alkyl substituents (ethyl groups) on the aryl thiocarbonates (compounds **13**−**15**) suggest that alkyl substituents in the *para* position may have a more positive effect on inhibitory activity than substitution in the *ortho* position. This is particularly true for the OFB AChE and may be due to steric factors in this enzyme, in that the larger molecular space swept out by the *ortho*-substituted compound could disallow a most correct arrangement of the molecule in the active site gorge.[31]

All of the enzymes employed in the inhibition studies are true acetylcholinesterases.[27,32] The compounds synthesized and studied in this investigation were designed such that, if significantly inhibitory to AChE activity, they, or their subsequent hydrolysis products, would not likely exhibit significant toxicity to mammalian systems. This goal was sought with the ultimate aim that new drugs for the treatment of AD might be developed. Certain of the compounds, particularly compounds **5**−**8** and **14**, are rather potent competitive AChE inhibitors; compound **5** compares favorably with procainamide in both IC_{50} and K_i values (Tables 1 and 2). The apparent, high specificities, coupled with the structural similarities of the new compounds to the

normal substrate, suggest that binding occurs with the catalytic site deep within, and not with the peripheral site near the entrance of, the active site gorge of AChEs.[13,15]

AChE inhibitors of the type synthesized in this study may well be capable of stimulating cholinergic activity in the brain by decreasing acetylcholine hydrolysis rates. Thus, they could have potential in the treatment of AD; however, there may still be significant barriers to that end use. For example, there may be rejection of the thiocarbonate inhibitors by the blood−brain barrier. *In vivo* studies may be required to determine whether or not these new inhibitors are passed through this barrier. Also, since the thiocarbonates are structural analogs of acetylcholine (and exhibit purely competitive inhibition of AChEs), it is possible that they are also effective competitors of acetylcholine at cholinergic receptor sites in the postsynaptic membrane. The effects of such competition could be either stimulation or inhibition of the normal ligand-binding response and thus result in either the opening or the closing of acetylcholine-gated channels.[33] It is our intention in future work to study cholinergic receptor binding of the thiocarbonates and other proposed AChE inhibitors, and consequent effects thereof, using cultured neuronal networks growing on multielectrode arrays.[34]

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