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NOVEL PYRIDINIUM DERIVATIVES AS INHIBITORS FOR ACETYLCHOLINESTERASE

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The carbamate 1-(methyl-3-(*N,N*-dimethylcarbamoyloxy)-2- pyridylmethylene)-4-(4-phenyl)diazinecarboxamide chloride (MHP 133) is the parent for a new class of pyridinium salts which inhibit acetylcholinesterase (AChE) *in vitro* as well as *in vivo*. Fourteen new derivatives of MHP 133 have been synthesized with the intention of improving their hydrophobicity while maintaining their propensity to inhibit acetylcholinesterase. Upon prolonged incubation with AChE, the pyridinium salts exhibit progressive time-dependent inhibition according to first order kinetics with $k_{obs}/[I]$ values ranging from 3 to 345 $M^{-1}s^{-1}$. The enzyme did not regain any activity after prolonged incubation with the inhibitors (1 day). The partition coefficients for each inhibitor were evaluated in octanol/water in order to determine their hydrophobic character as hydrophobicity is a key prerequisite for crossing the blood brain barrier.

KEY WORDS: Acetylcholinesterase, time-dependent inhibition, partition coefficient, pyridinium salts

INTRODUCTION

Acetylcholinesterase (AChE) is an enzyme which is primarily responsible for the termination of cholinergic nerve impulses by the rapid hydrolysis of the neurotransmitter acetycholine to choline and acetate.[1] Thus when the deterioration of brain cholinergic neurons occurs in conditions such as Alzheimer's disease the enzyme becomes an agent which limits normal cognitive processes especially memory, because of a gradual decrease in the availability of acetylcholine.[2] Several studies in Alzheimer's disease patients have attempted to assess the effectiveness of direct-acting muscarinic agonists in relieving symptoms. Agonists such as arecoline, oxotremorine and RS-86

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Abbreviations: AChE, acetylcholinesterase; bs, broad singlet; d, doublet; dd, doublet of doublets; DMF, *N,N*-dimethylformamide; DMSO, *N,N*-dimethylsulfoxide DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); m, multiplet; MHP 133, 1-(1-methyl-3-(*N,N*-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(4-phenyl) diazinecarboxamide chloride; MS, mass spectroscopy; P, partition coefficient; s, singlet; q, quartet.

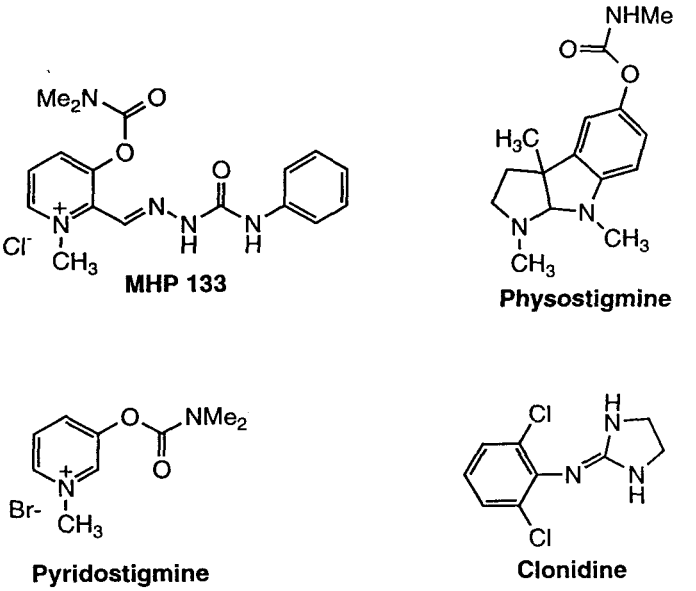


FIGURE 1 Structure of the acetylcholinesterase inhibitors MHP 133, physostigmine, pyridostigmine, and clonidine.

have been employed with no success, or very limited success. In many cases the lack of efficacy was related to the appearance of severe side effects.[3] A strategy which has hitherto been more promising has been the use of AChE inhibitors which would enable residual amounts of acetylcholine to remain unhydrolyzed and therefore permit more opportunities for normal communication between cholinergic neurons.

Numerous carbamates have been studied as AChE inhibitors.[4–7] Two of the most widely studied are physostigmine ($K_d = 3.14 \times 10^{-7}$ M) and pyridostigmine ($K_d = 5.50 \times 10^{-6}$) which are both being examined for their potential as Alzheimer's therapeutics.[5,8] The carbamate 5-(1,3,3-trimethylindolinyl) *N*-methylcarbamate which is a derivative of physostigmine has a K_d value of 7.14×10^{-8} M against electric eel acetylcholinesterase.[4] This value is the smallest value for any inhibitor of acetylcholinesterase which forms a covalent bond with the inhibitor including military toxins.[4]

Carbamates such as physostigmine and pyridostigmine (Figure 1) have found success as cholinergic agonists/AChE inhibitors, however, with important limitations. Both pyridostigmine and physostigmine have short durations of action and are highly toxic.[4,5] A simultaneous regimen of clonidine (Figure 1) and physostigmine given to young and aged macaque monkeys has been shown to decrease physostigmine toxicity while improving memory.[9] More recently, a new carbamylating AChE inhibitor (MHP 133, Figure 1) was shown to be a better Alzheimer's therapeutic than

physostigmine because of its lower toxicity and its ability to improve memory in Alzheimer's models at the μg/kg dose regimen (unpublished data).

Here we report the synthesis of fourteen new acetylcholinesterase inhibitors which are analogs of MHP 133. We have attempted to make MHP 133 more hydrophobic in order to increase the likelihood of penetration through the blood brain barrier as well as give the molecule clonidine-like features in some instances by adding substituents to the 2-and 6-positions of the phenyl ring. The dual strategy in these instances are to maintain MHP 133's propensity to inhibit acetylcholinesterase while decreasing the likelihood of its toxicity.

MATERIALS AND METHODS

Materials

All chemicals and reagents were purchased from the Aldrich Chemical Company, Milwaukee, WI. All common solvents were purchased from the Fisher Chemical Company, Fair Lawn, N.J. Each newly synthesized methiodide was analyzed by ^{1}H NMR, mass spectroscopy (FAB), melting point determinations, and elemental analysis. The NMR spectra were recorded on a Varian Gemini 300 MHz instrument in DMSO-d_6; teramethylsilane was used as an internal standard. Mass spectra (FAB) were measured on a VG Instrument 70-SE (8 KV, Xe atom, 1 mA ion current). Melting point determinations were made using a Swissco melting point apparatus, Swissco Research Equipment, Greenville, Illinois. Elemental analyses were performed by The Atlantic Microlabs, Norcross, Ga.

Synthesis

3-Hydroxy-2-pyridinecarboxaldehyde. The oxidation of 3-hydroxy-2-(hydroxymethyl) pyridine to 3-hydroxy-2-pyridine carboxaldehyde was carried out according to a modification of a previously published procedure.[10] Solid MnO_2 (0.65 mol) was added in portions over 30 min to a solution of 8.55 g (0.05 mol) of 3-hydroxy-2-(hydroxymethyl) pyridine in 303 mL of acetone at room temperature. The mixture was stirred for 24 h, the MnO_2 was removed by filtration using celite, and the filtrate was evaporated to dryness. Cyclohexane (2 mL) was added to the residue which was refrigerated for 24 h. The aldehyde (1.78 g, 28%) was obtained as yellow prisms, mp 78–80°C (lit. mp 80°C).[10]

2,6-Dichlorophenylsemicarbazide (General Procedure for Phenylsemicarbazide Synthesis). The synthesis of phenylsemicarbazides were carried out according to a previously published procedure.[11] To a stirred solution of 10 mL methylene chloride and 2 g (11 mmol) hydrazine monohydrate at 0°C was added dropwise a suspension of 5.5 g (110 mmol) of 2,6-dichlorophenyl isocyanate and 10 mL methylene chloride. The semicarbazide was formed instantaneously and collected by suction filtration as a white solid. The product was washed with 100 mL water and 100 mL methylene chloride; yield 600 mg (25%); mp 288–290°C; ^{1}H NMR (DMSO-d_6) δ: 8.30–8.26

(bs, 1H), 7.55 (s, 1H), 7.50 (dd, 2H), 7.23 (m, 1H), 4.42–4.40 (bs, 2H); FABMS m/e $(M+1)^+$ 220.1. Anal. calcd. for $C_7H_7N_3OCl_2$: C, 38.18; H, 3.20; N, 19.10; Cl, 32.23. Found: C, 38.04; H, 3.09; N, 18.93; Cl, 32.40%.

4-Methoxyphenylsemicarbazide. The synthesis of this compound by a different method has been reported.[12] This compound was prepared by the general procedure and collected as a white solid; yield 69%; mp 150–152°C (lit. mp 158–159°C)[12]; ^{1}H NMR (DMSO-d_6) δ: 8.62 (s, 1H), 7.44 (d, 2H), 7.41 (s, 1H), 6.87 (d, 2H), 4.33 (s, 2H), 3.69 (s, 3H); high resolution MS, m/e (M^+) calcd. for $C_8H_{11}N_3O$ 181.085. Found: 181.085.

4-Chlorophenylsemicarbazide. The synthesis of this compound by a different method has been reported.[13] This compound was prepared by the general procedure and collected as a white solid; yield 80%; mp 260–262°C (lit. mp 232°C)[13]; ^{1}H NMR (DMSO-d_6) δ: 8.80 (s, 1H), 7.60 (d, 2H), 7.50 (s, 1H), 7.78 (d, 2H), 4.39–4.30 (bs, 2H); high resolution MS, m/e (M^+) calcd. for $C_7H_8N_3OCI$ 185.035. Found: 185.035.

2,6-Dimethylphenylsemicarbazide. This compound was prepared by the general procedure and collected as a white solid; yield 77%; mp 270–272°C ^{1}H NMR (DMSO-d_6) δ: 7.85 (s, 1H), 7.24 (s, 1H), 7.03 (m, 3H), 4.40–4.33 (bs, 2H), 2.18 (s, 6H); high resolution MS, m/e (M^+) calcd. for $C_9H_{13}N_3O$ 179.105. Found: 179.106.

2,6-Difluorophenylsemicarbazide. This compound was prepared by the general procedure except that the product was washed with 50 mL H_2O, 50 mL ethyl acetate, and 50 mL ether, and collected as a white solid; yield 73%; mp 156–158°C ^{1}H NMR (DMS0-d_6) δ: 8.14 (s, 1H), 7.55 (s, 1H), 7.28 (m, 1H), 7.11 (dd, 2H), 4.42–4.39 (bs, 2H); FABMS m/e $(M+1)^+$ 188. Anal. calcd. for $C_7H_7N_3O_2 \cdot 0.6H_2O$: C, 42.47; H, 3.56; N, 21.22. Found: C, 42.93; H, 3.75; N, 20.96%.

2-Ethoxyphenylsemicarbazide. This compound was prepared by the general procedure and collected as a white solid; yield 93%; mp 133–135°C; ^{1}H NMR (DMSO-d_6) δ: 9.00–8.90 (bs, 1H), 8.18 (m, 1H), 7.63 (s, 1H), 6.97 (m, 1H), 6.87 (m, 2H), 4.47–4.51 (bs, 2H), 4.12 (q, 2H), 1.38 (t, 3H); MS m/e (M^+) 195.1. Anal. calcd. for $C_9H_{13}N_3O_2$: C, 55.37; H, 6.71; N, 21.52. Found: C, 55.38; H, 6.74; N, 21.47%.

3-Nitrophenylsemicarbazide. The synthesis of this compound by a different method has been reported.[12] This compound was prepared by the general procedure and collected as a yellow solid; yield 59%; mp 136–138°C (lit. mp 111–112 °C)[12]; ^{1}H NMR (DMSO-d_6) δ: 9.21 (s, 1H), 8.68 (s, 1H), 7.89 (d, 1H), 7.76 (m, 2H), 7.5 (m, 1H), 4.44–4.38 (bs, 2H), high resolution MS m/e (M^+) calcd. for $C_7H_8O_3N_4$ 196.059. Found: 196.058.

4-Bromophenylsemicarbazide. The synthesis of this compound by a different method has been reported.[14] This compound was prepared by the general procedure and collected as a white solid; yield 85%; mp 256–259°C (lit. mp 254°C)[14]; ^{1}H NMR (DMSO-d_6) δ: 8.78 (s, 1H), 7.48–7.55 (d and bs, 3H), 7.40 (d, 2H), 4.40–4.37 (bs, 2H); high resolution MS m/e (M^+) calcd. for $C_7H_8N_3OBr$ 228.985 Found: 228.984.

1-Napthylsemicarbazide. The synthesis of this compound by a different method has been reported.[15] This compound was prepared by the general procedure and collected

as a white solid; yield 71%; mp 165°C (lit. mp 165°C)[15]; ^{1}H NMR (DMSO-d_6) δ: 8.90–9.10 (bs, 1H), 7.90–8.10 (m, 3H), 7.67 (s, 1H), 7.61–7.45 (m, 4H), 4.53–4.55 (bs, 2H).

1-(3-Hydroxy-2-pyridylmethylene)-4-(2,6-dichlorophenyl)diazinecarboxamide (General Procedure for the Condensation of Phenylsemicarbazides or Phenylhydrazides with Pyridine Aldehydes). A solution of 500 mg (4 mmol) of 3-hydroxy-2-pyridinecarboxaldehyde and 876 mg (4 mmol) of 2,6-dichlorophenylsemicarbazide in 20 mL of ethanol was refluxed for 1 h. The mixture was cooled, filtered by gravity, washed several times with 25 mL portions of ethanol, and air dried. The product was obtained as a brown solid; yield 1.20 mg (95%); mp 262–265°C; ^{1}H NMR (DMSO-d_6) δ: 11.05 (s, 1H), 10.28–10.10 (bs, 1H), 9.05 (s, 1H), 8.30 (s, 1H), 8.18 (d, 1H), 7.58 (dd, 2H), 7.53 (m, 3H); MS m/e (M^+) 324.1.

The products of these reactions can be made in purer forms by the addition of 1 drop of H_2SO_4 to the reaction mixture before refluxing as in the case of the procedure for 1-(3-hydroxy-2-pyridylmethylene)-4-(2,6-dimethylphenyl)diazinecarboxamide given below.[16]

1-3-Hydroxy-2-pyridylmethylene)-4-(4-methoxyphenyl)diazinecarboxamide.
This compound was prepared by the general procedure except that the crude product was dissolved in hot methanol, concentrated *in vacuo*, and collected as a brown solid; yield 47%; mp 183–185°C; ^{1}H NMR (DMSO-d_6+D_2O) δ: 9.05 (s, 1H), 8.44 (s, 1H), 8.20 (s, 1H), 7.5 (d, 2H), 7.4 (s, 1H), 7.3 (d, 2H), 6.8 (d, 2H), 3.7 (s, 3H); FABMS m/e $(M+1)^+$ 287.

1-(3-Hydroxy-2-pyridylmethylene)-2-(2,4,6,trichlorophenyl)diazine. This compound was prepared by the general procedure. After cooling the product was obtained as crispy white needles; yield 84%; mp 185–187°C; ^{1}H NMR (DMSO-d_6) δ: 10.59 (s, 1H), 10.20 (s, 1H), 8.36 (s, 1H), 8.14 (d, 1H), 7.7 (s, 1H), 7.24 (m, 2H); MS m/e (M^+) 315.1.

1-(3-Hydroxy-2-pyridylmethylene)-4-(4-chlorophenyl)diazinecarboxamide.
This compound was prepared by the general procedure. The product was a brown solid; yield 33%; mp 216–218°C ^{1}H NMR (DMSO-d_6) δ: 10.60 (s, 1H), 10.60–10.50 (bs, 1H), 9.28 (s, 1H), 8.29 (s, 1H), 8.17 (d, 1H), 7.62 (dd, 2H), 7.2 (m, 4H); MS m/e (M^+) 290.1.

1-(3-Hydroxy-2-pyridylmethylene)-4-(2,6-dimethylphenyl)diazinecarboxamide.
This compound was prepared by the general procedure. The product was a brown solid; yield 87% mp 243–248°C ^{1}H NMR (DMSO-d_6) δ: 10.81 (s, 1H), 10.40–10.30 (bs, 1H), 8.53 (s, 1H), 8.27 (s, 1H), 8.16 (d, 1H), 7.33 (m, 2H), 7.10 (s, 3H), 2.20 (s, 6H); FABMS m/e $(M+1)^+$ 285.

1-(3-Hydroxy-2-pyridylmethylene)-4-(2,6-difluorophenyl)diazinecarboxamide.
This compound was prepared by the general procedure. The product was a brown solid; yield 56%; mp 238–240°C; ^{1}H NMR (DMSO-d_6) δ: 11.06 (s, 1H), 10.05–10.15 (bs, 1H), 8.88 (s, 1H), 8.31 (s, 1H), 8.18 (s, 1H), 7.35 (m, 3H), 7.18 (m, 2H); MS m/e (M^+) 292.

1-(3-Hydroxy-2-pyridylmethylene)-4-(2-ethoxyphenyl)diazinecarboxamide. This compound was prepared by the general procedure. The product was a brown solid; yield 58%; mp 197–198°C; ^{1}H NMR (DMSO-d_6) δ: 11.14 (s, 1H), 10.70–10.90 (bs, 1H), 8.73 (s, 1H), 8.27 (s, 1H), 8.15 (m, 2H), 7.32 (m, 2H), 7.05 (m, 3H), 4.13 (q, 2H), 1.45 (t, 3H); MS m/e (M^+) 300.2.

1-(3-Hydroxy-2-pyridylmethylene)-2-(4-fluorophenyl)diazine. This compound was prepared by the general procedure. The product was a red solid; yield 76%; mp 260–264°C (dec); ^{1}H NMR (DMSO-d_6) δ: 12.15–12.23 (bs, 1H), 12.07 (s, 1H), 8.24 (d, 1H), 8.12 (m, 2H), 7.66 (m, 1H), 7.62 (m, 2H), 7.20 (m, 2H); MS m/e (M^+) 231.1.

1-(3-Hydroxy-pyridylmethylene)-4-(3-nitrophenyl)diazinecarboxamide. This compound was prepared by the general procedure. The product was a brown solid; yield 73%; mp 223–225°C; ^{1}H NMR (DMSO-d_6) δ: 11.11 (s, 1H), 10.50–10.30 (bs, 1H), 9.68 (s, 1H), 8.62 (s, 1H), 8.33 (s, 1H), 8.20 (s, 1H), 8.19 (d, 1H), 7.97 (d, 1H), 7.88 (m, 1H), 7.61 (m, 2H); MS m/e (M^+) 300.1.

1-(3-Hydroxy-2-pyridylmethylene)-4-(4-bromophenyl)diazinecarboxamide. This compound was prepared by the general procedure. The product was a brown solid; yield 91%; mp 274°C (dec); ^{1}H NMR (DMSO-d_6) δ: 10.95 (s, 1H), 10.45–10.65 (bs, 1H), 9.29 (s, 1H), 8.30 (s, 1H), 8.19 (d, 1H), 7.50 (dd, 4H), 7.33 (m, 2H); MS m/e $(M+1)^+$ 335.

1-(3-Hydroxy-2-pyridylmethylene)-4-(1-napthyl)diazinecarboxamide. This compound was prepared by the general procedure. The product was a brown solid; yield 75%; mp 222°C ^{1}H NMR (DMSO-d_6) δ: 11.04 (s, 1H), 10.85–10.60 (bs, 1H), 9.29 (s, 1H), 8.32 (s, 1H), 8.19 (d, 1H), 8.18 (d, 1H), 8.06 (d, 1H), 7.98 (d, 1H), 7.77 (d, 1H), 7.59 (m, 3H), 7.37 (d, 1H), 7.30 (m, 1H); MS m/e (M^+) 306.

1-(3-Hydroxy-2-pyridylmethylene)-2-(3-trifluoromethylphenyl)diazine. This compound was prepared by the general procedure. After cooling overnight the product was isolated as a brown solid; yield 51%; mp 230°C; ^{1}H NMR (DMSO-d_6) δ: 11.09 (s, 1H), 10.80–10.60 (bs, 1H), 8.16 (s, 1H), 8.15 (d, 1H), 7.55 (t, 1H), 7.52 (d, 1H), 7.49 (m, 3H), 7.27 (d, 1H); MS m/e (M^+) 281.

1-(3-Hydroxy-2-pyridylmethylene)-2-(4-isopropylphenyl)diazine. This compound was prepared by the general procedure. After cooling overnight the product was collected as an orange solid; yield 80%; mp 246–248°C; ^{1}H NMR (DMSO-d_6) δ: 12.10–12.20 (bs, 1H), 11.88 (s, 1H), 8.21 (d, 1H), 8.19 (s, 1H), 8.01 (d, 1H), 7.50 (t, 1H), 7.33 (d, 2H), 7.21 (d, 2H), 2.84 (m, 1H), 1.20 (d, 6H); MS m/e (M^+) 255.

1-(3-Hydroxy-2-pyridylmethylene)-2-(2,3-dimethylphenyl)diazine. This compound was prepared by the general procedure. The product was rinsed thoroughly with an excess of water and ether and collected as an orange solid; yield 93% mp 258–260°C; ^{1}H NMR (DMSO-d_6) δ: 12.40–12.50 (bs, 1H), 11.04 (s, 1H), 8.50 (d, 1H), 8.22 (d, 1H), 7.77 (d, 1H), 7.62 (m, 1H), 7.27 (m, 1H), 6.82 (d, 1H), 2.25 (m, 6H); MS m/e (M^+) 241.

1-(3-(N,N)-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2,6- dichlorophenyl)diazinecarboxamide (General Procedure for the Synthesis of Pyridine Carbamates). The syn-

thesis of pyridine carbamates was carried out according to a previously published procedure.[17] A solution of 500 mg (1.5 mmol) of 1-(3-hydroxy-2-pyridylmethylene)-4-(2,6-dichlorophenyl) diazinecarboxamide and 1.65 g (15 mmol) of dimethylcarbamyl chloride in 15 mL of pyridine was allowed to stir for 14 h at room temperature. The reaction mixture was poured over crushed ice and the white solid which formed was washed thoroughly with water to remove the excess pyridine; yield 280 mg (47%); mp 225–229°C; ^{1}H NMR (DMSO-d_6) δ: 11.17 (s, 1H), 8.53 (d, 1H), 8.16 (s, 1H), 8.12 (s, 1H), 7.7 (d, 1H), 7.5 (d, 2H), 7.4 (dd, 1H), 7.3 (t, 1H), 3.0 (s, 3H), 2.7 (s, 3H); MS m/e (M^+) 395.2.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(4- methoxyphenyl)diazinecarboxamide. This compound was prepared by the general procedure and collected as a brown solid; yield 57%; mp 146–152°C; ^{1}H NMR (DMSO-d_6) δ: 10.91 (s, 1H), 8.52 (d, 1H), 8.34 (s, 1H), 8.09 (s, 1H), 7.67 (d, 1H), 7.46 (m, 3H), 3.73 (s, 3H), 3.08 (s, 3H), 2.85 (s, 3H); MS m/e $(M+1)^+$ 358.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(2,4,6,trichlorophenyl)diazine. This compound was prepared by the general procedure. The crude product recrystallizes from ethanol or methanol into a fluffy yellow solid, yield 58%; mp 145–147°C; ^{1}H NMR (DMSO-d_6) δ: 8.62 (d, 1H), 7.84 (d, 1H), 7.68 (s, 2H), 7.67 (dd, 1H), 7.47 (s, 1H), 3.14 (s, 3H), 2.95 (s, 3H); MS m/e $(M+1)^+$ 387.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(4-chlorophenyl)diazinecarboxamide. This compound was prepared by the general procedure except that the crude product was dissolved in hot ethyl acetate, concentrated *in vacuo* and collected as a brown solid; yield 36%; mp 170–175°C; ^{1}H NMR (DMSO-d_6) δ: 11.02 (s, 1H), 8.82 (s, 1H), 8.52 (d, 1H), 8.12 (s, 1H), 7.68 (m, 3H), 7.47 (dd, 1H), 7.37 (dd, 2H), 3.14 (s, 3H), 2.96 (s, 3H); MS m/e $(M+1)^+$ 362.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2,6-dimethylphenyl)diazinecarboxamide. This compound was prepared by the general procedure. The product was a brown solid; yield 78%; mp 215–217°C; ^{1}H NMR (DMSO-d_6) δ: 10.97 (s, 1H), 8.52 (d, 1H), 8.10 (s, 1H), 7.73 (s, 1H), 7.66 (d, 1H), 7,46 (m, 1H), 7.11 (s, 3H), 3.00 (s, 3H), 2.71 (s, 3H); MS m/e (M^+) 355.2.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2,6-difluorophenyl)diazinecarboxamide. This compound was prepared by the general procedure. The product is a white solid; yield 70%; mp 166–169°C; ^{1}H NMR (DMSO-d_6) δ: 11.17 (s, 1H), 8.53 (d, 1H), 8.13 (d, 2H), 7.70 (d, 1H), 7.49 (m, 1H), 7.40 (m, 1H), 7.21 (m, 2H), 3.07 (s, 3H), 2.84 (s, 3H); MS m/e (M^+) 363.1.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2-ethoxyphenyl)diazinecarboxamide. This compound was prepared by the general procedure. The product is a white solid; yield 70%; mp 166–169°C; ^{1}H NMR (DMSO-d_6) δ: 11.15 (s, 1H), 8.89 (s, 1H), 8.50 (d, 1H), 8.13 (m, 2H), 7.70 (m, 1H), 7.50 (m, 1H), 7.00 (m, 3H), 4.17 (m, 2H), 3.14 (s, 3H), 2.96 (s, 3H), 1.43 (m, 3H); MS m/e $(M+1)^+$ 372.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(4-fluorophenyl)diazine. This compound was prepared by the general procedure except that the crude material was washed three times with 50 mL of water and 50 mL of ether, and then dissolved in acetone. The acetone was concentrated *in vacuo* until the product crystallized in the form of brown prisms; yield 24%; mp 165–167°C; ^{1}H NMR (DMSO-d_6) δ: 10.63 (s, 1H), 8.45 (d, 1H), 7.91 (s, 1H), 7.59 (d, 1H), 7.33 (m, 1H), 7.13 (m, 2H), 7.01 (m, 2H), 3.10 (s, 3H), 2.94 (s, 3H); MS m/e $(M+1)^+$ 303.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(3-nitrophenyl)diazinecarboxamide. This compound was prepared by the general procedure. The product was washed thoroughly with 100 mL of ether and 100 mL of water and collected as a white solid; yield 90%; mp 195°C; ^{1}H NMR (DMSO-d_6) δ: 11.21 (s, 1H), 9.47 (s, 1H), 8.68 (s, 1H), 8.52 (d, 1H), 8.17 (s, 1H), 8.03 (d, 1H), 7.89 (d, 1H), 7.72 (d, 1H), 7.59 (m, 1H), 7.50 (m, 1H), 3.11 (s, 3H), 2.90 (s, 3H); FABMS m/e $(M+1)^+$ 373.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(4-bromophenyl)diazinecarboxamide. This compound was prepared by the general procedure and collected as a white solid; yield 89%; mp 176–179°C; ^{1}H NMR (DMSO-d_6) δ: 11.03 (s, 1H), 8.88 (s, 1H), 8.52 (d, 1H), 8.13 (s, 1H), 7.49 (m, 2H), 7.46 (m, 4H), 3.14 (s, 3H), 2.88 (s, 3H); MS m/e (M^+) 405.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(1-napthyl)diazinecarboxamide. This compound was prepared by the general procedure and collected as a white solid; yield 83%; mp 202–204°C; ^{1}H NMR (DMSO-d_6) δ: 11.15 (s, 1H), 8.63 (s, 1H), 8.54 (d, 1H), 8.17 (s, 1H), 7.98 (m, 2H), 7.71 (m, 3H), 7.50 (m, 4H), 3.0 (s, 3H), 2.70 (s, 3H); MS m/e (M^+) 377.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(3-trifluoromethylphenyl)diazine. This compound was prepared by the general procedure and collected as a white solid; yield 100% mp 178°C; ^{1}H NMR (DMSO-d_6) δ: 10.95 (s, 1H), 8.50 (d, 1H), 8.00 (s, 1H), 7.63 (d, 1H), 7.52 (t, 1H), 7.49 (m, 1H), 7.28 (m, 2H), 7.14 (d, 1H), 3.14 (s, 3H), 2.96 (s, 3H); FABMS m/e $(M+1)^+$ 353.

1-(3-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(4-isopropylphenyl)diazine. This compound was prepared by the general procedure. The crude product was recrystallized from a 10:1 solution of hexane and ethanol and collected as an orange solid; yield 77%; mp 112–116°C; ^{1}H NMR (DMSO-d_6) δ: 10.54 (s, 1H), 8.45 (d, 1H), 7.90 (s, 1H), 7.56 (d, 1H), 7.33 (m, 1H), 7.25 (m, 2H), 6.95 (d, 2H), 3.14 (s, 3H), 2.99 (s, 3H), 2.83 (m, 1H), 1.17 (d, 6H); MS m/e (M^+) 326.

1-(N,N-Dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(2,3-dimethylphenyl)diazine. This compound was prepared by the general procedure. The crude product was air dried, stirred in an excess of ether for 2 h, and collected as a yellow solid; yield 100%; mp 178°C; ^{1}H NMR (DMSO-d_6) δ: 9.72 (s, 1H), 8.36 (d, 1H), 8.12 (s, 1H), 7.47 (d, 1H), 7.22 (m, 1H), 7.07 (d, 1H), 6.92 (m, 1H), 6.60 (d, 1H), 2.96 (s, 3H), 2.86 (s, 3H), 2.17 (s, 3H), 2.03 (s, 3H); MS m/e (M^+) 312.

*1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2,6-dichlorophenyl) diazinecarboxmide Iodide (**7**) (General Procedure for the Synthesis of Pyridine Methiodides).* The synthesis of pyridine methiodides was carried out according to a previously published procedure.[17] A solution of 3 g (9 mmol) of 1-(3-(*N,N*-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2,6-dichlorophenyl)diazinecarboxamide, 6.39 g (45 mmol) of iodomethane, and 25 mL of DMF were sealed in a glass pressure tube and heated for 36 h at 65°C. The crude methiodide was isolated by evaporation of the DMF under reduced pressure and trituration of the residue with a 2:1 mixture of acetone and ether. The crude product was recrystallized from ethanol and collected as orange prisms; yield 0.69 g (19%); mp 132–140°C; ^{1}H NMR (DMSO-d_6) δ: 11.78 (s, 1H), 9.08 (d, 1H), 8.86 (s, 1H), 8.58 (d, 1H), 8.24 (s, 1H); 8.15 (dd, 1H), 7.60 (d, 2H), 7.50 (dd, 1H), 4.54 (s, 3H), 3.13 (s, 3H); 2.95 (s, 3H); FABMS m/e (M^+) 410. Anal. calcd. for $C_{17}H_{18}N_5O_3Cl_2I{\cdot}H_2O$: C, 36.71; H, 3.62; N, 12.59. Found: C, 36.67; H, 3.62; N, 12.51%.

*1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(4-methoxyphenyl) diazinecarboxamide Iodide (**12**).* This compound was prepared by the general procedure. The crude methiodide was precipitated by the addition of acetone, recrystallized from acetonitrile, and collected as orange prisms; yield 56%; mp 192–194°C: ^{1}H NMR (DMSO-d_6) δ: 11.58 (s, 1H), 9.1 (d, 1H), 8.79 (s, 1H), 8.55 (d, 1H), 8.18 (m, 2H), 7.46 (d, 2H), 6.89 (d, 2H), 4.51 (s, 3H), 3.73 (s, 3H), 3.09 (s, 3H), 2.92 (s, 3H); FABMS m/e (M^+) 372.2. Anal. calcd. for $C_{18}H_{22}N_5O_4I$: C, 43.29; H, 4.44; N, 14.02. Found: C, 43.16; H, 4.47; N, 14.03%.

*1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(2,4,6,richlorophenyl)diazine Iodide (**19**).* This compound was prepared by the general procedure. The crude methiodide was precipitated by the addition of acetone, recrystallized from ethanol, and collected as yellow needles; yield 90%; mp 198–200°C; NMR (DMSO-d_6) δ: 11.22 (s, 1H), 8.87 (d, 1H), 8.41 (d, 1H), 8.39 (s, 1H), 7.94 (dd, 1H), 7.85 (s, 2H), 4.3 (s, 3H), 2.89 (s, 3H), 2.83 (s, 3H); FABMS m/e (M^+) 401. Anal. calcd. for $C_{16}H_{16}N_4O_2Cl_3I$: C, 36.28; H, 3.04; N, 10.57; Cl, 20.08; I, 23.96. Found: C, 36.35; H, 3.07; N, 10.53; Cl, 20.04; I, 23.91%.

*1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(4-chlorophenyl)diazinecarboxamide Iodide (**11**).* This compound was prepared by the general procedure except that the crude methiodide was recrystallized from boiling methanol and collected as brown needles; yield 75%; mp 181–183°C; ^{1}H NMR (DMSO-d_6) δ: 10.63 (s, 1H), 8.04 (s, 1H), 7.98 (d, 1H), 7.55 (d, 1H), 7.11 (m, 2H), 6.57 (d, 2H), 6.34 (d, 2H), 2.02 (s, 3H), 1.87 (s, 3H), 1.45 (s, 3H); FABMS m/e (M^+) 376. Anal. calcd. for $C_{17}H_{16}N_5O_3ClI$: C, 40.53; H, 3.80; N, 13.90; Cl, 7.04; I, 25.20. Found: C, 40.64; H, 3.82; N, 13.91; Cl, 7.01; I, 25.09%.

*1-(1-Methyl-3-(N, N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2,6-dimethylphenyl) diazinecarboxamide Iodide (**9**).* This compound was prepared by the general procedure except that the crude methiodide was precipitated by the addition of 20 mL of ethyl acetate and 100 mL of water to the crude residue. The product was recrystallized from methanol and collected as orange prisms; yield 23%; mp 214–217°C; ^{1}H NMR

(DMSO-d_6) δ: 11.71 (s, 1H), 8.99 (d, 1H), 8.58 (d, 1H), 8.40 (s, 1H), 8.31 (s, 1H), 8.23 (m, 1H), 7.12 (s, 3H), 4.54 (s, 3H), 3.12 (s, 3H), 2.93 (s, 3H), 2.19 (s, 6H); FABMS m/e (M^+) 370. Anal. calcd. for $C_{19}H_{24}N_5O_3I \cdot I_2$: C, 30.36, H, 3.22; N, 9.32. Found: C, 30.68; H, 3.15, N, 9.36%.

1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2,6-difluorophenyl) diazinecarboxamide Iodide (**8**). This compound was prepared by the general procedure except that ice was added to the residue to precipitate the crude methiodide. The product was recrystallized from ethanol and collected as a red solid; yield 21%; mp 115–118°C; ^{1}H NMR (DMSO-d_6) δ: 11.79 (s, 1H), 9.01 (d, 1H), 8.68 (s, 1H), 8.60 (d, 1H), 8.34 (s, 1H), 8.22 (m, 1H), 7.50 (m, 1H), 7.22 (m, 2H), 4.5 (s, 3H), 3.12 (s, 3H), 2.95 (s, 3H); FABMS m/e (M^+) 378. Anal. calcd. for $C_{17}H_{18}N_5O_3IF_2 \cdot HI$: C, 32.24; H, 3.02; N, 11.06. Found: C, 32.07; H, 2.88; N, 10.83%.

1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2-ethoxyphenyl)diazinecarboxamide Iodide (**14**). This compound was prepared by the general procedure except that the crude methiodide was precipitated by the addition of ice to the residue. The product was then recrystallized from methanol and collected as yellow prisms; yield 91%; mp 194–196°C; ^{1}H NMR (DMSO-d_6) δ: 11.86 (s, 1H), 9.03 (d, 1H), 8.61 (d, 1H), 8.49 (s, 1H), 8.23 (s, 1H), 8.13 (m, 2H), 7.05 (m, 2H), 6.98 (m, 1H), 4.52 (s, 3H), 4.13 (q, 2H), 3.07 (s, 3H), 2.92 (s, 3H), 1.35 (t, 3H); FABMS m/e (M^+) 386. Anal. calcd. for $C_{19}H_{24}N_5O_4I$: C, 42.46; H, 4.50; N, 13.03. Found: C, 42.64; H, 4.59; N, 13.03%.

1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(4-fluorophenyl)diazine Iodide (**17**). This compound was prepared by the general procedure except that the crude methiodide was precipitated by ice. The product was then recrystallized from ethanol and collected as red prisms; yield, 94%; mp 265–267°C; ^{1}H NMR (DMSO-d_6) δ: 11.87 (s, 1H), 8.92 (d, 1H), 8.40 (d, 1H), 8.04 (s, 1H), 7.95 (dd, 1H), 7.21 (m, 4H), 4.42 (s, 3H), 3.06 (s, 3H), 2.98 (s, 3H); FABMS m/e (M^+) 317. Anal. calcd. for $C_{16}H_{18}O_2N_4FI.0.5H_2O$: C, 42.39; H, 4.22; N, 12.36. Found: C, 42.25; H, 4.22; N, 12.32%.

1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(3-nitrophenyl)diazinecarboxamide Iodide (**15**). This compound was prepared by the general procedure except that ice was used to precipitate the crude methiodide. The crude product was then dissolved in hot acetone, filtered and refrigerated. The analytically pure product was collected as a yellow solid; yield 25%; mp 200–202°C; ^{1}H NMR (DMSO-d_6) δ: 11.84 (s, 1H), 9.52 (s, 1H), 9.07 (d, 1H), 8.64 (d, and s, 2H), 8.22 (m, 2H), 8.05 (d, 1H), 7.94 (d, 1H), 7.63 (dd, 1H), 4.54 (s, 3H), 3.09 (s, 3H), 2.94 (s, 3H); FABMS m/e 387. Anal. calcd. for $C_{17}H_{19}O_5N_6I$: C, 39.70; H, 3.72; N, 16.34. Found: C, 39.92; H, 3.80, N, 16.13%.

1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(4-bromophenyl)diazinecarboxamide Iodide (**10**). This compound was prepared by the general procedure except that ice was added to the residue to precipitate the crude methiodide. The crude product was recrystallized from hot methanol and collected as red prisms; yield

86%; mp 202°C; ^{1}H NMR (DMSO-d_6) δ: 11.70 (s, 1H), 9.05 (s, 1H), 9.03 (d, 1H), 8.62 (d, 1H), 8.15 (m, 2H), 7.58 (m, 4H), 4.52 (s, 3H), 3.16 (s, 3H), 2.93 (s, 3H); FABMS m/e (M^+) 420. Anal. calcd. for $C_{17}H_{19}N_5O_3BrI$: C, 37.24; H, 3.49; N, 12.77. Found: C, 37.42; H, 3.59; 12.60%.

*1-(1-Methyl-3-)(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(1-napthyl)diazine-carboxamide Iodide (**13**).* This compound was prepared by the general procedure except that a 1:1 solution of methanol and diethyl ether was used to precipitate the crude methiodide. The crude product was recrystallized from hot methanol and collected as brown prisms; yield 30%; mp 190°C; ^{1}H NMR (DMSO-d_6) δ: 11.76 (s, 1H), 9.07 (m, 2H), 8.58 (d, 1H), 8.29 (s, 1H), 8.14 (m, 1H), 7.99 (m, 2H), 7.97 (d, 1H), 7.82 (d, 1H), 7.69 (m, 3H), 4.5 (s, 3H), 3.16 (s, 3H), 2.95 (s, 3H); FABMS m/e (M^+) 392. Anal. calcd. for $C_{21}H_{21}N_5O_3I$. $0.25H_2O$: C, 48.23; H, 4.43; N, 13.93; I, 24.26. Found: C, 48.10; H, 4.29; N, 13.36; I, 24.17%.

*1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(3-trifluoromethylphenyl)diazine Iodide (**20**).* This compound was prepared by the general procedure except that ice was used to precipitate the crude methiodide. The crude product was recrystallized from ethanol and collected as yellow prisms; yield 81%; mp 190–192°C; ^{1}H NMR (DMSO-d_6) δ: 11.87 (s, 1H), 8.82 (d, 1H), 8.35 (d, 1H), 8.01 (s, 1H), 7.88 (t, 1H), 7.50 (t, 1H), 7.30 (m, 3H), 4.34 (s, 3H), 2.94 (s, 3H), 2.85 (s, 3H); FABMS m/e (M^+) 367. Anal. calcd. for $C_{17}H_{18}N_4O_2F_3I$: C, 41.31; H, 3.67; N, 11.33; I, 25.67. Found: C, 41.55; H, 3.69; N, 11.29; I, 25.50%.

*1-(1-Methyl-3-(N,N-dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(4-isopropylphenyl) diazine Iodide (**18**).* This compound was prepared by the general procedure except that ice was used to precipitate the crude methiodide. The crude product was recrystallized from ethanol and collected as a bright orange solid; yield 71%; mp 204°C; ^{1}H NMR (DMSO-d_6) δ: 11.85 (s, 1H), 8.84 (d, 1H), 8.38 (d, 1H) 8.05 (s, 1H), 7.87 (m, 1H), 7.27 (d, 2H), 7.11 (d, 2H), 4.42 (s, 3H), 3.08 (s, 3H), 2.89 (s, 3H), 2.87 (m, 1H), 1.18 (d, 6H); FABMS m/e (M^+) 341. Anal. calcd. for $C_{19}H_{25}N_4O_2I$: C, 48.72; H, 5.38; N, 11.96; I, 27.09. Found: C, 48.78; H, 5.39; N, 11.92; I, 27.15%.

*1-(1-Methyl-3-(N, N-dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(2,3-dimethylphenyl) diazine Iodide (**16**).* This compound was prepared by the general procedure except that ice was used to precipitate the crude methiodide. The crude product was recrystallized from ethanol at rt and collected as orange prisms; yield 78%; mp 222°C; ^{1}H NMR (DMSO-d_6) δ: 11.09 (s, 1H), 8.85 (d, 1H), 8.38 (m, 2H), 7.87 (m, 1H), 7.14 (m, 2H), 6.90 (d, 1H), 4.39 (s, 3H), 3.01 (s, 3H) 2.95 (s, 3H), 2.27 (s, 3H), 2.22 (s, 3H); FABMS m/e (M^+) 327. Anal. calcd. for $C_{18}H_{23}N_4O_2I$: C, 47.58; H, 5.10; N, 12.33; I, 27.93. Found: C, 47.66; H, 5.13; N, 12.32; I, 28.00%.

Enzyme kinetics

Materials and Methods. Electric eel acetylcholinesterase and acetylthiocholine were purchased from Boehringer Mannheim, Indianapolis, IN. Ellman's Reagent or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from the Aldrich Chemical Co.,

Milwaukee, WI. Optical densities and initial rates were determined using a Beckman DU 650 spectrophotometer, Beckman Instruments Inc., Fullerton, California.

Enzymatic Studies. AChE was stored and assayed for the enzyme activity in a 0.1 M pH 7.0 phosphate buffer. A concentrated stock solution of the enzyme was prepared in this buffer and stored at 0°C. The acetylthiocholine stock solution (0.1 M) was prepared in the 0.1 M pH 7.0 phosphate buffer and was stored at 0°C. The DTNB (0.5 M stock solution prepared daily) and the inhibitors (0.02 M stock solutions) were dissolved in DMSO. Fresh inhibitor solutions were typically stored for no longer than 48 h at 0–5°C.

Preincubation Assay. The enzyme inhibition was analyzed either by the preincubation method or by the progress curve method.[18,19] In the preincubation method, AChE inhibition was initiated by adding 50 μL of the inhibitor solution in DMSO to 550 μL of an AChE solution prepared by diluting the stock AChE solution into the 0.1 M phosphate pH 7.0 buffer so that the enzyme solution contained 200–2850 units/mL of AChE. A unit of AChE is the amount of AChE necessary to hydrolyze 1 μM of acetylthiocholine per min at saturating concentration of substrate at 25°C and pH 7.0. Inhibitor concentrations in the inhibition mixture were 0.2 (**6**), 0.5 (**7**), 0.5, (**10**), 0.2 and 0.5 (**13**), 0.2 (**16**), 4 (**17**), 0.2 and 0.5 (**18**), 0.05 (**19**), 0.2 (**20**) mM. Aliquots (40 μL) were withdrawn at various time intervals and the reaction was stopped by 50-fold dilution into the 0.1 M phosphate pH 7.0 buffer solution (2 mL) containing the substrate acetylthiocholine (2 mM), DTNB (10 mM) and 2% DMSO. Residual activity was measured spectrophotometrically by following the change in absorbance at 412 nm at 25°C. Controls containing the same concentration of DMSO but no inhibitor showed no significant decrease in activity during the time course of the experiments. The inactivation pseudo first order rate constants k_{obs} were obtained from least-squares analysis of the semilogarithmic plots of the remaining activity vs. time. The results were analyzed according to the simplified model described by Kitz and Wilson (Kitz and Wilson, 1962) which implicates the formation of an enzyme-inhibitor complex (E·I) between the enzyme E and the inhibitor I prior to the formation of the covalently inactivated enzyme E-I (equation 1).

$$E + I \overset{K_I}{\leftrightarrows} E{\cdot}I \overset{k_2}{\leftarrow} E\text{–}I \tag{1}$$

This expression is obtained from the general reaction of an acylating agent with AChE, neglecting the deacylation rate k_3, where E-I is the acyl enzyme (equation 2).

$$E + I \underset{k_1}{\overset{k_{-1}}{\leftrightarrows}} E{\cdot}I \overset{k_2}{\leftarrow} E\text{–}I \overset{k_3}{\leftarrow} E + I' \tag{2}$$

The value for k_2/K_1 is approximated by $k_{obs}/[I]$ when [I] is less than K_1 (equation 3).

$$k_{obs} = k_2 \times [I]/(K_1 + [1]) \tag{3}$$

Progress Curve Method. The progress curves for the enzyme inactivation run in the presence of a chromogenic substrate were analyzed as described previously (Hart and O'Brien, 1973) according to equation 4.

$$E + P \overset{k_{cat}}{\leftarrow} E{\cdot}S \overset{K_M}{\leftrightarrows} S + E + I \overset{K_I}{\leftrightarrows} E{\cdot}I \overset{k_2}{\rightarrow} E\text{–}I \tag{4}$$

A 50 μL aliquot of AChE (20–30 units/mL final concentration), was added to a cuvet containing DTNB (10 mM), acetylthiocholine (2 mM), the inhibitor (**8**, 70–500 μM; **9**, 100–700 μM; **19**, 100–450 μM) and 7–9% DMSO in a 0.1 M pH 7.0 phosphate buffer. Controls containing no inhibitor showed no loss of activity during the time course of the experiment. The rate (v) of change in aborbance at 412 nm due to hydrolysis of acetylthiocholine and subsequent reduction of DTNB was obtained continuously using a computer-controlled spectrophotometer. The kinetic constants K_I and k_2 were obtained from a plot of $\Delta \ln v/\Delta t$ *vs.* $[I]/(I+[S]/K_M)$ using equation 5 where $-\Delta \ln v/\Delta t$ is the slope of the straight line formed by plotting $\ln v$ *vs.* time at a given inhibitor concentration.

$$\Delta \ln v/\Delta t = k_2 \times [I]'/(K_I + [I]') \tag{5}$$

where

$$[I]' = [I]/(I+[S]/K_M)$$

In equation 5 [I] and [S] represent the initial concentrations of the inhibitor and substrate. The Michaelis constant (K_M) for the substrate was determined and found to be 1.09 ($\pm$0.17) mM at pH 7.0 and 25°C. The least-squares fit of the data points to a $\Delta \ln v/\Delta t$ *vs.* $[I]/(I+[S]/K_M)$ hyperbolic plot was calculated using Kaleidagraph (version 3.0.1, Abelbeck Software). The best-fit values of K_I and k_2/K_I were obtained and are reported in the Tables (k_2/K_I values are reported as $k_{obs}/[I]$).

Some of the inhibitors appeared to be reversible and competitive during the initial assay period. We observed this when the enzyme's initial velocity was constant during the assay time period (0–3 min) and less than the substrate hydrolysis rate in the same assay without inhibitor (control). The dissociation constants (K_I) of the enzyme/inhibitor complexes were determined using the following mathematical treatment. The initial velocity of substrate hydrolysis in the presence of a competitive inhibitor is represented by (equation 6). A plot of inverse initial velocity vs. inhibitor concentration gives a straight line (equation 7).

$$v = \frac{V_M \times [S]}{K_M \times (1 + [I]/K_I + [S])} \tag{6}$$

$$\frac{1}{v} = \frac{K_M}{V_M}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V_M} \tag{7}$$

The K_I values were calculated by an iterative least squares fit of the experimental data to equation 7 using fixed values for V_M and K_M, which were determined in a

separate experiment by standard methods. Experimental conditions in those assays were: 5–30 units/mL AChE; inhihitors, 0.08–0.4 (**7**), 0.1–0.7 (**10**), 0.2–1.0 (**11**), 0.4–2.0 (**12**), 0.1–0.7 (**14**) mM; 7–9% DMSO, 0.1 M pH 7.0 phosphate buffer at 25°C.

Hydroxylamine reactivation assays were performed by treatment of the inactivated enzyme solution with the nucleophile hydroxylamine (0.5 M, final concentration) at pH 7.0 and 25°C for 30 min. The enzyme activity of a control solution containing no inhibitor and the enzyme-inhibitor solution were assayed as described above.

Partition coefficient analysis

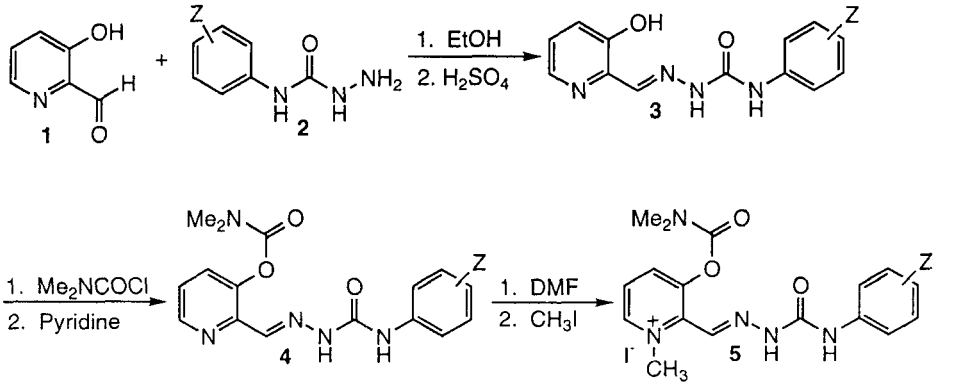
Materials and Methods. The partition coefficient analysis for all of the methiodides were determined according to a modification of previously published procedures using a Beckman DU 650 spectrophotometer.[20] A 50 μM solution of the inhibitor (10 μL) aliquot of a 5 mM stock solution in DMSO was added ts 990 μL solution of octanol) was prepared and the wavelength of the maximum absorbance was determined. The value for the maximum absorbance was then substituted into the equation $A=\epsilon lc$ (Beer-Lambert Law) and the extinction coefficient was determined. A 50 μM solution of the inhibitor (100 μL aliquot of the stock solution) was added to a separatory funnel which contained 5 mL of water and 5 mL of octanol. The separatory funnel was shaken vigorously. The absorbance of a 1000 μL sample of the octanol layer was determined at the previously chosen wavelength The partition coefficient was then calculated as the ratio [A]octanol/[A]water.

RESULTS AND DISCUSSION

Numerous carbamylated inhibitors of AChE have been reported previously.[4–7] In this study, we have synthesized and tested fourteen novel carbamates which are analogs of **6** (MHP 133). Those compounds were studied enzymatically and found to be irreversible inhibitors of AChE. The influence that substituents on the phenyl moiety of **6** have on its inhibitory potency and hydrophobicity have been investigated. We have simultaneously attempted to add the structural features of clonidine to some of our new carbamate analogs.

Chemistry

The synthesis of phenylsemicarbazone analogs of **6** is outlined in Scheme 1. The synthesis of 3-hydroxy-2-pyridinecarboxaldehyde **1** has been previously reported.[10] Substituted phenylsemicarbazides **2** were prepared by a modification of published procedures which involves the reaction of the appropriate substituted phenylisocyanate with excess hydrazine monohydrate.[11] The semicarbazide was condensed with **1** to give **3**.[16] The pyridylhydroxy group of **3** was carbamylated by reacting a 3–5 molar excess of dimethylcarbamyl chloride and pyridine to give **4**.[17] The pyridyl methiodides (**5**) were prepared by reacting **4** with a 5–10 molar excess of iodomethane in DMF.[17] The analytically pure products were obtained by recrystallization from various solvents.



SCHEME 1 Synthesis of novel pyridinium acetylcholinesterase inhibitors.

Inhibitory potency

The values of the inhibition constants K_I and second order rate constants $k_{obs}/[I]$ for phenylsemicarbazones are shown in Table 1. The data for the phenylhydrazone derivatives is shown in Table 2. The novel pyridinium carbamates were analyzed for their inhibitory potency against electric eel acetylcholinesterase by the progress curve assay, the dilution assay or both and compared to compound **6** (K_I=69 μM, MHP 133) as a means to predict their relative efficacy. Overall the phenylsemicarbazone derivatives are a more reactive family of inhibitors than the phenylhydrazones.

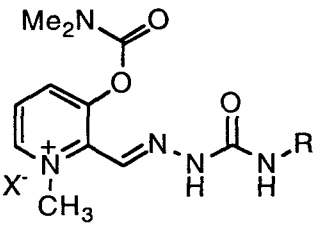
Three compounds have clonidine like structural features and contain 2,6-disubstituted phenyl groups. Of those three compounds, compound **8** (K_I=39μM) was the most reactive *in vitro* compared to **7** (K_I = 290 μM) and **9** (K_I=>240 μM). Compound **8** is a 2,6-difluoro substituted analog which has differing halogen atoms compared to the more similar clonidine analog **7**.

Other reactive inhibitors were compounds **14** (K_I=68 μM) and **15** (K_I=25 μM). Both compounds are endowed with single substituents at positions on their phenyl ring that no other compounds in the family contain. A better assessment of the importance of substitutions at the 2 and 3 positions of pyridinium phenyl semicarbazones for optimal AChE inhibition could be made by synthesizing derivatives such as the 2-halogen and 3-halogen analogs.

Compounds **10**, **11**, **12**, and **13** are all substituted at the para positions. Of these four analogs compound **10** was the best (K_I=130 μM) and **12** was the worst (K_I=660 μM). Compound **10** is comparable in reactivity to **11** but nevertheless neither of these analogs improve the inhibitory potency of **6** *in vitro*.

The second order rate constants of compounds **16**, **18**, and **20** were poor. Compound **19** (K_I=120 μM) was the most reactive phenylhydrazone inhibitor as well as being one of the more reactive inhibitors overall. The K_I of the remaining phenylhydrazones were unobtainable under the reaction conditions described.

TABLE 1
Inhibition of electric eel acetylcolinesterase by pyridinium phenylsemicarbazones.



Compound	R	K_I (μM)	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	p[a]
6	phenyl (MHP 133)	69[b]	2.5[c]	0.091
7	2,6-dichlorophenyl	290[b]	3.2[c]	0.020
8	2,6-difluorophenyl	39[b]	345.0[b]	0.121
9	2,6-dimethylphenyl	>240[b]	20.0[b]	0.144
10	4-bromophenyl	130[b]	3.0[c]	0 087
11	4-chlorophenyl	137[b]		0.012
12	4-methoxyphenyl	660[b]		0.062
13	l-napthylphenyl	490[c]	65.0[c]	0.087
14	2-ethoxyphenyl	68[b]		0.123
15	3-nitrophenyl	25[b]		0.078

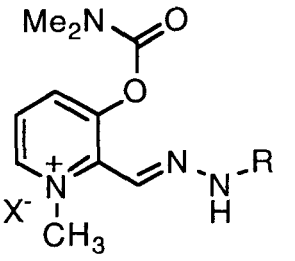
For compound **6X** = Cl^-; for compounds **7–15X** = I^-.

[a]P = [drug]lipid/ [drug]water. [b]Reaction conditions for the progress curve assay were 0.1 M sodium monophosphate buffer pH 7.0, 2 mM acetylthiocholine, 10 mM DTNB, 50–700 μM of the inhibitor and 5–10% DMSO at 25°C. [c]Reaction conditions for the inhibition mixture in the preincubation method were: inhibitor (0.05–5 mM), AChE (200–2850 units/mL) in 0.1 M sodium phosphate buffer at pH 7.0 containing 12% DMSO. In the assay mixture the concentrations were: AChE (4/57 units/mL), acetylthiocholine (2 mM), DTNB (10 mM) and 2% DMSO in 2 mL of a 0.1 M sodium phosphate buffer at pH 7.0.

Partition coefficient

The partition coefficient of a drug is defined as the equilibrium constant: P=[drug] lipid/[drug]water.[21] Partition coefficients are shown in Tables 1 and 2 for each phenylsemicarbazone and phenylhydrazone. In general the phenylhydrazones were more hydrophobic than the phenylsemicarbazones. The partition coefficients of the phenylsemicarbazones ranged from 0.012, for the 4-chloro analog; to 0.122, for the 2,6-difluoro; to 0.123 for the 2-ethoxy analog. The partition coefficient of the phenylhydrazones ranged from 0.121, for the 4-fluoro analog (NI at 1 mM); to 0.492 for the 4-isopropyl analog. The partition coefficients for each of the most reactive compounds were greater than **6** (P=0.091) except for the 3-nitro analog (P=0.078).

TABLE 2
Inhibition of electric eel acetylcholinesterase by pyridinium phenylhydrazones.



Compound	R	K_I (μM)	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	p[a]
16	2,3-dimethylphenyl		2.0[c]	0.159
17	4-fluorophenyl		NI[c]	0.121
18	4-isopropylphenyl		1.0[c]	0.492
19	2,4,6-trichlorophenyl	120.0[b]	15.0[c]	0.434
20	3-trifluoromethylphenyl		1.5[c]	0.282

For compounds **16–20** X = I^-.

[a]P=[drug]lipid/ [drug] water. [b]Reaction conditions for the progress curve assay were 0.1 M sodium monophosphate buffer pH 7.0, 2 mM acetylthiocholine, 10 mM DTNB, 50–700 μM of the inhibitor and 5–10% DMSO at 25°C. [c]Reaction conditions for the inhibition mixture in the preincubation method were: inhibitor (0.05–5 mM), AChE (200–2850 units/mL) in 0.1 M sodium phosphate buffer at pH 7.0 containing 12% DMSO. In the assay mixture the concentrations were: AChE (4/57 units/mL), acetylthiocholine (2 mM), DTNB (10 mM) and 2% DMSO in 2 mL of a 0.1 M sodium phosphate buffer at pH 7.0.

Mechanism of inhibition

Carbamates inhibit AChE by reacting covalently with the active site serine.[7] Furthermore, the active site triad of Glu-Ser-His, of electric eel AChE has been described as being near the bottom of a deep and narrow "aromatic gorge" (consisting of approximately 40% aromatic residues) which has an electrostatic dipole that facilitates the movement of the positively charged subrate (acetylcholine) down the gorge through charge-charge interactions with the π systems of the aromatic residues.[22]

We propose the movement of our analogs down the active site gorge is due to charge-charge interactions as well as simultaneous preferred flat surface-flat surface aromatic interactions. The enzyme is apparently more able to adapt to the greater electron withdrawing 2,6-difluoro group than to the 2,6-dichloro analog; possibly because of the degree to which fluorine increases the charge on the aromatic ring making the inhibitor more electrostatically attractive to the bottom of the "aromatic gorge" where the active site machinery is located. The inhibition constant ($k_{obs}/[I]$) of the 2,6-dichloro analog does not however differ substantially from the unsubstituted analog, which may indicate steric conflicts between the 2,6-dichloro derivative and the

enzyme. The enzyme also has an affinity for phenylsemicarbazones substituted at the 2- or 3-position i.e. the 2-ethoxy and 3-nitro analogs. It is difficult though to make an argument for either position as being preferred over the other because these two compounds were the only ones of their family substituted solely at those positions. In general, the phenylhydrazones are much less reactive than the phenylsemicarbazones.

Summary

The synthesis of fourteen novel irreversible inhibitors of AChE, which are analogs of **6**, has been accomplished in 4–5 steps. The inhibitory potency of these new compounds are described by the inactivation rate constant ($k_{obs}/[I]$), the enzyme-inhibitor dissociation constant (K_I), or both. The partition coefficients of these analogs were determined in order to assess the ease with which these compounds might cross the blood brain barrier.

Compound **8** or 1-(1-methyl-3-(*N,N*-dimethylcarbamoyloxy)-2-pyridylmethylene)-4-(2,6-difluorophenyl)diazinecarboxamide iodide was the most potent compound which we discovered with a K_I=39 μM and $k_{obs}/[I]$=345 $M^{-1}s^{-1}$ and was slightly more hydrophobic than **6** (P=0.121). The phenylhydrazone **19**, or 1-(1-methyl-3-(*N,N*-dimethylcarbamoyloxy)-2-pyridylmethylene)-2-(2,4,6,trichlorophenyl)diazine iodide (K_I=120 μM, and $k_{obs}/[I]$=15 $M^{-1}s^{-1}$ is an effective inhibitor and also very hydrophobic (approximately 5 fold greater than **6**, P=0.434). Neither of our analogs are as potent as physostigmine or pyridostigmine *in vitro*, which are both highly toxic. However, we are more concerned with improving the efficacy of analogs of compound **6** in Alzheimer's models since the effectiveness of **6** appears not to be solely related to its ability to inhibit AChE. We plan to test some of the novel carbamate inhibitors in Alzheimer's disease models since they are more hydrophobic than **6** and are also more potent AChE inhibitors.

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