Anticholinesterase Activity of Compounds Related to Geneserine Tautomers. *N*-Oxides and 1,2-Oxazines[||]

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A series of phenylcarbamate analogues of geneserine (**8**, **10**, **12**, **14**) were synthesized from their counterparts, the phenylcarbamate analogues of physostigmine (**2**−**5**), by oxidation. The geneserine analogues can undergo tautomerism between *N*-oxide and 1,2-oxazine structures in a pH- and time-dependent manner. Assessment by ^{1}H NMR indicated that the *N*-oxide structure is adopted at neutral pH and that the compound exists in an equilibrium between several epimers. Evaluation of their biological action to inhibit human acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), ex vivo, demonstrated that the *N*-oxide (**7**, **9**, **11**, **13**, **15**) and 1,2-oxazine (**6**, **8**, **10**, **12**, **14**) structures possessed similar potencies against AChE, but the latter structures were more potent against BChE. With the exception of the BChE selective inhibitor, **12**, none of the geneserine analogues were as potent or enzyme subtype selective as their physostigmine analogue counterparts.

Introduction

Although the classical anticholinesterase, physostigmine (**1**), is the primary and best known alkaloid isolated from the Calabar bean, the dried ripe seed of the *Physostigma venenosum* vine of Tropical West Africa; the same seed produces the structurally related alkaloids, geneserine (**6**) and physosvenine, that likewise possess anticholinesterase activity.[1−3] Geneserine (**6**) has, as its free base, a 1,2-oxazine structure.[4,5] This readily converts into the *N*-oxide tautomer (**7**) under acidic conditions.[5]

A recent report that the *N*-oxide hydrochloride of the potent and acetylcholinesterase (AChE) selective inhibitor, eseroline 2′-ethylphenylcarbamate (**5**), 2′-ethylphenylgeneserine (**15**) (CHF 2819, Chiesi Farmaceutici, Parma, Italy), showing sufficient anticholinesterase activity to merit its clinical evaluation in Alzheimer's disease (AD) patients,[6] prompted us to take a further look at *N*-oxides and derived 1,2-oxazines ex vivo. Our initial synthesis of eseroline 2′-ethylphenylcarbamate (**5**) was undertaken in the development of physostigmine (**1**) analogues that were long-acting and capable of differentially inhibiting the two subtypes of cholinesterase enzyme,[7,8] AChE (EC 3.1.1.7) from butyrylcholinesterase (BChE, EC 3.1.1.8).[8,9] Both enzymes are disparately localized throughout the body and possess a multitude of functions, depending on their site. In the brain, AChE is primarily localized at the postsynaptic element of cholinergic neurons, whereas BChE is primarily associated with glial cells.[10−12] Their levels are variably altered in specific diseases. For example, AChE is regionally reduced in the AD brain by as much as 65%, whereas BChE levels are elevated.[13,14]

Because of the early involvement of the cholinergic system in the development and progression of AD and the association of the cholinergic system with memory processing, anticholinesterases have been used and, thus far, have proved to be the most effective drug class in AD treatment.[15,16] In light of the contrasting increase and decrease of AChE and BChE, respectively, in the AD brain, their site-dependent selective inhibition likely is critical for optimal therapeutic intervention.[10]

The results of the current study, including a comparison between the synthesized agents and the highly selective AChE inhibitors phenserine (**2**),[7] tolserine (**3**),[7] and homotolserine (eseroline 2′-ethylphenylcarbamate) (**5**)[7] and the highly selective BChE inhibitor cymserine (**4**),[7] are reported herein. The *N*-oxide HCl salts were prepared from the corresponding carbamates with 3-chloroperbenzoic acid as the oxazine tautomers, as described in the literature,[5] and thereafter were converted into the *N*-oxide hydrochloride with HCl ether solution. Both the oxazine structures and those of their *N*-oxide tautomers, present in the HCl salt, were apparent from ^{1}H NMR analyses. However, it proved to be difficult to assess the purity of these compounds by silica gel TLC (silica gel and CH_2Cl_2/MeOH system) because it showed streaking, suggesting that the tautomers are in a constant equilibrium. Biological evaluation of the anticholinesterase activity of compounds **6**−**15** demonstrated that they were less potent than their analogous eseroline carbamates.

Results

Chemistry. Physostigmine (**1**), phenserine (**2**), tolserine (**3**), cymserine (**4**), and 2′-ethylphenserine (**5**)

[||] This paper is dedicated to Professor Nelson J. Leonard, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, on the occasion of his 85th birthday.

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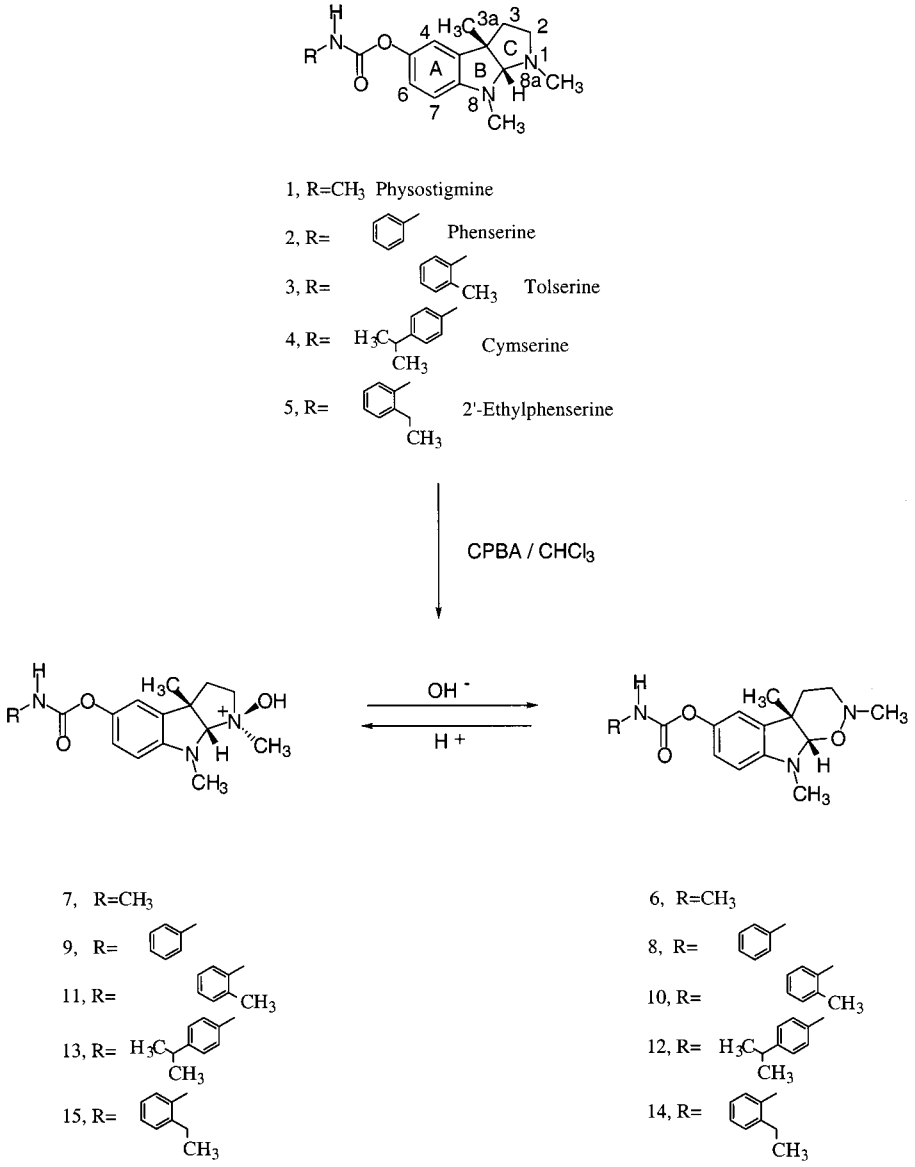
Scheme 1



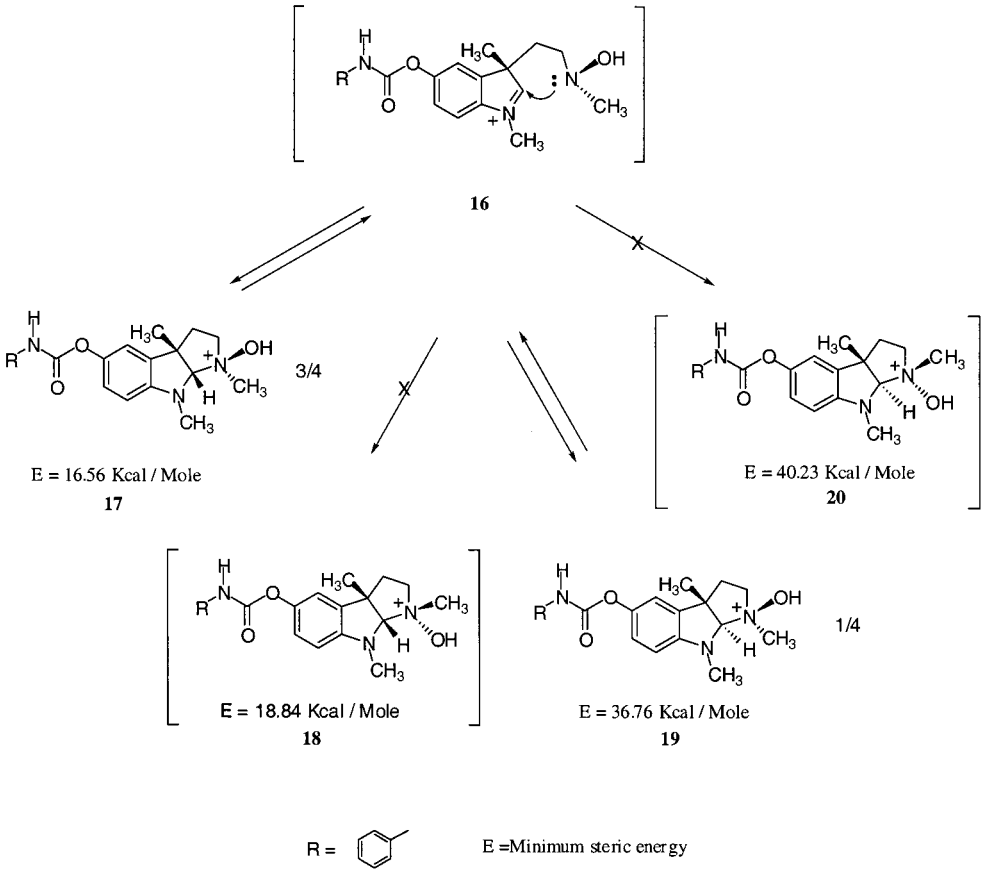
were oxidized by 3-chloroperbenzoic acid in chloroform to give the corresponding hexahydro-1,2-oxizino-[5,6-*b*]-indoles **6**, **8**, **10**, **12**, and **14**, respectively, according to the procedure for making geneserine from physostigmine.[5] The ether solution of the 1,2-oxizines was then subjected to acidification by addition 1.0 M hydrochloric acid in ether to provide the precipitated HCl salts that were the corresponding hexahydropyroloindole *N*-oxides **7**, **9**, **11**, **13**, and **15**, respectively. During this process, initially, a Meisenheimer rearrangement occurred; C8a migrated from N1 to O. Then, on acidification, a retro-Meisenheimer rearrangement took place; C8a migrated from O to N (Scheme 1).

The structure and absolute configuration of compounds **6** and **7** have been well established by ^{1}H NMR[5] and X-ray crystallography.[5,17] An inspection of ^{1}H NMR spectra of compounds **7**, **9**, **11**, **13**, and **15** indicated that similar impurities existed in them, all in the same ratio, when comparing them to the spectra of their main products. However, assessment of the ^{1}H NMR (DMSO-d_6) of geneserine (**6**) fumarate found no existence of an impurity. Its chemical shifts were in accord with the 1,2-oxazine structure (**6**). This phenomenon implies that in the case of the HCl salt the strong mineral acid induces ring-C opening to form a ring-C open intermediate, **16**, which then closes to form the main *N*-oxide structure, **17**, and concomitantly produces a minor ring-closure product (Scheme 2).

Theoretically, the N1 atom of intermediate **16** could attack C8 from either of two sides, which, together with the reversion of the N1 atom itself, allows the formation of four epimerides, **17**, **18**, **19**, and **20**. An analysis of X-ray crystallography and ^{1}H NMR proved that the main product possesses the structure of compound **17**, with C3a−CH_3 and C8−H in a cis configuration. Using R = phenyl as an example, the minimum steric energy of the four possible configurations (Scheme 2) was computed by using the MM-2 program of Chem 3D.[18]

The structure of **17** possesses the lowest of the computed steric energies among the configurations and hence corresponds to the most thermodynamically stable structure and represents the structure of all crystalline HCl salts. In solution, however, **17** denotes the structure of some 75% of the compound, whereas the remaining

Scheme 2



25% is in the form of the three other possible epimerides, which coexist with structure **17** in an equilibrium state.

A 2D ^{1}H NMR NOESY spectrum of the HCl salt of 4′-isopropylphenylgeneserine (**13**) (DMSO-d_6) (Figure 1) shows that the minor structure has a trans configuration between C3a−CH_3 and C8a−H, which are on the opposing sides of the B−C ring. The magnified part of the described NOESY spectrum (Figure 1, lower panel) indicates that for the minor structure there is no NOE effect between the trans C3a−CH_3 and C8a−H. In contrast, a significant NOE effect can be found between the cis C3a−CH_3 and C8a−H in the main structure. Hence, the minor structure is either compound **19** or **20**. Although data are lacking to directly define the N1 configuration, we nevertheless propose that the minor compound likely has the structure of **19**. We support this presumption, first, by the lower computed steric energy of structure **19** versus **20** and, second, by the likelihood of the N1 configuration remaining the same as for the main structure. The hypothetical process to account for this is that when the HCl salts, **7**, **9**, **11**, **13** and **15**, are in solution (at a neutral pH as per the condition during NMR analysis), the ring-open intermediate, **16**, is produced. This immediately undergoes closure to form **17** and the trans structure **19** in a 3:1 ratio. The process occurs so rapidly that (i) indolium intermediate **16** was unable to be detected by NMR at room temperature and (ii) the N1 atom could not be reversed prior to ring closure.

Biological Evaluation. Table 1 illustrates the biological activity of compounds **6**−**15** to inhibit freshly prepared human AChE and BChE, ex vivo, in comparison to their parent compounds **1**−**5**, whose measured values are in accord with results from our previous reports.[5,8,9,19,20] On an equimolar basis, both tautomers of all geneserine analogues were equipotent (within 2-fold) in their ability to inhibit AChE. All, however, were less potent than their respective physostigmine analogues with respect to AChE inhibitory activity ($p < 0.05$), from as little as 4-fold for geneserine (**6**) and its HCL salt (**7**), versus physostigmine (**1**), to as much as 17-fold for the 2′methylphenyl analogues **10** and **11**, versus tolserine (**3**).

With regard to BChE inhibitory action, the 1,2-oxazine structure proved to be the most potent of the two tautomers, which proved to be from 5-fold for the 2′ethylphenyl analogues (**14**, **15**) to as much as 200-fold for the 4′-isopropylphenyl analogues (**12**, **13**), more potent than the *N*-oxide tautomer. In general, the BChE potency of the 1,2-oxazine analogues was similar, within 2-fold, to that of their respective physostigmine counterparts, with the exception of **14** that proved to be mildly BChE potent, and 6-fold-more active than **5**.

As a consequence of the reduced AChE potency of the geneserine analogues versus their physostigmine counterparts and their approximately similar BChE activity, their enzyme subtype selectivity was marginal. For example, 2′-ethylphenylgeneserine, in either of its structural forms (**14** or**15**), was between 5- and 14-fold selective for AChE versus BChE, whereas 2′-ethylphenserine was 290-fold selective in this regard. The exception to this was the highly BChE selective inhibitor 4′-isopropylphenylgeneserine in its oxazine (**12**) rather than *N*-oxide form (**13**).

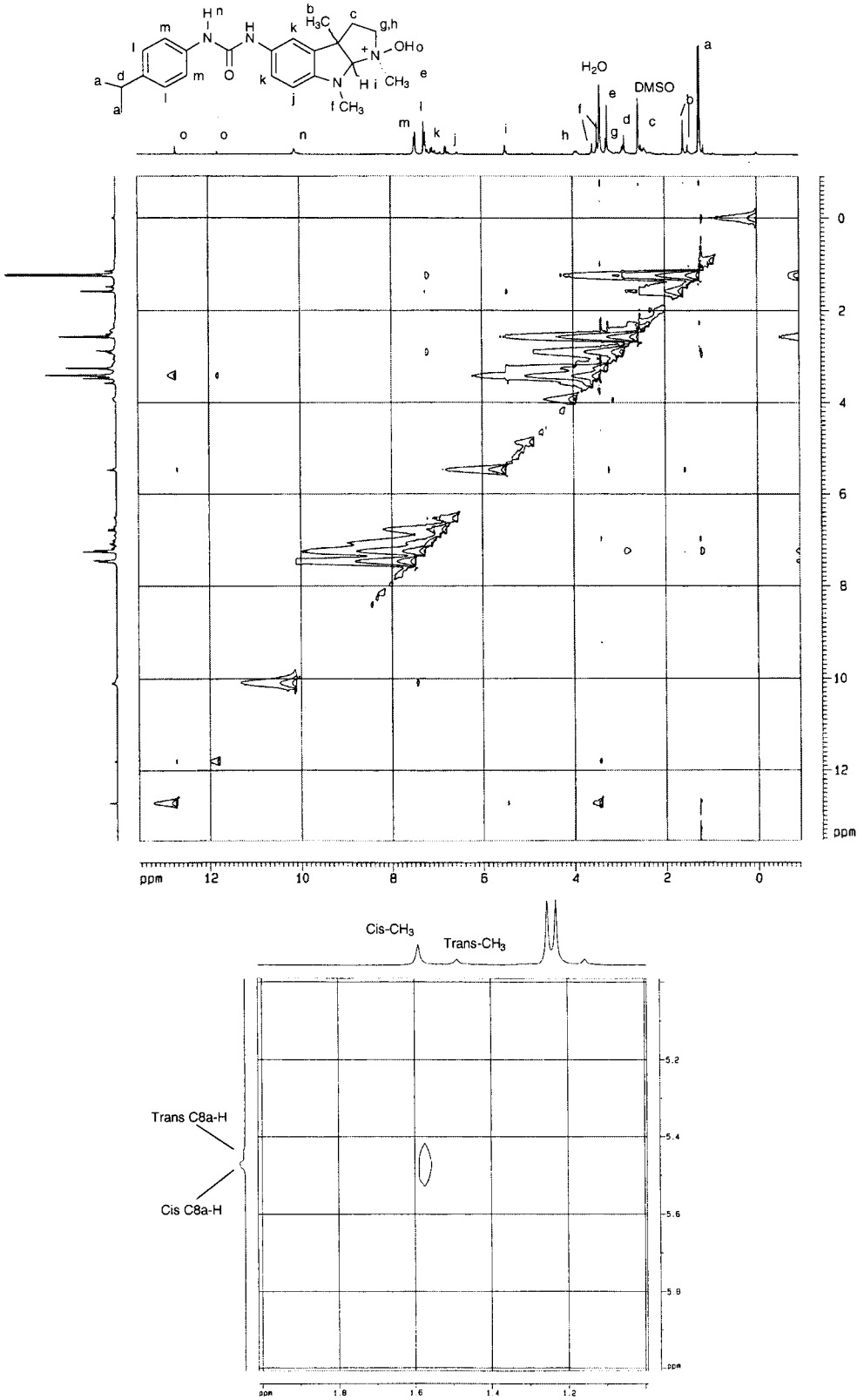


Figure 1. 2D ^{1}H NMR, NOE spectrum of HCl salt of 4′-isopropylphenylgeneserine (DMSO-d_6).

In theory, in aqueous solution at a specific pH, the *N*-oxides (**7**, **9**, **11**, **13**, **15**) and their respective 1,2-oxazine (**6**, **8**, **10**, **12**, **14**) should possess the same anticholinesterase activity, since the same tautomeric ratio should eventually be obtained starting from either. Clearly, however, this is not the case, as measured at the optimal working pH, 8.0, of AChE and BChE (Table 1). To clarify why their biological activities differed, the ^{1}H NMR spectra of geneserine oxazine (**6**) and *N*-oxide (**7**) were examined, with (**1**) as a control, in a time-dependent manner. In this regard, equal amounts of the agents (5 mg) were dissolved in 150 μL of DMSO-d_6, separately. Thereafter, each solution was diluted with 1 mL of 0.1 M Na_3PO_4 in deuterium oxide (pH 8.0), and ^{1}H NMR analysis was undertaken at 2 and some 20 h later. The former time was chosen to coincide with the

Table 1. 50% Inhibitory Concentration of Compounds versus Human Erythrocyte AChE and Plasma BChE

		IC_{50}[a] ± SEM (nM)		
no.	compound	AChE	BChE	selectivity
1	physostigmine·salicylic acid	28 ± 2.4	16 ± 2.9	2-fold BChE
2	phenserine·tartaric acid[b]	24 ± 6	1560 ± 45	65-fold AChE
3	tolserine·tartaric acid	10 ± 1.6	1950 ± 240	195-fold AChE
4	cymserine·tartaric acid	760 ± 21	50 ± 1	15-fold BChE
5	2′-ethylphenserine·tartaric acid	10 ± 0.7	2900 ± 540	290-fold AChE
6	geneserine oxazine[c]	150 ± 28	12 ± 0.2	12-fold BChE
7	geneserine·HCl[c]	100 ± 7	660 ± 280	7-fold AChE
8	phenylgeneserine oxazine	130 ± 17	815 ± 135	6-fold AChE
9	phenylgeneserine·HCl	200 ± 19	>10 000	>50-fold AChE
10	2′-methylphenylgeneserine oxazine	175 ± 10	1570 ± 390	2-fold AChE
11	2′-methylphenylgeneserine·HCl	180 ± 10	>10 000	55-fold AChE
12	4′-isopropylphenylgeneserine oxazine	>10 000	25 ± 6	400-fold BChE
13	4′-isopropylphenylgeneserine·HCl	>10 000	5200 ± 485	2-fold BChE
14	2′-ethylphenylgeneserine oxazine	65 ± 9	310 ± 67	5-fold AChE
15	2′-ethylphenylgeneserine·HCl	125 ± 23	1700 ± 385	14-fold AChE

[a] The IC_{50} data of compounds **1**−**5** were cited from refs 5, 19, and 20. [b] Geneserine hydrochlorides represent the genserine *N*-oxide structures. The oxazine structures are indicated by name. [c] Tartaric acid refers to L-(+)-tartaric acid.

Table 2. Comparison of the IC_{50} Values of Phenserine against Human Erythrocyte AChE and Plasma BChE with Those of Several ChEIs of Clinical Interest

	IC_{50}[a] ± SEM (nM)		
compound	AChE	BChE	selectivity
physostigmine (**1**)	28 ± 2.4	16 ± 2.9	2-fold BChE
phenserine (**2**)	22 ± 1.4	1560 ± 45	70-fold AChE
2′-ethylphenylgeneserine *N*-oxide·HCl (CHF 2819) (**15**)	125 ± 23	1700 ± 385	14-fold AChE
heptylphysostigmine (Eptastigmine)	22 ± 2.0	5.0 ± 0.1	4-fold BChE
tacrine (Cognex)	190 ± 40	47.0 ± 10	4-fold BChE
donepezil (Aricept)	22 ± 8.1	4150 ± 1700	188-fold AChE
huperzine A	47 ± 22	>10,000	>212-fold AChE
galanthamine (Reminyl)	800 ± 60	7300 ± 830	9-fold AChE
rivastigmine (Exelon)	4150 ± 160[b]	37 ± 5	122-fold BChE

[a] IC_{50} values were determined in duplicate on a minimum of four different occasions. [b] Rivastigmine is unusual in that its activity against brain-derived AChE is far more potent than against erythrocyte-derived enzyme.[34] The measured value therefore dramatically underestimates its activity in brain, and the agent has been reported to be nonselective between AChE and BChE.[34]

time that the cholinesterase study was undertaken on solutions of the agents.

All of spectra are available in the Supporting Information. A comparison of these spectra provided the following information. Physostigmine (**1**) clearly existed as a single stable form; the C3a−CH_3 was at δ 1.20. After 20 h, its spectrum was unchanged. Geneserine oxazine (**6**), however, existed as three forms. The main form was the 1,2-dioxazine tautomer (C3a−CH_3 at δ1.10), with the remaining forms as the two epimers of the *N*-oxide tautomer (C3a−CH_3 at δ 1.40 and δ 1.30). After 20 h, the three forms remained but in a different ratio. The main forms became epimers of the *N*-oxide tautomer.

In contrast, geneserine hydrochloride (**7**) likewise existed as the three forms, but the two epimers of the *N*-oxide tautomer proved to be the main components (C3a−CH_3 at δ 1.40 and δ 1.30). After 20 h, the ratio of the forms had changed and the forms were similar to those of **6** at 20 h. Hence, geneserine analogues clearly undergo tautomerism between *N*-oxide and 1,2-oxazine structures in both a pH- and time-dependent manner.

Discussion

Table 2 illustrates a side-by-side comparison of the anticholinesterase activity of a number of compounds that either are currently in clinical assessment or have been approved by the FDA for use in subjects with Alzheimer's disease. Each agent was assayed against AChE and BChE, ex vivo, freshly obtained from the same individual. In this regard, 2′-ethylphenylgeneserine *N*-oxide HCl (**15**) possesses moderate AChE inhibitory activity that is approximately equipotent to tacrine, and it also possesses a negligible BChE activity that is equipotent to that of phenserine (**2**). Our results are not in accord with those of Pietra and colleagues,[21,22] who reported a lower (approximately a log-fold) AChE potency for **15** and a greater differential selectivity (IC_{50} of AChE, 477 ± 63 nM; IC_{50} of BChE, 55 170 ± 6430 nM; 115-fold AChE selective). In this study, however, all the geneserine analogues were less potent than those reported in our evaluation, as was physostigmine (**1**) whose value was a log-fold less active than both ours and its accepted value.[23]

In comparison to their physostigmine counterparts (Table 1) and in accord with Robinson[3] and with Pietra et al.,[21] the geneserine analogues are both less potent and less enzyme subtype selective, irrespective of their state of tautomerization. The equilibrium between the *N*-oxide and 1,2-oxazine, and epimers associated with the former, is pH-dependent and, as shown Supporting Information, does not occur instantaneously but in a time-dependent manner. Whereas our ex vivo quantification of their anticholinesterase activity was assessed at pH 8.0, the optimal working pH of both AChE and BChE,[11,12] when administered to humans or animals, the pH of their environment will be dependent on their body location. After oral administration, which is the

preferred route of most drugs and the likely route for **15** in clinical trials,[6] the environment of the stomach is pH 1.4, in which the compound would exist in its charged *N*-oxide form. As it travels through the small and large intestines, however, the pH of the gastric contents gradually rises to 7.4. This likely would affect the equilibrium between the tautomers, as would absorption from the gastrointestinal tract into the blood and eventual delivery to the brain, with a pH of 7.4. The predominant structural form of the agent in each of these locations likely is different and may thus provide different activities, toxicities, and metabolic products. All of these would need to be monitored and may change during aging, during any disease process, or with concomitant food or fluid intake[24,25] and may thus complicate the eventual FDA approval process of a geneserine analogue.

Irrespective of the structural form of geneserine and analogues following their in vivo administration, studies in rats clearly indicate that, similar to other anticholinesterases, they significantly inhibit AChE levels in the brain[21] and thereby elevate extracellular levels of ACh.[22] For example, oral administration of **15** at a dose of 1.5 mg/kg to rats caused a 50% inhibition of brain AChE and optimally overcame a scopolamine-induced impairment of passive avoidance behavior.[21,22] This dose is approximately one-fifth of its LD_{50} (6.5 mg/kg).[21] In accord with the higher potency of **2** versus **15**, a 0.25 mg/kg intraperitoneal dose of phenserine (**2**), which is roughly equivalent to an oral dose because the agent is 100% bioavailable, induces a 70% inhibition in CSF AChE and optimally improves cognitive performance in rats.[26,27] Phenserine (**2**) has a lower toxicity than that reported for **15**, with a LD_{50} of approximately 25 mg/kg following oral administration to male rats.

Recent attention has focused on the potential role of BChE in health, aging, and AD.[10] In contrast to the increasing loss of cholinergic markers, and particularly of choline acetyl transferase, acetylcholine, and AChE, during the progression of AD, levels of BChE become elevated.[10,13,14] In addition, BChE is found in association with both of the neuropathological hallmarks of AD, β-amyloid plaques, and neurofibrillary tangles and may be involved in the seeding process associated with the former.[28,29] As a consequence, there is current interest in assessing (i) whether selective BChE inhibitors may offer therapeutic potential and (ii) the value of the BChE component in effective unselective anticholinesterases.[10] In this regard, 4′-substituted analogues of phenserine are proving to be useful tools in defining the enzyme's function.[19,20] Likewise, compound **12**, should it remain preferentially in its 1,2-oxazine form in vivo, may also prove to be useful in this respect. In addition, because specific anticholinesterases, such as phenserine and analogues, have been shown to reduce β-amyloid levels in animals and tissue culture studies,[30−32] genserine analogues may possess similar, potentially useful actions.

In light of the effectiveness of many of the anticholinesterases listed in Table 2, we await the outcome of ongoing clinical trials[33] to see how the preclinical activity of compounds **2** and **15** translates into efficacy in individuals afflicted with AD.

Experimental Section

Chemistry. Melting points (uncorrected) were measured with a Fisher-Johns apparatus. ^{1}H NMR spectra were recorded on a Bruker (Avance, 300 mHz) spectrometer, and MS (*m*/*z*) data were recorded on a Hewlett-Packard 5890 GC−MS (EI) and on a Finnigan-1015D mass spectrometer. Optical rotations were measured by a JASCO, model DIP-370 (Japan, Spectroscopic Co., Ltd.), and elemental analyses were performed by Atlantic Microlab, Inc.

Geneserine (6). Geneserine was synthesized according to a procedure reported in the literature.[5] New and previously unreported analytical data are reported herein.

Geneserine Fumarate Salt (6). Foam; ^{1}H NMR (DMSO-d_6) δ 1.00 (s, 3H, C3a−CH_3), 2.10 (m, 2H, C3−H_2), 2.35 (s, 3H, CH3−N1), 2.55 (br. 3H, CH_3−NH−CO−), 2.60 (m, 2H, C2−H_2), 2.70 (s, 3H, CH_3−N8), 4.68 (s, 1H, C8a−H), 4.90 (br, 1H, NH), 6.30 (d, J = 8.0 Hz, 1H, C7−H), 6.65 (m, 1H, C6−H), 6.75 (m, 1H, C4−H).

Geneserine HCl Salt (7). White crystal; mp 151−152 °C; $[\alpha]_D^{20}$ −124.3° (*c* 0.5, EtOH); ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 1/4 × 3H, trans C3a−CH_3), 1.58 (s, 3/4 × 3H, cis C3a−CH_3), 2.50 (m, 2H, C3−H_2), 2.85 (m, 1H, C2−H), 3.10 (br, 3H, CH_3−NH−CO−), 3.24 (s, 3/4 × 3H, CH_3−N1), 3.28 (s, 1/4 × 3H, CH_3−N1), 3.45(s, 3/4 × 3H, CH_3−N8), 3.55 (s, 1/4 × 3H, CH_3−N8), 3.90 (m, 1H, C2−H), 5.40 (s, 1/4H, trans C8a−H), 5.45 (s, 3/4H, cisC8a−H), 6.75−6.80 (m, 1H, C7−H), 7.10−7.30 (m, 2H, C6−H and C4−H), 9.90 (br, 1H, N−H), 11.40 (s, 1/4H, OH), 12.20 (s, 3/4H, OH).

Phenylgeneserine (8). Phenserine (107 mg, 0.317 mmol) was dissolved in $CHCl_3$ (10 mL), and 3-chloroperbenzoic acid (78.5 mg, 0.39 mmol of 85% purity) in $CHCl_3$ (1 mL) was added slowly at 0−5 °C. The reaction mixture was stirred at room temperature overnight. The $CHCl_3$ solution was washed with a saturated aqueous solution of $NaHCO_3$ until the aqueous layer became basic. Thereafter, it was washed with brine and dried over Na_2SO_4. After removing the solvent by vacuum, the residue was dissolved in Et_2O and washed with a saturated aqueous solution of $NaHCO_3$, followed by brine, and then was dried over Na_2SO_4. Evaporation of solvent by vacuum gave phenylgeneserine (**8**) as an amorphous solid (89.6 mg, 80%): mp 131−132 °C; $[\alpha]_D^{20}$ −150.0° (*c* 0.2, EtOH); ^{1}H NMR ($CHCl_3$) δ 1.15 (s, 3H, C3a−CH_3), 1.90−2.10 (m, 2H, C3−H_2), 2.45−2.65 (m, 2H, C2−H_2), 2.50 (s, 3H, CH_3−N1), 2.80 (s, 3H, CH_3−N8), 4.65 (s, 1H, C8a−H), 6.35 (d, J = 8.0 Hz, 1H, C7−H), 6.80 (m, 1H, C6−H), 6.85 (m, 1H, C4−H), 7.10−7.45 (m, 5H, phenyl−H); CI-MS (NH_3) *m*/*z* 354 ($M^+ + 1$); HR-MS *m*/*z* calcd for $C_{20}H_{23}N_3O_2$ 353.1792, found 353.1747.

Phenylgeneserine HCl Salt (9). Compond **8** in Et_2O was acidified with anhydrous 1.0 M HCl in Et_2O to give crude HCl salt as an amorphous solid, which then was crystallized from THF. Mp 171−172 °C; $[\alpha]_D^{20}$ −87.0° (*c* 0.1, EtOH); ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 1/4 × 3H, trans C3a−CH_3), 1.58 (s, 3/4 × 3H, cis C3a−CH_3), 2.35−2.55 (m, 2H, C3−H_2), 3.10 (m, 1H, C2−H), 3.20 (s, 3/4 × 3H, CH_3−N1), 3.22 (s, 1/4 × 3H, CH_3−N1), 3.45(s, 3/4 × 3H, CH_3−N8), 3.55 (s, 1/4 × 3H, CH_3−N8), 3.90 (m, 1H, C2−H), 5.40 (s, 1/4H, trans C8a−H), 5.42 (s, 3/4H, cisC8a−H), 6.70 (d, J = 8 Hz, 1/4H, C7−H), 6.75 (d, J = 8.0 Hz, 3/4H, C7−H), 7.05−7.10 (m, 2H, C6−H and C4−H), 7.10−7.45 (m, 5H, phenyl−H), 10.55 (br, 1H, N−H), 11.80 (s, 1/4H, −OH), 12.75 (s, 3/4H, −OH). Anal. ($C_{21}H_{25}N_3O_3$·HCl) C, H, N.

Compounds **10**−**15** were synthesized according to the above procedure in similar yields. The following are their characteristic data.

2′-Methylphenylgeneserine (10). Amorphous solid; $[\alpha]_D^{20}$ −94.7° (*c* 0.5, EtOH); ^{1}H NMR ($CHCl_3$) δ 1.15 (s, 3H, C3a−CH_3), 1.90−2.10 (m, 2H, C3−H_2), 2.30 (s, 3H, phenyl−CH_3), 2.45−2.65 (m, 2H, C2−H_2), 2.50 (s, 3H, CH_3−N1), 2.80 (s, 3H, CH_3−N8), 4.65 (s, 1H, C8a−H), 6.35 (d, J = 8.0 Hz, 1H, C7−H), 6.80 (m. 1H, C6−H), 6.85 (m, 1H, C4−H), 6.90−7.20 (m, 4H, phenyl−H), 7.80 (br, 1H, −NH); CI-MS (NH_3) *m*/*z* 368 ($M^+ + 1$); HR-MS *m*/*z* calcd for $C_{21}H_{25}N_3O_2$ 367.1898, found 367.1905.

2′-Methyphenylgeneserine HCl Salt (11). Mp 135−140 °C; $[\alpha]_D^{20}$ −67.2° (*c* 0.4, EtOH); ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 1/4 × 3H, trans C3a−CH_3), 1.58 (s, 3/4 × 3H, cis C3a−CH_3), 2.30 (s, 3H, phenyl-CH_3), 2.35−2.55 (m, 2H, C3−H_2), 3.10 (m, 1H, C2−H), 3.16 (s, 3/4 × 3H, CH_3−N1), 3.18 (s, 1/4 × 3H, CH_3−N1), 3.38 (s, 3/4 × 3H, CH_3−N8), 3.40 (s, 1/4 × 3H, CH_3−N8), 3.90 (m, 1H, C2−H), 5.40 (s, 1/4H, trans C8a−H), 5.45 (s, 3/4H, cisC8a−H), 6.70 (d, J = 8 Hz, 1/4H, C7−H), 6.75 (d, J = 8.0 Hz, 3/4H, C7−H), 7.00−0.7.10 (m, 2H, C6−H and C4−H), 7.20−7.40 (m, 4H, phenyl−H), 9.40 (br, 1H, N−H), 11.80 (s, 1/4H, −OH), 12.75 (s, 3/4H, −OH). Anal. ($C_{21}H_{25}N_3O_3$·HCl) C, H, N.

4′-Isopropylphenylgeneserine (12). Amorphous solid; mp 165−167 °C; $[\alpha]_D^{20}$ −103.0° (*c* 0.4, EtOH); ^{1}H NMR ($CHCl_3$) δ 1.15 (s, 3H, C3a−CH_3), 1.20 (d, J = 11 Hz, 2 isopropyl−CH_3), 1.90−2.10 (m, 2H, C3−H_2), 2.45−2.65 (m, 2H, C2−H_2), 2.50 (s, 3H, CH_3−N1), 2.80 (s, 3H, CH_3−N8), 4.65 (s, 1H, C8a−H), 6.35 (d, J = 8.0 Hz, 1H, C7−H), 6.80−6.90 (m, 2H, C4−H and C6−H), 7.15 (d, J = 8.0 Hz, 2H, C3′-H and C5′-H), 7.30 (d, J = 8.0 Hz, 2H, C2′-H and C6′-H), 7.80 (br, 1H, −NH); CI-MS (NH_3) *m*/*z* 368 (M^+ + 1); HR-MS *m*/*z* calcd for $C_{21}H_{25}N_3O_2$ 367.1898, found 367.1905.

4′-Isopropylphenylgeneserine HCl Salt (13). Mp 151−152 °C; $[\alpha]_D^{20}$ −95.0° (*c* 0.3, EtOH); ^{1}H NMR (DMSO-d_6) δ 1.20 (d, J = 11 Hz, 2 isopropyl CH_3), 1.40 (s, 1/4 × 3H, trans C3a−CH_3), 1.58 (s, 3/4 × 3H, cis C3a−CH_3), 2.30 (s, 3H, phenyl-CH_3), 2.35−2.55(m, 2H, C3−H_2), 3.10 (m, 1H, C2−H), 3.16 (s, 3/4 × 3H, CH_3−N1), 3.18 (s, 1/4 × 3H, CH_3−N1), 3.38 (s, 3/4 × 3H, CH_3−N8), 3.40 (s, 1/4 × 3H, CH_3−N8), 3.90 (m, 1H, C2−H), 5.40 (s, 1/4H, trans C8a−H), 5.45 (s, 3/4H, cis C8a−H), 6.70 (d, J = 8 Hz, 1/4H, C7−H), 6.75 (d, J = 8.0 Hz, 3/4H, C7−H), 7.00−7.10 (m, 2H, C6−H and C4−H), 7.20 (d, J = 8.0 Hz, 2H, C3′-H and C5′-H), 7.45 (d, J = 8 Hz, C2′-H and C6′-H), 10.10 (br, 1H, N−H), 11.80 (s, 1/4H, −OH), 12.75 (s, 3/4H, −OH). Anal. ($C_{21}H_{25}N_3O_3$·HCl·0.25H_2O) C, H, N.

2′-Ethylphenylgeneserine (14). Amorphous solid; $[\alpha]_D^{20}$ −127.2° (*c* 0.1, EtOH); ^{1}H NMR ($CHCl_3$) δ 1.15 (s, 3H, C3a−CH_3), 1.20 (t, 3H, CH_3-benzyl), 1.90−2.10 (m, 2H, C3−H_2), 2.45−2.65 (m, 2H, C2−H_2), 2.50 (s, 3H, CH_3−N1), 2.65 (q, 2H, −CH_2−phenyl), 2.80 (s, 3H, CH_3−N8), 4.65 (s, 1H, C8a−H), 6.35 (d, J = 8.0 Hz, 1H, C7−H), 6.80−6.85 (m, 2H, C4−H and C6−H), 6.90−7.20 (m, 4H, aromatic H), 7.80 (br, 1H, −NH); CI-MS (NH_3) *m*/*z* 382 (M^+ + 1); HR-MS *m*/*z* calcd for ($C_{22}H_{27}N_3O_2$ + 1) 382.2132, found 382.2130.

2′-Ethylphenylgeneserine HCl Salt (15). Amorpous solid; $[\alpha]_D^{20}$ −139.9° (*c* 0.5, EtOH); ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3H, CH_3−benzyl), 1.40 (s, 1/4 × 3H, trans C3a−CH_3), 1.58 (s, 3/4 × 3H, cis C3a−CH_3), 2.35−2.55(m, 2H, C3−H_2), 2.75 (q, 2H, −CH_2−phenyl), 3.10 (m, 1H, C2−H), 3.16 (s, 3/4 × 3H, CH_3−N1), 3.18 (s, 1/4 × 3H, CH_3−N1), 3.38 (s, 3/4 × 3H, CH_3−N8), 3.40 (s, 1/4 × 3H, CH_3−N8), 3.90 (m, 1H, C2−H), 5.40 (s, 1/4H, trans C8a−H), 5.45 (s, 3/4H, cis C8a−H), 6.70 (d, J = 8 Hz, 1/4H, C7−H), 6.75 (d, J = 8.0 Hz, 3/4H, C7−H), 6.90−7.05 (m, 2H, C6−H and C4−H), 7.10−7.40 (m, 4H, aromatic H), 9.40 (br, 1H, N−H), 11.80 (s, 1/4H, −OH), 12.75 (s, 3/4H).

Quantitation of Anticholinesterase Activity. The action of compounds **1**−**15** to inhibit the ability of freshly prepared human AChE and BChE to enzymatically degrade the specific substrates acetyl(β-methyl)thiocholine and *s*-butyrylthiocholine (0.5 mmol/L) (Sigma Chemical Co., St. Louis, MO), respectively, was quantified.[7−9,19,20,23] Samples of AChE and BChE were derived from whole red blood cells and plasma, respectively. Compounds were dissolved in Tween 80/EtOH 3:1 (v/v, <150 μL total volume) and were diluted in 0.1 M Na_3PO_4 buffer (pH 8.0) in half-log concentrations to provide a final concentration range that spanned 0.3 nM to 10 mM. Tween 80/EtOH was diluted to a concentration in excess of 1 in 5000, and no inhibitory action on either AChE or BChE was detected in separate prior experiments.

For the preparation of BChE, freshly collected blood was centrifuged (10000*g*, 10 min, 4 °C), and plasma was removed and diluted to 1:125 with 0.1 M Na_3PO_4 buffer (pH 7.4). For AChE preparation, whole red blood cells were washed five times in isotonic saline, lysed in nine volumes of 0.1 M Na_3PO_4 buffer (pH 7.4) containing 0.5% Triton-X (Sigma), and then were diluted with an additional 19 volumes of buffer to a final dilution of 1:200.

Analysis of anticholinesterase activity, utilizing a 25 μL sample of each enzyme preparation, was undertaken at their optimal working pH, 8.0, in 0.1 M Na_3PO_4 buffer (0.75 mL total volume). Compounds were preincubated with enzymes (30 min, room temp) and then were incubated with their respective substrates and with 5,5′-dithiobis-2-nitrobenzoic acid (25 min, 37 °C). Production of a yellow thionitrobenzoate anion was measured by spectrophotometer at λ = 412 nm. To correct for nonspecific substrate hydrolysis, aliquots were co-incubated under conditions of absolute enzyme inhibition (by the addition of 1 × 10^{-5} M physostigmine (**1**)), and the associated alteration in absorbance was subtracted from that observed through the concentration range of each test compound. Each agent was analyzed on four separate occasions and assayed alongside phenserine (**2**) and physostigmine (**1**) as control and external standards whose activity we have previously reported.[7−9,19,20,23]

The enzyme activity at each concentration of test compound was expressed as a percent of the activity in the absence of compound. This was transformed into a logit format [logit = (% activity)/(100 − % activity)] and then was plotted as a function of its log concentration vs ln(logit). Inhibitory activity was calculated as an IC_{50}, defined as the concentration of compound (nM) required to inhibit 50% of enzymatic activity, and determined from a correlation between log of the concentration and ln(logit activity), where $r^2 \geq 0.98$.

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Supporting Information Available: ^{1}H NMR spectra of physostigmine, geneserine base, and geneserine hydrochloride. This material is available free of charge via the Internet at http://pubs.acs.org.

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