Pyridophens: Binary Pyridostigmine−Aprophen Prodrugs with Differential Inhibition of Acetylcholinesterase, Butyrylcholinesterase, and Muscarinic Receptors

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A series of "binary prodrugs" called carbaphens,[1] carbamylated derivatives on one or both of the aromatic rings of the muscarinic receptor antagonist aprophen [(*N*,*N*-diethylamino)ethyl 2,2-diphenylpropionate], were synthesized to develop binary prophylactic agents against organophosphorus intoxication. As a group, the carbaphens retained the muscarinic receptor antagonist properties of aprophen but also preferentially inhibited butyrylcholinesterase (BChE) in contrast to acetylcholinesterase (AChE). Therefore, a new series of compounds named pyridophens were designed and synthesized to achieve binary prodrugs to preferentially inhibit AChE over BChE, while still retaining the muscarinic receptor antagonism of aprophen. The pyridophens consist of the basic pyridostigmine skeleton combined with the 2,2-diphenylpropionate portion of aprophen by replacement of the diethylamino group. Three compounds, **9** (a tertiary pyridine), **10** (a quaternary pyridine), and **12** (a tertiary tetrahydropyridine), were found to be effective inhibitors of both BChE and AChE. However, **10**, *N*-methyl-3-[[(dimethylamino)carbonyl]oxy]-2-(2′2′-diphenylpropionoxy-methyl)pyridinium iodide, inhibited AChE selectively over BChE, with a bimolecular rate constant similar to pyridostigmine. In contrast to their potent cholinesterase inhibitory activity, all of the pyridophen analogues were less potent antagonists of the muscarinic receptor than aprophen.

Introduction

Current prophylactic and therapeutic regimens for the protection against organophosphate (OP) poisoning include a pretreatment regimen consisting of (i) pyridostigmine, the reversible, covalent acetylcholinesterase (EC 3.1.1.7; AChE) inhibitor,[2−4] (ii) the muscarinic receptor antagonist atropine (ATR),[5] and (iii) the AChE reactivator pralidoxime chloride.[3,6] Additional treatment with diazepam has proven advantageous in attempts to prevent convulsions.[7−9] These regimens provide extensive protection against OP intoxication in animals. However, treatment might be considerably simplified if drugs that possessed multiple protective functions were used. For example, a single drug that exhibited both reversible antimuscarinic (in place of ATR) and reversible anticholinesterase (in place of pyridostigmine) properties might be of unusual utility and efficacy, simultaneously protecting AChE from an irreversible OP inhibition and the occupation of muscarinic receptors by accumulated acetylcholine. To test this hypothesis, a group of experimental binary prodrugs, the carbaphens, were synthesized and evaluated.[1] These compounds, which contain a carbamyl substituent on one or the other aromatic rings of aprophen, a muscarinic receptor antagonist,[11] retained the antimuscarinic activity of aprophen but inhibited butyrylcholinesterase (EC 3.1.1.8; BChE) preferentially in comparison with AChE.[10] In view of these findings, the pyridophen analogues

were designed and synthesized to achieve preferential inhibition of AChE over BChE, while retaining antimuscarinic activity. We describe here (i) the synthesis of pyridophens, (ii) their anticholinesterase properties, and (iii) their antimuscarinic activity.

Chemistry

To synthesize the pyridophens, the pyridostigmine skeleton was introduced and combined with the basic structure of aprophen, leading to the quaternary compound **10** and to the tertiary tetrahydropyridine analogue **12**. The synthesis is outlined in Figure 1. The key intermediate, 2-hydroxymethyl-3-dimethylaminocarbonyl-oxypyridine (**2**), was isolated by column chromatography as the major product from the carbamylation of 3-hydroxy-2-hydroxymethylpyridine HCl (**1**) with dimethylcarbamyl chloride. The other two products isolated from the reaction mixture were found to be the isomeric carbamate **3** and the bis-carbamate **4**. Structural elucidation of the isomers **2** and **3** was based on the ¹H nuclear magnetic resonance (NMR) spectrum of these two compounds.

Acylation of **2** with 2,2-diphenylpropionyl chloride (**6**) afforded the carbamyl-ester (**9**) in moderate yield. However, **9** could be obtained through an alternative pathway by acylation of **1** with the acyl chloride **6**, followed by carbamylation of the hydroxy ester **7** by dimethylcarbamyl chloride; this alternative route was found to give a higher yield. Carbamylation of **7** with methylisocyanate afforded the monomethylcarbamate **8**.

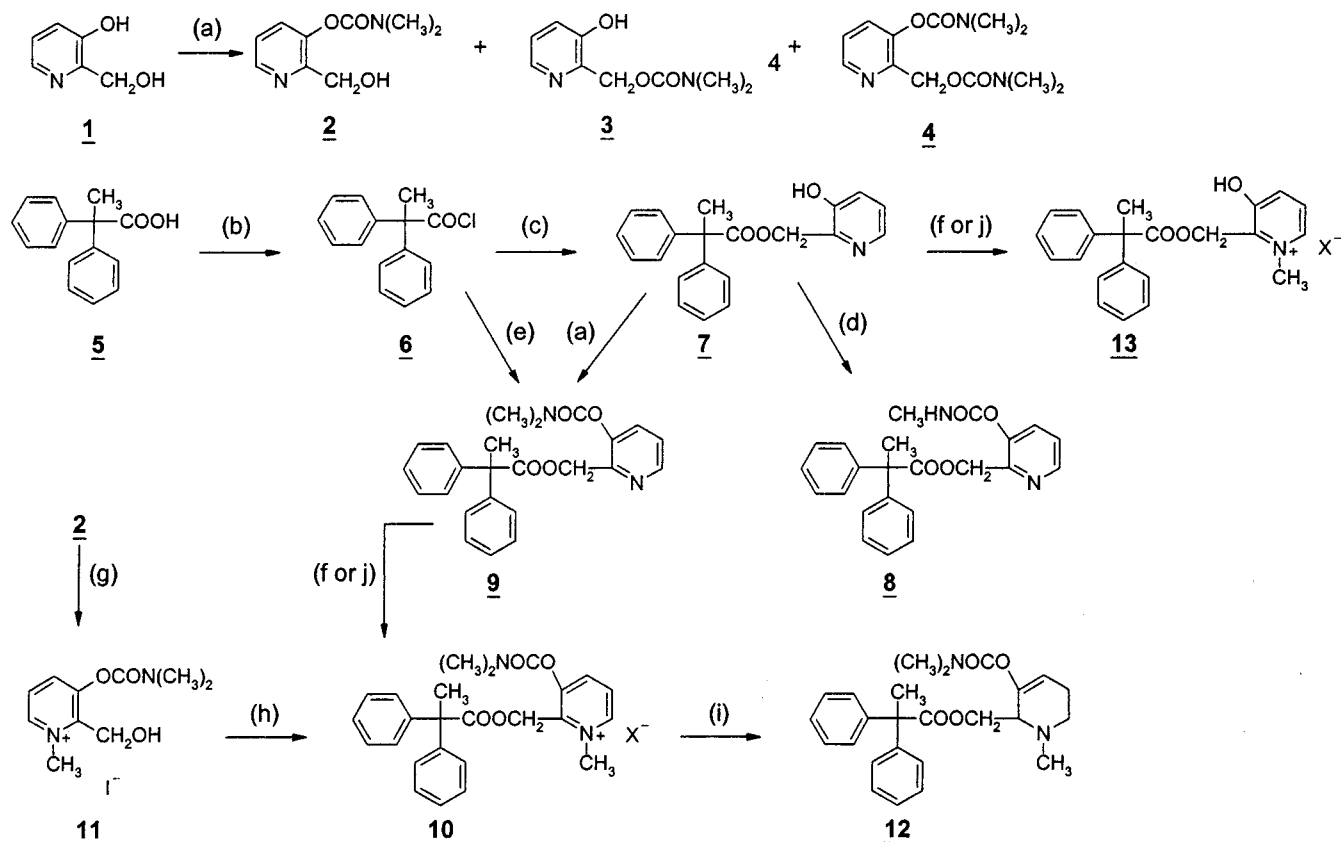The quaternary carbamate **10** (X = I⁻) was obtained by methylation of **9** with methyl iodide in dry acetone.

Figure 1. Synthetic pathways for the pyridophen analogues. Key: (a) $(CH_3)_2NCOCl$. (b) $SOCl_2$. (c) **1**, triethylamine, benzene. (d) CH_3NCO, benzene. (e) **2**, pyridine. (f) CH_3I or $(CH_3O)_2SO_2$, acetone. (g) CH_3I, acetone. (h) **6**, pyridine. (i) $NaBH_4$, methanol. (j) $(CH_3)_2SO_2$, acetone, X^- is I^- or $[CH_3SO_4]^-$.

The same quaternary compound could be obtained directly from the methyl pyridinium carbamate **11** (obtained by methylation of **2** with methyl iodide) with the acyl chloride **6** in pyridine. The two products were identical, thus verifying the structural elucidation of the carbamates **2**, **9**, and **11**. Also, the quaternary carbamate **10** ($X = ^-OSO_2OCH_3$) was obtained by methylation of **9** with $(CH_3O)_2SO_2$ in dry acetone. The latter product was identical in all biological assays and offered two advantages: the quaternization was faster, and the product was more stable in solution.

Reduction of the carbamylated methylpyridinium iodide **10** with $NaBH_4$ in methanol afforded the tetrahydropyridine analogue **12**. Here again, the ^1H NMR spectrum, including homodecoupling irradiation techniques, was the major basis for the elucidation of the structure and assignment for the location of the double bond in the tetrahydropyridine structure.

Biological Activities

I. AChE and BChE Inhibition. A comparison of the inhibitory potency of the respective compounds (Figure 1) with AChE (hatched bar) and BChE (solid bar) is illustrated in Figure 2. In this comparison, each enzyme was incubated with a concentration of 10^{-4} M of the appropriate compound for 1 h at 2 °C. AChE was inhibited most strongly by **10**, while BChE was inhibited most strongly by **9** and **12**. Compounds **2**, **3**, and **11** also inhibited AChE preferentially in comparison with BChE, although such inhibition was considerably less than that exhibited by **10** (Figure 2). Compounds **7** and **13**, which lack the pyridostigmine skeleton, were without effect
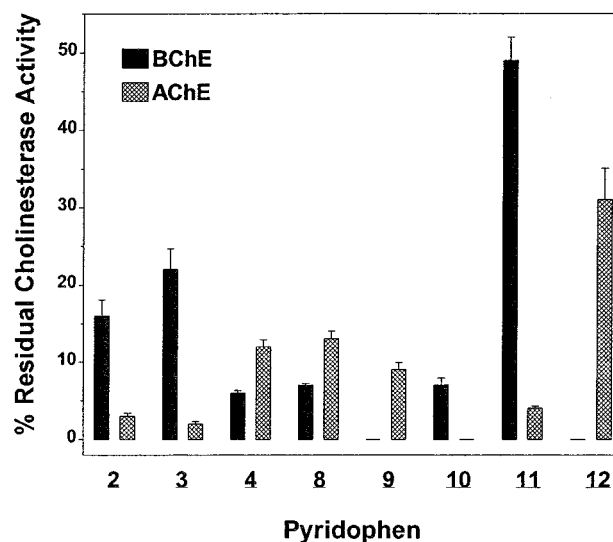

Figure 2. Pyridophen inhibition of BChE and AChE. This histogram compares the inhibitory activity of the pyridophens with AChE (hatched) and BChE (solid). The ordinate represents the percent residual cholinesterase activity. Assay conditions were as follows: compound concentrations, 10^{-4} M; enzyme concentrations, AChE = 10 U and BChE = 15 U; incubation, 1 h at 25 °C in a final volume of 100 μL of 0.05 M phosphate buffer, pH 8.0. Each value represents the mean of 3–5 independent assays ± SEM.

at these concentrations. Table 1 presents a summary of the kinetic and affinity constants derived from (i) time-dependent enzyme incubation with graded concentrations of **2**, **9**, **10**, **12**, and pyridostigmine (i.e.,

Table 1. Kinetic and Equilibrium Constants for AChE and BChE[a]

compound	K_i (M)	k_2' ($M^{-1}\,min^{-1}$)	k_{2c} (min^{-1})
	AChE		
2	5.21×10^{-5}	1.25×10^3	0.07
9	3.88×10^{-5}	2.78×10^4	1.08
10	5.62×10^{-6}	2.28×10^5	1.28
12	4.59×10^{-5}	8.45×10^3	0.39
pyridostigmine[b]	1.97×10^{-5}	2.91×10^5	5.78
	BChE		
2	1.38×10^{-3}	7.87×10^2	1.08
9	8.51×10^{-6}	3.43×10^5	2.91
10	3.14×10^{-6}	9.38×10^3	0.03
12	5.53×10^{-6}	2.55×10^5	1.41
pyridostigmine	1.14×10^{-4}	4.56×10^3	0.52

[a] Each constant represents the mean of 3−5 independent experiments, and $r^2 \geq 0.95$ for all primary and secondary plots from which the constants were derived (SEM ≤ 13%). [b] Data from ref 11.
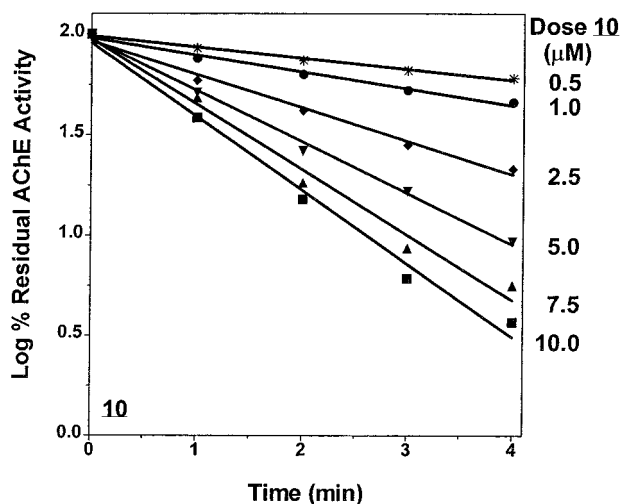

Figure 4. Typical secondary plots of pseudo-first reaction curves for doses of **10** (upper curve) and pyridostigmine[12] (lower curve); linear regression coefficients were ≥0.99. Kinetic and affinity constants derived from eq 2 are reported in Table 1. *Y*-axis, reciprocal of the reaction velocity × the concentration of the inhibitor; *X*-axis, reciprocal of the apparent equilibrium constant (molarity). AChE concentration for **10** and pyridostigmine was 11 and 40 U/mL, respectively.

Figure 3. Time-dependent inhibition of AChE by **10**. *Y*-axis, log percent residual activity; *X*-axis, time in minutes. The linear regression coefficients for the six doses in this typical experiment were ≥0.99.

Figure 3 illustrates the inhibition of AChE by **10**) and (ii) secondary plots[11] of such data (i.e., Figure 4 shows the effect of **10** and pyridostigmine on AChE). Compound **10** preferentially inhibited AChE in comparison with BChE and inhibited both cholinesterases at least as strongly as pyridostigmine.[12] A comparison of the affinity constant (K_i) values for the initial reaction (eq 1, Table 1) of **10** and pyridostigmine with both enzymes shows **10** to bind more tightly to these enzymes than pyridostigmine, whereas differences in inhibition occur through differences in the carbamylation rates (k_{2c}; release of the alcoholic moieties from the enzyme−inhibitor [E·I] complexes). The rate of carbamylation of AChE by **10** was 44 times greater than observed with BChE.

Bimolecular rate constants (k_2' = affinity constant/carbamylation rate; eq 1, Table 1) for **9** and **12** revealed that these compounds react 10 and 3 times more strongly with BChE than with AChE, respectively. The affinity of **9** was 20 times greater for BChE than for AChE, while its carbamylation rate with BChE was twice its carbamylation rate for AChE. The K_i of **12** was 10-fold greater for BChE than for AChE, while its carbamylation rate was 3.5-fold greater with BChE than AChE.
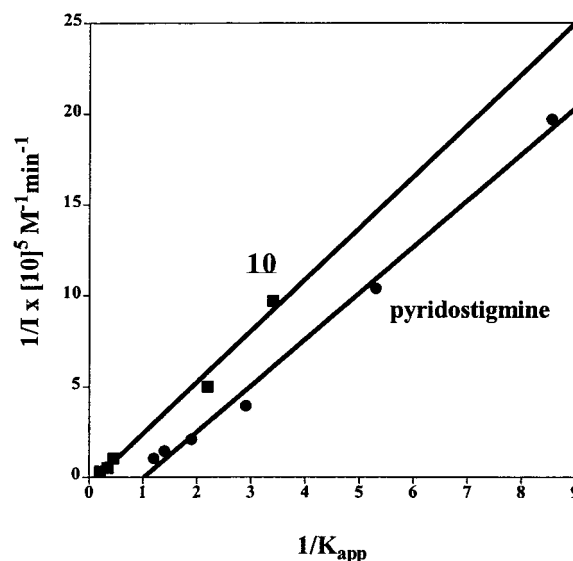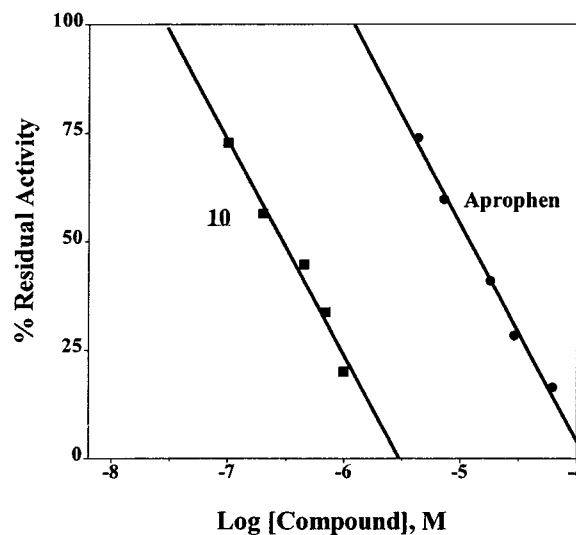

Figure 5. Dose response inhibition curve of BChE by **10** (left) and aprophen (right). The reaction was initiated by the addition of 15 U of BChE to Ellman assay mixtures. *Y*-axis, percent residual enzyme activity; *X*-axis, log of the molar concentration of **10** or aprophen. The representative experiment yielded linear regression coefficients of ≥0.98.

To detect noncovalent, reversible enzyme inhibition by the compounds, they were evaluated by inclusion in the colorimetric assay solution,[13] rather than by preincubation with enzyme. Aprophen is a BChE inhibitor of this type;[14] it lacks a carbamyl function; therefore, its ability to inhibit BChE depends on ionic and hydrophobic interactions. BChE was completely inhibited by **10** in this assay, whereas **9** and **12** inhibited BChE to the same extent as aprophen. Figure 5 compares dose response curves for reversible BChE inhibition by **10** and aprophen: the 50% inhibitory concentration (I_{50}) of **10** was 1.7×10^{-7} M, while the I_{50} of aprophen was 6.7×10^{-6} M.[14] Thus, the apparent hydrophobic and

ionic affinity of **10** for BChE was 40-fold greater than that of aprophen, and the observed I_{50} for aprophen was in agreement with a previous report.[14] For these compounds, only results with BChE are shown, since AChE failed to undergo this type of inhibition.

Strong, preferential inhibition of AChE occurred with compound **10**, and in structure and activity, **10** more closely resembled its progenitors, pyridostigmine and aprophen, than other compounds in this series. Compound **10** binds strongly to AChE through a time-dependent carbamylation reaction similar in magnitude to the one between AChE and pyridostigmine. Also, **10** binds strongly to BChE both covalently, as does pyridostigmine, and noncovalently through hydrophobic and ionic interactions similar to the binding of aprophen to BChE. Likewise, **9** and **12** inhibited BChE noncovalently (not shown), with affinities similar to aprophen.

The basis for preferential BChE inhibition by aprophen and its analogues now appears explicable based on the structure and properties of chimeric AChE mutants.[15–17] In one group of mutants, the conversion of TYR_{337} (mouse AChE) or PHE_{330} (*Torpedo californica* AChE) to ALA was accompanied by a concomitant increase in the affinity of acridines and phenothiazines to that which occurs with BChE.[16] In other mutants, replacement of PHE_{295} and PHE_{297} with aliphatic residues releases constraints upon the acyl pocket, bestowing BChE-like properties upon the chimeras.[15–17] Such mutants provide the basis for an experimental resolution of the preferential inhibition of BChE as opposed to AChE by aprophen and other benzilic acid esters.

Compound **10** contains both the *N*-methyl pyridinium ring and the dimethylaminocarbamyl substituent of pyridostigmine in contrast to **8**, which contains a monomethylamino carbamyl substituent but lacks a quaternary ring nitrogen, while **9** lacks a quaternary ring nitrogen. Replacement of the pyridinium ring of **10** with a partially saturated structure in **12** also resulted in the absence of a quaternary ring nitrogen. These changes indicate that both the quaternary ring nitrogen and the presence and position of the dimethylaminocarbamyl substituent of pyridostigmine found in **10** are necessary for a strong inhibition of AChE, and each change in this structure reduces activity against AChE (Figure 2). Thus, the presence in **11** of a hydroxymethyl group in the 2-position of pyridostigmine reduced activity. While **11** preferentially inhibited AChE, this inhibition was very weak in comparison with **10**. The presence of the 2,2-diphenylpropionic acid substituent of aprophen in **8**–**10** and **12** also caused a significant increase in hydrophobic, noncovalent reversible inhibition of BChE.

II. Muscarinic Receptor Inhibition. Table 2 lists the K_i values obtained for the inhibition of [³H]-NMS (*N*-[³H]methylscopolamine) binding to mAChR by compounds **7**–**10**, **12**, and **13**; the K_i values were 75, 29, 42, 4.7, 8.1, and 2.6 μM, respectively. The Hill slopes for the inhibition curves were not significantly different from 1, as shown in Figure 6 for **10** and **12**. In contrast to these pyridophen analogues, both **11** and pyridostigmine at a dose of 100 μM minimally inhibited [³H]-NMS binding to mAChR (less than 20%). Also shown for reference in Table 2 are the K_i values for the inhibition of mAChR obtained for aprophen and the muscarinic

Table 2. Antimuscarinic Activity of Pyridophen Analogues and Standard Compounds

compound	K_i (M) $\pm$ SD[a]	compound	K_i (M) $\pm$ SD[a]
7	$7.5 \pm 1.1 \times 10^{-5}$	**12**	$8.1 \pm 1.8 \times 10^{-6}$
8	$2.9 \pm 1.6 \times 10^{-5}$	**13**	$2.6 \pm 0.9 \times 10^{-6}$
9	$4.2 \pm 1.7 \times 10^{-5}$	pyridostigmine	$> 10^{-4}$
10	$4.7 \pm 1.4 \times 10^{-6}$	aprophen[b]	$5.1 \pm 1.0 \times 10^{-8}$
11	$> 10^{-4}$	ATR[b]	$2.4 \pm 0.7 \times 10^{-9}$

[a] [³H]-NMS binding to cerebral cortex membranes. Each inhibition constant represents the mean of 3–6 independent experiments $\pm$ SD of the mean. The Hill slopes for all compounds were not significantly different from 1. [b] From ref 1.
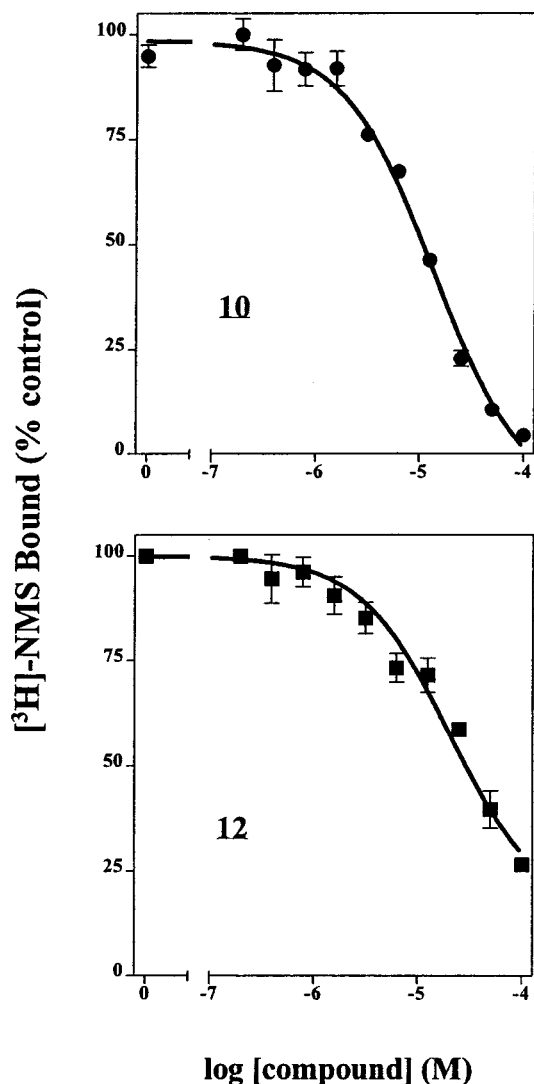

Figure 6. Displacement of [³H]-NMS bound to cortex mAChR by pyridophes **10** (top) and **12** (bottom). *Y*-axis, percent control [³H]-NMS bound; *X*-axis, log concentration (M) of pyridophen **10** or **12**. Each point represents the mean $\pm$ SD of three replicates.

antagonist ATR.[1,10] Both the iodide and the methyl sulfate of **10** and **13** yielded similar K_i values and are not reported separately.

Next, the possible effects of **10** and **12** on the dissociation kinetics of [³H]-NMS binding to the mAChR in cortex tissue were evaluated. A decrease in the rate of dissociation of [³H]-NMS from mAChR would indicate an allosteric interaction on mAChR by these compounds in addition to inhibition at the antagonist binding site.[18–20] The dissociation of bound [³H]-NMS in the
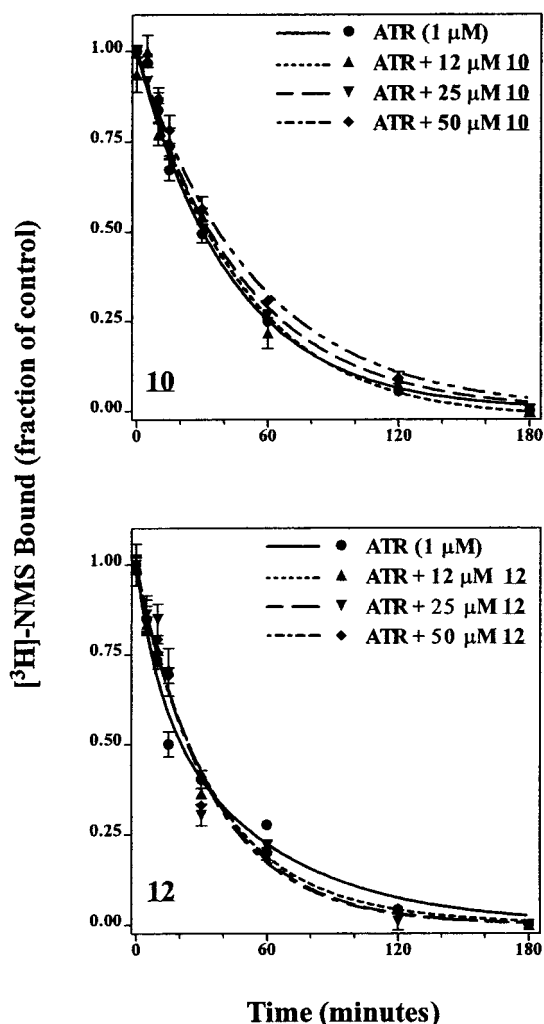
Figure 7. Time course for the dissociation of bound [3H]-NMS from cortex mAChR. Dissociation was measured in the presence of 1 μM ATR alone or 1 μM ATR and indicated doses of **10** or **12**. Each point represents the mean $\pm$ SD of duplicate experiments each in triplicate; (top) **10**, (bottom) **12**. *Y*-axis, fraction of control [3H]-NMS bound; *X*-axis, time in minutes.

presence of ATR alone or ATR combined with graded doses of **10** or **12** are shown in Figure 7, top and bottom, respectively. The dissociation rate constants obtained from these figures by nonlinear curve fitting are reported in Table 3. The dissociation of the [3H]-NMS–receptor complex in the presence of ATR alone was biphasic in the cortex membrane (Table 3; the portion of muscarinic receptors possessing a fast dissociation constant is the A fraction, as previously observed).[20] However, the dissociation curves obtained when **10** or **12** were added together with 1 μM ATR remained unaltered (Figure 7, Table 3) since the curves did not shift. An upward shift would indicate a slowing of the dissociation of [3H]-NMS from the muscarinic receptor.[18,20] Because neither **10** nor **12** had an affect on the k_{-1} or k_{-2} dissociation rate constants (Table 3) and the Hill slopes (Figure 6) were not significantly different from 1, these compounds did not allosterically modulate [3H]-NMS binding in cortex but rather interacted only at the [3H]-NMS antagonist binding site.

The potency order of the six pyridophen analogues as antagonists of mAChR was as follows: **13** $\sim$ **10** $\sim$ **12** > **8** $\sim$ **9** $\sim$ **7** (Table 2). The potency of the pyridophen

Table 3. Dissociation Constants for [3H]-NMS Binding to Guinea Pig Cerebral Cortex Muscarinic Receptors[a]

| | | dissociation rate constants (min^{-1}) | | |
compd(s)	μM	k_{-1}	k_{-2}	A fraction
ATR	0	0.095 $\pm$ 0.011	0.018 $\pm$ 0.005	0.44 $\pm$ 0.07
ATR + **10**	12	0.082 $\pm$ 0.005	0.015 $\pm$ 0.007	0.47 $\pm$ 0.10
ATR + **10**	25	0.079 $\pm$ 0.007	0.013 $\pm$ 0.009	0.49 $\pm$ 0.09
ATR + **10**	50	0.089 $\pm$ 0.013	0.020 $\pm$ 0.009	0.49 $\pm$ 0.09
ATR	0	0.108 $\pm$ 0.002	0.025 $\pm$ 0.008	0.51 $\pm$ 0.05
ATR + **12**	12	0.102 $\pm$ 0.006	0.021 $\pm$ 0.003	0.51 $\pm$ 0.09
ATR + **12**	25	0.096 $\pm$ 0.010	0.019 $\pm$ 0.004	0.47 $\pm$ 0.07
ATR + **12**	50	0.108 $\pm$ 0.006	0.022 $\pm$ 0.009	0.48 $\pm$ 0.07

[a] After a 90 min incubation with 2 nM [3H]-NMS, 1 μM ATR was added to each sample along with the indicated concentration (0, 12, 25, or 50 μM) of the pyridophen analogue (**10** or **12**). The means $\pm$ SD are listed for two experiments, each value in triplicate. As described in the Experimental Section, the dissociation constants were determined using the equation:[18] $(T) = Ae^{-k_{-1}T} + Be^{-k_{-2}T}$. The A fraction is that portion of the bound ligand–receptor complex with a fast dissociating constant, k_{-1}; B is the fraction with a slow dissociation constant, k_{-2} (i.e., $1 - A = B$).

analogues contrast with ATR and aprophen, which have K_i values of 2.4 and 51 nM, respectively (Table 2). Thus, the pyridophens as a group were all more than 50-fold less potent than the parent compound aprophen. In contrast, pyridostigmine, which carbamylates cholinesterases, had a minimal effect on [3H]-NMS binding to mAChR at 100 μM (Table 2).

We have previously demonstrated that monomethylcarbaphen (β-(N,N-diethylamino)ethyl 2-phenyl-2-[4-[[(methyl-amino)carbonyl]oxy]phenyl]propionate), where a carbamyl group is substituted at the para position of the phenyl ring of aprophen at the bulky hydrophobic portion of the molecule, retained antimuscarinic potency similar to the parent compound aprophen (28 vs 51 nM, respectively).[1] Unlike the carbaphen analogues, all of the pyridophens were significantly less potent than aprophen. The most potent compound in the series with a K_i of 2.6 μM was **13**, which represents the leaving group after **10** carbamylates the cholinesterases. Structure–activity relationship data suggest that the ionic binding by the mAChR to the protonated nitrogen of aprophen, ATR, or the carbaphens must be significantly different than found in the pyridophens. The location of the added carbamate moiety in the pyridophen analogues, e.g., **10**, might prevent a proper interaction of its quaternary nitrogen with the anionic site of the mAChR.[21,22] However, **13** contains a hydroxyl group in place of the carbamate moiety and is only about 2-fold more potent. The mechanism by which the carbamate and/or hydroxyl moiety in the pyridophens prevented high mAChR binding is not clear. Several possible explanations include (i) the carbamate ester positioned the pyridophen in a location normally reserved for the ester of the 2,2-diphenylpropionate group, (ii) the OH or carbamate disrupted ionic bonding to the protonated nitrogen, and (iii) the size of the OH or carbamate group excluded proper alignment within the antagonist binding site. There is evidence for carbamate-induced misalignment within the hydrophobic portion of antimuscarinic compounds. A single carbamate, but not two carbamate groups, added to the phenyl moiety of 2,2-diphenylpropionate is accommodated into the large hydrophobic pocket of the receptor without altering potency.[1] However, note that in the latter case, the carbamate(s) were added to the bulky phenyl groups

rather than to the opposite end of the compound containing the quaternary amine.

An alternate explanation for the decreased potency of the pyridophens in comparison with aprophen might be the result of the rigidity of the pyridostigmine moiety replacing the very flexible diethylaminoethanol found in aprophen.[21,22] The flexibility or rigidity of an antagonist has been proposed as a mechanism by which a compound can attain the conformation necessary to interact with mAChRs in general and subtypes of mAChRs specifically.[21] In this study, aliphatic and therefore less rigid compounds with the quaternary nitrogen three carbon atoms distant from the ester group of the 2,2-diphenylpropionate yielded K_i values of about 140 and 500 nM for *N*-methyl-2-piperidinemethyl-2,2-diphenylpropionate and *N*-ethyl-3-piperidyl-2,2-diphenylpropionate, respectively. These results suggest that the rigidity of the pyridinium ring containing either a hydroxyl or a carbamate moiety as found in **13** or **10** decreases potency 5−25-fold over the aliphatic ring conformation.

Conclusions

The series of pyridophens resulted in compounds, notably **9** and **12**, which inhibited BChE specifically, similar to the previously synthesized carbaphens.[1] More important, the pyridophens **10** and to a lesser extent **2**, **3**, and **11** preferentially inhibited AChE over BChE and contrasts with the previously described carbaphens that showed no AChE specificity.[1] With respect to muscarinic receptors, all of the pyridophens displayed competitive antagonism to the binding of [³H]-NMS to cortex receptors based on Hill values and dissociation kinetics. The pyridophens, however, were less potent muscarinic receptor inhibitors than the parent compound aprophen.

Ideally, the pyridophens (**10**, **2**, **3**, and **11**) should act minimally in the periphery and penetrate the blood brain barrier. There, the pyridophens would carbamylate AChE, which is consequently sequestered and protected from OP binding. Also upon carbamylation, the release of the mAChR antagonist metabolite occurs. This metabolite of cholinesterase carbamylation would be in the ideal location to function as a muscarinic antagonist to protect these receptors from elevated ACh levels. High potency mAChR antagonism would not be desirable before interaction with AChE but rather only after AChE protection. Therefore, the decrease in muscarinic potency observed for the pyridophens is compatible with the goal of protecting AChE first and then the mAChR.

Experimental Section

I. Chemistry. Melting points were determined on a Thomas−Hoover melting point apparatus and are uncorrected. ¹H NMR spectra were obtained on a Varian XL 300 (Me₄Si). Mass spectra (MS) were obtained on a Finnigen 1015 mass spectrometer (chemical ionization-NH₃). For purity tests, thin-layer chromatography (TLC) was performed on fluorescent silica gel plates (Polygram Sil G/UV254), and for each of the compounds, only one spot (visualized by UV light and I₂ vapor) was obtained. All new compounds gave satisfactory microanalyses for C, H, and N within ±0.4% and/or mass spectra consistent with the assigned structures.

2-Hydroxymethyl-3-[[(dimethylamino)carbonyl]oxy]-pyridine (2), 2-[[(Dimethylamino)carbonyl]oxy]methyl-3-hydroxypyridine (3), and 2-[[(Dimethylamino)carbonyl]-oxy]methyl-3-[[(dimethylamino)carbonyl]oxy]pyridine (4). Dimethylcarbamyl chloride (5 mL) was added to a warm solution (50 °C) of 3-hydroxy-2-(hydroxymethyl)pyridine hydrochloride (**1**, 3.2 g, 0.02 mol) in 50 mL of dry pyridine. The mixture was heated (50 °C) with stirring for another 30 min and cooled to 20 °C, and the pyridine was evaporated under vacuum. The crude reaction mixture was dissolved in H₂O (100 mL), excess K₂CO₃ was added, and the basic aqueous solution was extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with brine (2 × 50 mL) and dried (MgSO₄), and the solvent was evaporated to give 3.1 g of a brown viscous oil, which was found to be a mixture of **2**−**4** (60:30:10) according to TLC and ¹H NMR analysis. Separation and purification of **2**−**4** were achieved by column chromatography (silica, 5% MeOH−CHCl₃).

Compound 2. Yielded 2.10 g, viscous oil, solidified on standing, mp 55 °C, TLC (silica, 5% MeOH−CHCl₃) $R_f =$ 0.50. ¹H NMR (CDCl₃): δ 8.42 (d.d, 1H, *J* = 4.8, 1.3 Hz), 7.55 (d.d, 1H, *J* = 8.1, 1.3 Hz), 7.28 (d.d, 1H, *J* = 8.1, 4.8 Hz), 4.73 (s, 2H), 3.12 and 3.03 (2 s, 6H, Me₂N−). Anal. (C₉H₁₂N₂O₃) C, H, N.

Compound 3. Yielded 0.66 g, mp 118−119 °C (dec, from ethyl acetate; ether), TLC (silica, 5% MeOH−CHCl₃) $R_f =$ 0.65. ¹H NMR (CDCl₃): δ 8.16 (d.d, H, *J* = 4.4, 1.5 Hz), 7.28 (d.d, 1H, *J* = 6.4, 1.5 Hz), 7.23 (d.d, 1H *J* = 8.3, 4.4 Hz), 5.22 (s, 2H), 2.94 (s, 6H, Me₂N−). Anal. (C₉H₁₂N₂O₃) C, H, N.

Compound 4. Yielded 0.15 g of viscous oil, TLC (Silica, 5% MeOH−CHCl₃) $R_f =$ 0.58. ¹H NMR (CDCl₃): δ 8.41 (d.d, 1H, *J* = 4.6, 1.5 Hz), 7.5 (d.d, 1H, *J* = 8.2, 1.5 Hz), 7.26 (d.d, 1H, *J* = 8.2, 4.8), 5.22 (s, 2H), 3.08 and 2.98 (2s, 6H, −N(CH₃)₂), 2.87 (s, 6H, −N(CH₃)₂). Anal. (C₁₂H₁₇N₃O₄) C, H, N.

2,2-Diphenylpropionyl Chloride, (6). 2,2-Diphenylpropionic acid (**5**, 3.0 g) was dispersed in freshly distilled thionyl chloride (20 mL), and the mixture was refluxed for 2 h. The thionyl chloride was removed under vacuum, and the residue was dissolved in *n*-hexane and filtered, and the solvent was evaporated to leave 3.0 g of colorless oil.

Compound 6. ¹H NMR (CDCl₃): δ 7.27 (m, 6H), 7.17 (m, 4H), 2.01 (s, 3H). This acid chloride was used without any further purification.

3-Hydroxy-2-(2′,2′-diphenylpropionoxymethyl)pyridine (7). A solution of the acid chloride **6** (1.22 g, 0.005 mol) in dry benzene (10 mL) was added to a solution of 3-hydroxy-2-(hydroxymethyl)pyridine hydrochloride (0.9 g, 0.006 mol) in dry pyridine (30 mL). The mixture was warmed (55 °C) with stirring for 3 h and cooled to room temperature, and the benzene and pyridine were evaporated under reduced pressure. The semisolid residue was suspended in H₂O (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with brine and dried (MgSO₄), and the solvent was evaporated under vacuum to give 1.5 g of white solid. During the ethyl acetate extraction, some solid (100−200 mg) was found in the interphase between the organic and the aqueous phases. This solid was filtered and found to be identical to the major solid product.

Compound 7. The compound was purified by chromatography (silica, CHCl₃) to give 1.35 g (80% yield) of white crystals, mp 164−165 °C. ¹H NMR (CDCl₃ + 1%CD₃OD): δ 8.06 (d.d, 1H, *J* = 4.92 Hz, 1.76 Hz), 7.16 (m, 12H), 5.24 (s, 2H), 1.87 (s, 3H). Anal. (C₂₁H₁₉NO₃) C, H, N.

3-[[(Methylamino)carbonyl]oxy]-2-(2′,2′-diphenylpropionoxymethyl)pyridine (8). A small piece of sodium (∼5 mg) was added to a solution of **7** (0.2 g, 0.6 mmol) and methylisocyanate (0.25 g, excess) in dry benzene (100 mL). The reaction mixture was stirred for 72 h at room temperature. The solvent was removed under reduced pressure, and the viscous residue was chromatographed (silica, 2% MeOH−CHCl₃) to give 0.18 g (77% yield) of **8** as a colorless viscous oil.

Compound 8. ¹H NMR (CDCl₃): δ 8.39 (d, *J* = 4.4 Hz, 1H), 7.58 (d, *J* = 8.1 Hz, 1H), 7.26 (m, 11H), 5.24 (s, 2H), 4.6 (bs, 1H, −NH), 2.66 (d, *J* = 4.4 Hz, 3H), 1.84 (s, 3H). Anal. (C₂₃H₂₂N₂O₄) C, H, N.

3-[[(Dimethylamino)carbonyl]oxy]-2-(2′,2′-diphenyl-propionoxymethyl)pyridine (9). a. By Carbamylation of the Hydroxypyridine (7). Dimethylcarbamyl chloride (3 mL, excess) was added to a solution of the hydroxypyridine **7** (0.33 g, 0.001 mol) in dry pyridine (20 mL). The solution was warmed with stirring to 55 °C for 30 min and left overnight at room temperature. The pyridine was removed under vacuum, and the residue was dissolved in ethyl acetate (50 mL). The ethyl acetate solution was washed with brine (2 × 20 mL) and dried (MgSO$_4$), and the solvent was evaporated to give a pale brown viscous oil, which was purified by column chromatography (silica, 2% MeOH−CHCl$_3$), to yield a colorless oil, 0.28 g (70% yield).

Compound 9. ^{1}H NMR (CDCl$_3$): δ 8.37 (d.d, 1H, J = 4.6, 1.53 Hz), 7.51 (d.d, 1H, J = 8.24, 1.22 Hz), 7.20 (m, 11H), 5.26 (s, 2H), 2.89, 2.82 (2s, Me$_2$N−), 1.87 (s, 3H). Anal. (C$_{24}$H$_{24}$N$_2$O$_4$) C, H, N.

b. By Esterification of the Carbamate 3. A solution of acyl chloride **6** (0.244 g, 0.001 mol) in dry benzene (5 mL) was added dropwise to a stirred solution of the carbamate **3** (0.196 g, 0.001 mol) and triethylamine (0.2 g, 0.002 mol) in dry benzene (15 mL). The reaction mixture was refluxed for 3 h, the triethylamine HCl was filtered, and the organic solution was washed with brine (2 × 20 mL), dried (MgSO$_4$), and evaporated to leave a yellow viscous oil; according to TLC and ^{1}H NMR analysis, the oil was a mixture of **9** and starting materials. The desired compound **9** (0.1 g, 25% yield) was isolated and purified by column chromatography (silica, 5% MeOH−CHCl$_3$). ^{1}H NMR was identical to the ^{1}H NMR spectrum of **9** obtained by method a, by carbamylation of the hydroxypyridine **7**.

N-Methyl-2-hydroxymethyl-3-[[(dimethlamino)carbonyl]oxy]pyridinium Iodide (11). A solution of **3** (70 mg) and methyl iodide (100 mg) in dry acetone was warmed to 50 °C for 10 h. The methyl pyridinium iodide **11** that formed separated out from the acetone solution as a pale brown viscous oil (110 mg, 90% yield).

Compound 11. ^{1}H NMR ((CD$_3$)$_2$CO): δ 9.17 (d, 1H, J = 5.7 Hz), 8.56 (d, 1H, 8.8 Hz), 8.18 (d.d, 1H, J = 8.5, 6.1 Hz) 5.06 (s, 2H), 4.72 (s, 3H), 3.22, 3.01 (2s, 6H, Me$_2$N−). Anal. (C$_{10}$H$_{15}$N$_2$O$_3$I) C, H, N.

N-Methyl-3-[[(Dimethylamino)carbonyl]oxy]-2-(2′,2′-diphenylpropionoxymethyl)pyridinium Iodide (10, X = I$^-$). a. By Esterification of the Hydroxymethylpyridinium Iodide (11). A solution of **11** (0.112 g, 0.3 mmol) and the acid chloride **6** (0.140 g, 0.6 mmol) in 2 mL of deuterated pyridine (C$_6$D$_5$N) was kept at room temperature for 4 days. Progress of the reaction was monitored by ^{1}H NMR. The pyridine was removed under vacuum (10^{-3} mm Hg), and the viscous residue was extracted with ether (3 × 10 mL) to remove the excess of the acid chloride **7**. The pale brown viscous oil was purified by column chromatography (silica, 5−10% MeOH−CHCl$_3$) to give a pale yellow viscous oil (85 mg, 60%).

Compound 10 (X = I$^-$). ^{1}H NMR (C$_6$D$_5$N): δ 9.90 (d, 1H, J = 6.1 Hz), 8.86 (d, 1H, J = 8.8 Hz), 8.41 (d.d, J = 8.8, 6.1 Hz), 7.5−7.1 (m, 10H), 6.18 (s, 2H), 4.85 (s, 3H, N$^+$−CH$_3$), 2.94 and 2.87 (2s, 6H, −N(CH$_3$)$_2$), 2.01 (s, 3H). ^{1}H NMR (CD$_3$COCD$_3$): δ 9.29 (d, 1H, J = 6.1 Hz), 8.63 (d, 1H, J = 8.8 Hz), 8.25 (d.d, 1H, J = 8.8, 6.1 Hz), 7.4−7.2 (m, 10H), 5.81 (s, 2H), 4.50 (s, 3H, N$^+$−CH$_3$), 3.08 and 2.97 (2s, 6H, −N(CH$_3$)), 1.94 (s, 3H). Anal. (C$_{25}$H$_{27}$N$_2$O$_4$I) C, H, N. MS fast atom bombardment (FAB): 419 ([intact cation]$^+$).

b. By Methylation of the Carbamate Ester 9 with Methyl Iodide. A solution of **9** (100 mg) and methyl iodide (100 mg, excess) in deuterated acetone ((CD$_3$)$_2$CO, 2 mL), was kept at room temperature. The reaction progress was monitored by ^{1}H NMR. After 7 days, ~95% of the tertiary pyridine compound was converted to the quaternary methyl pyridinium iodide **10 (X = I$^-$)**. ^{1}H NMR was identical with that of **10 (X = I$^-$)** obtained in method a, by esterification of the hydroxymethylpyridinium iodide **11**, as described above.

N-Methyl-3-[[(Dimethylamino)carbonyl]oxy]-2-(2′,2′-diphenylpropionoxymethyl)pyridinium Methyl Sulfate (10, X = [CH$_3$SO$_4$]$^-$). A solution of **9** (100 mg) and dimethyl sulfate (120 mg, excess) in deuterated acetone ((CD$_3$)$_2$CO, 2 mL) was kept at room temperature. The reaction progress was monitored by ^{1}H NMR. After 5 days, ~90% of the tertiary pyridine compound was converted to the quaternary methylpyridinium methyl sulfate **10, X = [CH$_3$SO$_4$]$^-$**. The deuterated acetone was removed under vacuum, and the residual viscous oil was triturated 3 times with hot dry ether (reflux, 3 × 50 mL) and dried under vacuum for 24 h at room temperature.

Compound 10, X = [CH$_3$SO$_4$]$^-$. ^{1}H NMR (1% CD$_3$OD−CDCL$_3$): δ 8.73 (d, 1H, J = 6.0), 8.23 (d, 1H, J = 8.7), 7.92 (d.d, J = 8.7, 6.0), 7.23−7.05 (m, 10H), 5.58 (s, 2H), 4.12 (s, 3H, N + −CH$_3$), 3.57 (s, 3H, −OCH$_3$), 3.05 and 2.94 (2s, 6H, −N(CH$_3$)$_2$), 1.86 (s, 3H, C−CH$_3$). Anal. calcd for ([C$_{25}$H$_{27}$N$_2$O$_4$]$^+$-[CH$_3$SO$_4$]$^-$): C, 58.86; H, 5.66; N, 5.28. Found: C, 58.05; H, 5.50; N, 5.05. According to the ^{1}H NMR spectrum, this compound contained a small amount (~3%) of dimethyl sulfate. MS (FAB): 419 ([intact cation]$^+$).

N-Methyl-3-[[(dimethylamino)carbonyl]oxy]-2-(2′2′-diphenylpropionoxymethyl)-1,2,5,6-tetrahydropyridine (12). Sodium borohydride (75 mg, 1.8 mmol) was added in small portions to a cold solution (5 °C) of **10** (250 mg, 0.45 mmol) in methanol (high-performance liquid chromatography (HPLC) grade). Stirring was continued for 6 h at 5−10 °C. Water was added (~10 mL), and the mixture was extracted with ethyl acetate (3 × 20 mL). After it was dried, the solvent was evaporated under reduced pressure, and the pale yellow viscous oil residue was chromatographed (silica, ethyl acetate) to give **12** as a colorless viscous oil (120 mg, 62% yield).

Compound 12. ^{1}H NMR (CDCl$_3$): δ 7.4−7.2 (m, 10 H), 5.51 (d.t, 1 H, J = 4.0, 1.1 Hz), 4.41 (d.d, 1 H, J = 12.1, 5.5 Hz), 4.32 (d.d, 1 H, J = 12.1, 2.3 Hz), 3.28 (m, 1H), 2.90 and 2.86 (2s, 6H, −N(Me)$_2$), 2.75 (m, 1H), 2.55 (m, 1H), 2.39 (s, 3H, N−CH$_3$), 2.25 (m, 1H), 2.0 (m, 1H), 1.92 (s, 3H). Anal. (C$_{25}$H$_{30}$N$_2$O$_4$) C, H, N.

N-Methyl-3-hydroxy-2-(2′2′-diphenylpropionoxymethyl)pyridinium Iodide (13, X = I$^-$). A solution of **7** (0.1 g) and methyl iodide (0.1 g, excess) in deuterated acetone ((CD$_3$)$_2$CO, 4 mL) was kept at room temperature. Reaction progress was monitored by ^{1}H NMR. After 48 h, all of the tertiary pyridine compound was converted to the quaternary methyl pyridinium iodide **13**, X = I$^-$. The acetone was removed under reduced pressure, and the crude viscous brown oil was triturated in dry ether to give yellow crystals (65 mg), mp 112−114 °C (dec).

Compound 13, X = I$^-$. ^{1}H NMR ((CD$_3$)$_2$CO): δ (relative to CD$_2$ H = 2.10 ppm) 8.50 (d, 1H, J = 6.0 Hz), 8.41 (d, 1H, J = 8.7 Hz), 7.80 (d.d, 1H, J = 8.7, 6.0 Hz), 7.4−7.2 (m, 10H), 5.77 (s, 2H), 4.31 (s, 3H, −N$^+$CH$_3$), 2.00 (s, 3H, C−CH$_3$). Anal. ([C$_{22}$H$_{22}$NO$_3$]$^+$I$^-$) C, H, N. MS (FAB): 348 ([intact cation]$^+$).

N-Methyl-3-hydroxy-2-(2′2′-diphenylpropionoxymethyl)pyridinium Methyl Sulfate (13, X = $^-$OSO$_2$OCH$_3$). A solution of **7** (0.1 g) and dimethyl sulfate (0.1 g) in deuterated acetone ((CD$_3$)$_2$CO, 5 mL) was kept at room temperature. After 48 h, the quaternization of the tertiary pyridine compound was completed (^{1}H NMR). The acetone was removed under reduced pressure, and the viscous residue was triturated 3 times with dry ether and acetone to give white crystals, mp 164−166 °C.

Compound 13 (X = $^-$OSO$_2$OCH$_3$). ^{1}H NMR (D$_2$O): δ (relative to DHO = 4.8 ppm) 8.23 (d, 1H, J = 6.0 Hz), 7.97 (d, 1H, J = 8.7 Hz), 7.79 (d.d, 1H, J = 8.7, 6.0 Hz), 5.65 (s, 2H), 4.84 (s, 3H, $^-$OCH$_3$), 4.04 (s, 3H, −N$^+$CH$_3$), 2.00 (s, 3H, C−CH$_3$). Anal. ([C$_{22}$H$_{22}$NO$_3$]$^+$[CH$_3$SO$_4$]$^-$) C, H, N. MS (FAB): 348 ([intact cation]$^+$).

II. Biological Activities. Cholinesterase Purification and Kinetic Assays. Both fetal bovine serum (GIBCO, Grand Island, NY) AChE and horse serum BChE (Sigma Chemical Co., St. Louis, MO) were purified to electrophoretic homogeneity by procainamide affinity chromatography.[23,24] Pyridostigmine was purchased from Sigma Chemical Co., and aprophen was synthesized at Walter Reed using the method of Zuagg and Horrom.[25]

Both enzymes were assayed colorimetrically,[13] using a concentration of either 1 mM acetylthiocholine as substrate for AChE or 1 mM butyrylthiocholine as substrate for BChE in a 3 mL solution of 0.1 M phosphate buffer, pH 8.0, containing 3.11×10^{-4} M 5,5′-dithio-bis-(2-nitrobenzoic acid). Assays were conducted at 25 °C. The specific activity of AChE was 6700 U/mg, and the specific activity of BChE was 700 U/mg. Time-dependent inhibition reactions contained 15 U AChE or BChE in 100 μL of 0.05 M phosphate buffer, pH 8.0, and graded carbamate concentrations. Aliquots of 5 or 10 μL were withdrawn at the indicated time intervals, and enzyme activity was assayed. Kinetic constants were calculated from secondary plots of the slopes of pseudo-first-order progress curves as functions of the inhibitor concentrations as described by Main.[12] In this treatment, the reaction between an inhibitor and an enzyme is described by equation 1:

$$I + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_{2c}} E' \xrightarrow{k_3} E + \text{carbamic acid} \qquad (1)$$
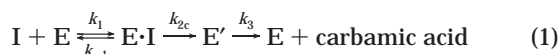
and leads to a graphic solution for the desired kinetic constants through the derived equation 2:

$$[I] \, \Delta t / 2.3 \, \Delta \log v = [I]/k_{2c} + 1/k_2' \qquad (2)$$

where the reciprocal of the slope yields k_{2c}, the carbamylation constant; the intercept on the X-axis yields $(-K_i)$, the equilibrium affinity constant; and the reciprocal of the intercept on the Y-axis yields $1/k_2'$, the bimolecular rate constant.

Guinea Pig Cortex Membrane Preparations. Brain (male guinea pig, 200−400 g) was obtained after decapitation[21] and processed at 4 °C. Briefly, the cortex was homogenized in 20 volumes of 50 mM potassium phosphate buffer, pH 7.4, and centrifuged two times at 39 000g for 20 min. The brain was then resuspended in 4 volumes of buffer and frozen (−20 °C) in aliquots until use.

Binding Assays. Equilibrium Binding Assays. As previously described, equilibrium binding assays were performed in triplicates containing 200−400 μg of protein and 1−2 nM [^{3}H]-NMS (72 Ci/mmol, NEN Life Science Products, Inc., Boston, MA) in 50 mM potassium phosphate buffer, pH 7.4, at 25 °C.[21] ATR (1 μM) was added to determine nonspecific binding, which typically was about 10% of the total counts. The assays were terminated after 1 h by collecting the samples with a cell harvester (Brandel, Gaithersburg, MD) on to glass-fiber filters.

Specific [^{3}H]-NMS binding to muscarinic receptors was determined by subtracting the total from the nonspecific counts. The K_i values were derived from the IC$_{50}$ values by correcting for receptor occupancy by the [^{3}H]-NMS;[20] the K_D value determined for cortex was 0.48 nM.

Dissociation Binding Assays. Cortex membranes were preincubated with 1−2 nM [^{3}H]-NMS for 90 min at 25 EC with gentle mixing.[20] Then, 1 μM ATR was added simultaneously with graded doses of **10** or **12** for time intervals between 0 and 180 min. Dissociation curve kinetic parameters were determined using the equation[18] where [^{3}H]-NMS bound to each tissue receptor at a measured time, T:

$$(T) = A_e^{-k_{-1}T} + B_e^{-k_{-2}T}$$

The portion of the bound ligand−receptor complex with a fast dissociating constant k_{-1} is denoted the "A fraction". B is the fraction with a slow dissociating constant k_{-2}. The parameters were fitted to curves using nonlinear curve fitting; the r^2 values (goodness of curve fit) were all >0.97 (Prism version 3.02, GraphPad Software, San Diego, CA).

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