Neurochemistry of Aging. 1. Toxins for an Animal Model of Alzheimer's Disease

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A chronic deficiency in central cholinergic function has been implicated in a number of neuropsychiatric diseases including Alzheimer's disease. Until recently, animal models that simulate the neurochemical conditions that appear to cause these diseases in humans, as a result of a direct manipulation of the central cholinergic system, were not available. Over the past few years, however, we have been successful in developing a cholinotoxin, 1-ethyl-1-(2-hydroxyethyl)aziridinium chloride (AF64A), which has the potential to serve as a novel compound in developing animal models of human brain disorders in which a cholinergic hypofunction has been implicated. In this paper are described the design, synthesis, and testing of several structural analogues of AF64A as potential cholinotoxins, by evaluating them for their ability to inhibit high-affinity choline transport and their affinity toward brain muscarinic receptors. One of the compounds, 1-cyclopropyl-1-(2-hydroxyethyl)aziridinium chloride (i.e. aziridine analogue of 13) was found to have a remarkably high affinity (about 40 times higher than AF64A) toward brain muscarinic receptors.

The central cholinergic system is important in the regulation of memory and learning processes.[1-3] Impairment of memory has been shown to occur following blockade of cholinergic function with antimuscarinic agents.[4] There is also a large body of evidence that implicates a central cholinergic deficit in a variety of neurologic and psychiatric disorders such as Alzheimer's disease,[5-10] tardive dyskinesia,[11-14] Huntington's chorea,[15-23] and Down's syndrome.[24]

If one were to develop an *animal model* in which one could simulate the neurochemical conditions that appear to cause these diseases and impairments in humans, it would be feasible to explore methods to reverse these biochemical abnormalities. One approach would be to develop a neurotoxin, more specifically, a cholinotoxin, that would selectively be targeted toward cholinergic nerve terminals, and subsequently permanently disturb the normal equilibrium of chemical processes in the affected nerve terminals.

Most known neurotransmitter-specific neurotoxins are cytotoxic analogues of the affected neurotransmitter. For example, 6-hydroxydopamine and 5,6-dihydroxytryptamine are two specific neurotoxins, which act as degenerating agents for the catecholaminergic and serotonergic nerve terminals respectively.[25,26] Another neurotoxin, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine, active at noradrenergic neurons, has also been reported.[27] Its persistent neurotoxic activity in vivo is due to a reactive aziridinium structure. Until recently, central acetylcholine-containing neurons had constituted the one major neurotransmitter system that has not been susceptible to *selective attack* in vivo by any of the known neurotoxins. This is due to the fact that the drugs known to affect central cholinergic function have been either short lasting [e.g. hemicholinium-3, which when centrally administered, is a potent high-affinity choline transport inhibitor] or not cholinospecific (e.g. venoms such as β-bungarotoxin). Also, unlike the other neurotransmitter systems, the cholinergic system is unique in that acetylcholine undergoes very rapid hydrolysis, coupled with remarkable regenerative powers.[28] Therefore, originally it appeared that an irreversible disruption of central cholinergic transmission would be very difficult, if not an impossible goal to achieve.

With the above in mind, we set out to develop cholinotoxins that could ultimately be used in vivo to develop animal models of disease states of cholinergic hypofunction, as well as to serve as unique and valuable tools to probe the dynamics of cholinergic mechanisms and function. The reduced central cholinergic activity in Alzheimer's disease, as well as in the other hypofunctional cholinergic disorders mentioned, could be attributed

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(1) Glick, S. D.; Mittag, T. W.; Green, J. P. *Neuropharmacology* **1973**, *12*, 291-296.
(2) Beatty, W. W.; Carbone, C. P. *Physiol. Behav.* **1980**, *24*, 675-678.
(3) Bartus, R. T. *Science* **1979**, *206*, 1087-1089.
(4) Drachman, D. A. "Banbury Report: 15, Biological Aspects of Alzheimer's Disease"; Katzman, R., Ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY 1983; pp 363-370.
(5) Davies, P.; Maloney, A. J. F. *Lancet* **1976**, *2*, 1403.
(6) Davis, K. L.; Yesavage, J. A. "Brain Acetylcholine and Neuropsychiatric Disease"; Davis, K. L., Berger, P. A., Eds.; Plenum Press: New York, 1978; pp 205-213.
(7) Corkin, S. *Trends Neurosci.* **1981**, *12*, 287-290.
(8) Whitehouse, P. J.; Price, D.; Clark, A.; Coyle, J. T.; Delong, M. *Ann. Neurol.* **1981**, *10*, 122-126.
(9) McKinney, M.; Hedreen, J.; Coyle, J. T. "Alzheimer's Disease: A Report of Progress in Research"; Katzman, R., Ed.; Raven Press: New York, 1982; Vol. 19, pp 259-265.
(10) Bartus, R. T.; Dean, R. L.; Beer, B.; Lippa, A. S. *Science* **1982**, *217*, 408-417.
(11) Casey, D. E. *Dis. Nerv. Sys.* **1977**, *38*, 7-15.
(12) Growdon, J. H.; Hirsch, M. J.; Wurtman, R. J.; Wiener, W. *N. Engl. J. Med.* **1977**, *297*, 524-527.
(13) Growdon, J. H.; Gelenberg, A. J.; Doller, J.; Hirsch, M. J.; Wurtman, R. J. *N. Engl. J. Med.* **1978**, *298*, 1029-1030.
(14) Penovich, P.; Morgan, J. P.; Kerzner, B.; Karch, F.; Goldblatt, D. *J. Am. Med. Assoc.* **1978**, *239*, 1997-1998.
(15) Davis, K. L.; Berger, P. A.; Hollister, L. E.; Barchas, J. D. *Life Sci.* **1978**, *22*, 1865-1872.
(16) Aquilonius, S. M.; Eckernas, S. A. *Neurology* **1977**, *27*, 887.
(17) Coyle, J. T.; Schwarcz, R.; Bennet, J. P.; Campochiaro, P. *Prog. Neuro-Psychopharmacol.* **1977**, *1*, 13-30.
(18) Growdon, J. H.; Cohen, E. L.; Wurtman, R. J. *Ann. Neurol.* **1977**, *1*, 418-422.
(19) Tarsy, D. "Brain Acetylcholine and Neuropsychiatric Disease"; Davis, K. L., Berger, P. A., Eds.; Plenum Press: New York, 1979; pp 395-424.
(20) Barbeau, A. *Can. Sci. Neurol.* **1978**, *5(1)*, 157-160.
(21) Barbeau, A. *N. Engl. J. Med.* **1978**, *299*, 200-201.
(22) Nutt, J. G.; Rosin, A.; Chase, T. N. *Neurology* **1978**, *28*, 1061-1064.
(23) Sadeh, M.; Brahman, J. *Isr. J. Med. Sci.* **1979**, *15*, 288 (abstract).
(24) Yates, C. M.; Simpson, J.; Maloney, A. F. J.; Gordan, A. *J. Neurol. Sci.* **1980**, *48*, 257-263.
(25) Rotman, A. *Life Sci.* **1977**, *21*, 891-900.
(26) Breese, G. R.; Vogel, R. A.; Muller, R. A. *J. Pharmacol. Exp. Ther.* **1978**, *205*, 587-595.
(27) Jaim-Etcheverry, G.; Zieher, L. M. *Brain Res.* **1980**, *188*, 513-523.
(28) Fisher, A.; Hanin, I. *Life Sci.* **1980**, *27(18)*, 1615-1634.

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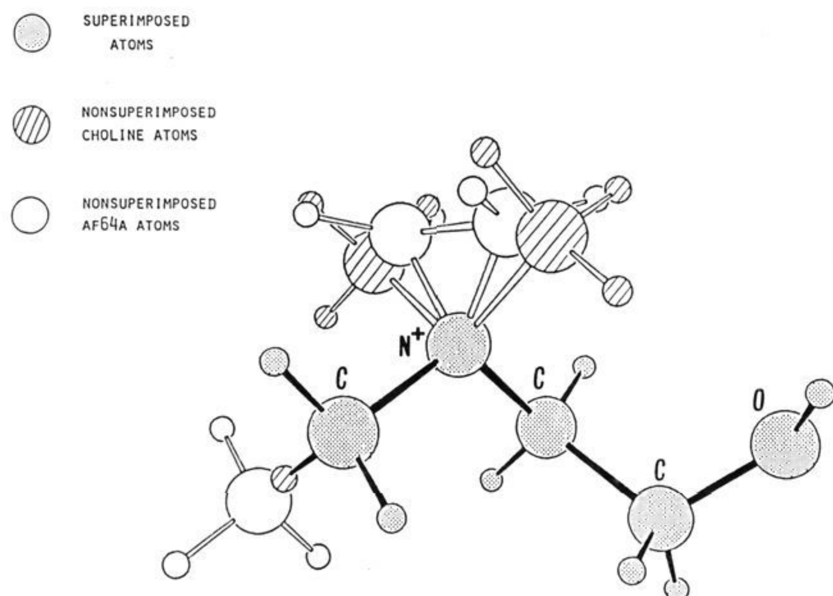


Figure 1. Overlap of AF64A with choline. Coordinates for the non-hydrogen atoms of choline were taken from the X-ray structure of choline chloride (Senko, M. E.; Templeton, D. H. *Acta Crystallogr.* **1960**, *13*, 281). The coordinates for AF64A were obtained by model building with use of CHEMGRAF (Chemgraf, created by E. K. Davies, Chemical Crystallography Laboratory, Oxford University; developed and distributed by Chemical Design Ltd., Oxford), starting with the X-ray coordinates of choline. The solid bonds join exactly superimposed atoms and the open bonds join the nonsuperimposed atoms.

mainly to a disruption in acetylcholine metabolism due to persistent presynaptic nerve terminal dysfunction.

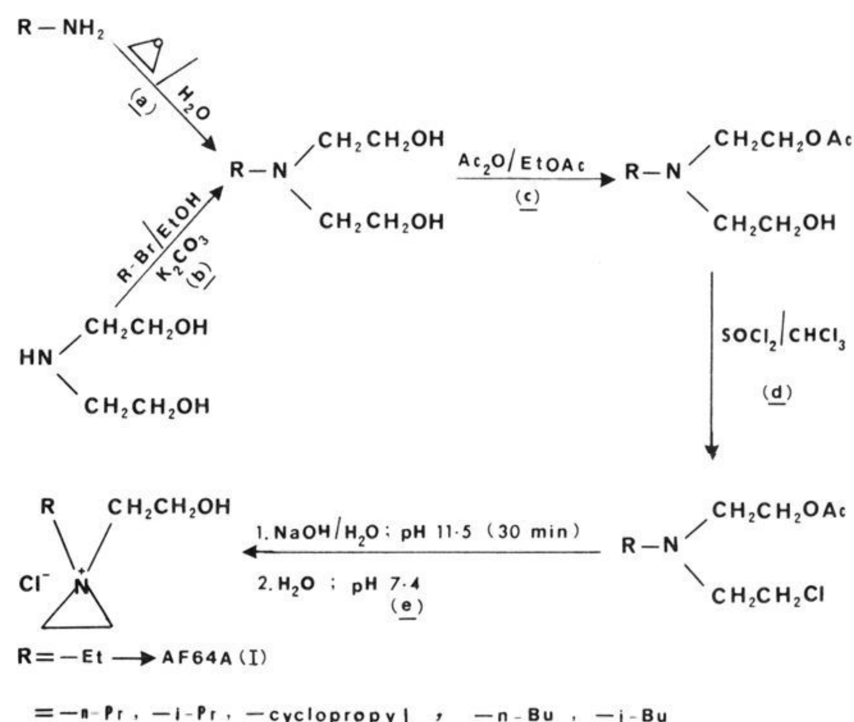
Hence, our initial attempts were focused on designing cholinotoxins, to be specifically targeted toward the Na^+-dependent, active transport system, i.e. the "high affinity choline transport system", the rate-limiting step in acetylcholine synthesis.[28,29] This system is uniquely localized to cholinergic nerve terminal areas and is responsible for transporting choline into nerve terminals for acetylcholine synthesis. Therefore, it was thought that agents that interfere with the transport system would be especially effective in blocking acetylcholine synthesis and release[28] by selectively "poisoning" cholinergic neurons.

During the past few years, we have developed a specific and selective cholinotoxin, 1-ethyl-1-(2-hydroxyethyl)-aziridinium chloride (AF64A) (**1**).[30,31] Originally, we designed **1** to be a mustard-like toxin, which when cyclized mimics, as close as possible, the natural substrate choline. For example, a look at the structures of choline and the aziridinium ion of AF64 reveals a similar molecular volume (75% common surface area) when one superimposes the two molecules (see Figure 1). The CH_2CH_2OH and the positive nitrogen atom of the two molecules superimpose exactly if they are kept in the same conformation. The aziridinium ring of **1** is constructed in the same plane as two of the methyl groups of choline, permitting good overlap in volume of the aziridinium ring of **1** with the two methyl groups of choline. The methylene group of the ethyl moiety of **1** superimposes with the remaining methyl group of choline. The terminal methyl of **1** is the only group that has no overlapping volume with choline, except for one methyl hydrogen on choline, which is substituted by the methyl group of **1**.

Compound **1** induces, in vivo, a persistent central cholinergic hypofunction of presynaptic origin, to the apparent exclusion of any effect on other neurotransmitter systems in the same brain areas.[30-33] It should be noted that

(29) Jope, R. *Brain Res. Rev.* **1979**, *1*, 313–344.
(30) Mantione, C. R.; Fisher, A.; Hanin, I. *Science* **1981**, *213*, 579–580.
(31) Fisher, A.; Mantione, C. R.; Abraham, D. J.; Hanin, I. *J. Pharmacol. Exp. Ther.* **1982**, *222*, 140–145.

Scheme I



AF64A has been reported as being nonspecific for cholinergic neurons by some workers.[34-37] This phenomenon, in our hands, appears to be one of dose-related response, with specificities occurring at very low concentrations of AF64A.

Although AF64A is a strikingly promising cholinotoxin, we decided to design, synthesize, and screen other potential cholinotoxic agents that might possess even more selective activity, or demonstrate other effects not exhibited by AF64A. It was also anticipated that synthesis of close structural analogues of AF64A would help us identify the optimal structural features required for the future development of other selective in vivo cholinergic neurotoxins.

This paper therefore describes the synthesis of several structural analogues of AF64A, and reports preliminary neurochemical findings vis a vis their potential as specific cholinotoxins. Attempts have also been made in this paper to rationalize the structure–activity relationships of these compounds, by molecular structure comparisons with choline and acetylcholine.

Chemistry. Several alkyl derivatives of AF64A were prepared as shown in Scheme I.[1]

(AF64A was originally synthesized by Dr. A. Fisher, and the synthetic scheme described here is modelled after his original route.)

Step (a) was used to convert the cyclopropyl-, isopropyl-, isobutylamines to the corresponding 2,2′-(alkylimino)bisethanols. When the step (b) was used to prepare the bisethanolamine, for R = *n*-Pr, the yields were considerably increased by the selective extraction procedure mentioned in the Experimental Section. However, when cyclopropyl bromide was used for N-alkylation, the reaction failed. Preparation of the 2-[alkyl(2-hydroxyethyl)amino]ethyl acetates (compounds **6–10**) from the corresponding bis-

(32) Mantione, C. R.; Fisher, A.; Hanin, I. *Life Sci.* **1984**, *35*, 33–41.
(33) Hanin, I.; DeGroat, W. C.; Mantione, C. R.; Coyle, J. T.; Fisher, A. "Banbury Report 15: Biological Aspects of Alzheimer's Disease"; Katzman, R., Ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1983; pp 243–253.
(34) Asante, J. W.; Cross, A. J.; Deakin, J. F. W.; Johnson, J. A.; Slater, H. R. *Br. J. Pharmacol.* **1983**, *80*, 573 (abstract).
(35) Levy, A.; Kant, G. J.; Meyerhoff, J. L.; Jarrard, L. E. *Brain Res.* **1984**, *305*, 169–172.
(36) Jarrard, L. E.; Kant, G. J.; Meyerhoff, J. L.; Levy, A. *Pharmacol., Biochem. Behav.* **1984**, *22*, 273–280.
(37) Villani, L.; Contestabile, A.; Poli, A.; Migani, P.; Fonnum, F. *Neurosci. Lett.* **1984**, *Supplement 18*, S228 (abstract).

Table I. IC_{50} Values for HAChT System

R	IC_{50},[a] μM	R	IC_{50},[a] μM
Et	1.5	cyclopropyl	3.9
n-Pr	7.5	*i*-Bu	31
i-Pr	2.6	*n*-Bu	>1000

[a] IC_{50} value for choline = 1.6 μM.

ethanolamines [step (c)] invariably resulted in production of some diacetates and unreacted starting material. Tedious work-up procedures were employed to selectively obtain 2-[alkyl(2-hydroxyethyl)amino]ethyl acetates, leading to low yields of the desired intermediates. It is very important to obtain compounds **6–10** in pure form, devoid of the bisethanols, since the next step (d) would convert the bisethanols into bis-mustards. These mustard-like impurities would be highly toxic, nonselective compounds.

It was discovered that compounds **6–10** were unstable and had to be used for the next step immediately after purification. The 2-[alkyl(2-chloroethyl)amino]ethyl acetate hydrochloride salts (compounds **11–15**) were easily precipitated after step (d), in pure form, by using the solvent system dry THF/dry diethyl ether. These salts were stable. Because of their instability, aziridinium compounds were freshly prepared as 10 mM solutions, 1–2 h before being used in the biological assays, according to step (e).

Biological Results and Discussion

A. High-Affinity Choline Transport. The inhibitory potency of a toxin on the high-affinity choline transport system in vitro was determined in terms of concentration of the compound required to inhibit 50% of the transport of [^{3}H]choline (IC_{50}) into synaptosomes. The IC_{50} values for the various cholinotoxins were determined by plotting the concentration of toxin vs. percent inhibition on semilog paper. The results are presented in Table I. It is clear that increasing the chain length reduced the activity (increased IC_{50}) whereas branching or cyclization of propyl analogues (R = *i*-Pr, cyclopropyl) produced compounds with activity (IC_{50}) similar to that of choline and AF64A. This suggests that the high-affinity choline transport system does possess structural requirements for the inhibitors.

Although the butyl analogues are less potent on the high-affinity choline transport system, it is possible that their potency on the low-affinity choline transport system may be insignificant. If this is the case, the butyl analogues would be of interest since the specific presynaptic cholinotoxicity, defined as the ratio of IC_{50}(HAChT)/IC_{50}(LAChT), could be higher than with the Et and Pr analogues. This is currently being investigated in our laboratories.

B. QNB Binding. Quinuclidinyl benzilate (QNB) is a potent inhibitor of muscarinic cholinergic receptors and is considered to be a suitable agent for receptor labeling because of its potency, specificity, and persistence of action.[38] QNB binds to muscarinic receptors as well as to tissues; the former is referred to as *specific* binding and the latter as *nonspecific* binding. The sum of the two is referred to as *total* binding. Muscarinic agonists and antagonists displace [^{3}H]QNB from *specific* binding sites while nicotonic and noncholinergic drugs possess little affinity for [^{3}H]QNB binding sites.

The affinity of a toxin to bind to brain muscarinic receptors in vitro was determined in terms of the percent QNB displaced from the receptors at 50 μM concentration

(38) Yamamura, H. I.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 1725–1729.

Table II. Data for QNB Binding Studies

R	% QNB displaced[a]
Et	18
n-Pr	16
i-Pr	9
cyclopropyl	90[b]
i-Bu	6
n-Bu	9

[a] At 50 μM concentration of the corresponding toxin. [b] IC_{50} = 6.25 μM.

of a compound. A graph of μM of toxin vs. percent QNB displacement was plotted on semilog paper, and percent QNB displaced at 50 μM concentration of the toxin was determined from the graph. This method of presenting the data, rather than the conventional approach of reporting IC_{50} values, is due to the fact that in all cases but one (see below) extremely high concentrations of the substance (>50 μM) had minimal effects on QNB binding; hence, an IC_{50} evaluation was not practical in most cases. Table II compares the toxins under investigation for their ability to displace QNB in vitro from brain muscarinic receptors.

Results from Table II indicate, however, that the cyclopropyl analogue of AF64A did in fact have a high affinity for the postsynaptic muscarinic brain receptors, while the other analogues have very low affinity. The cyclopropyl analogue displaced 50% of the QNB from the receptors, at a concentration as low as 6.25 μM (=IC_{50}), which is equivalent to the activity of the highly potent muscarinic agonist oxotremorine.

This dual effect of the cyclopropyl analogue, both on high-affinity choline transport (see above) and on cholinergic receptors, could be advantageous, since the net result should produce a more extensive cholinergic hypofunction within the CNS.

Conclusions. From the reported[28] SAR for the high-affinity choline transport system, one can deduce the following biological requirements needed for this class of inhibitors: (1) Whereas *choline* is the natural substrate for the high affinity choline transport system, *acetylcholine* cannot be transported by the system. This suggests the importance of a *free* OH group in the substrate molecule. (2) Spatial separation between N and OH cannot be larger than three methylene groups. (3) Some *N*-alkyl substitution is possible without significant loss of activity. (4) The aziridinium ring appears to be important for induction of prolonged inhibition of choline transport, presumably by interacting irreversibly with the nucleophilic sites on high-affinity choline transport receptor.

With reference to the above, these studies indicate that an increasing alkyl chain length on our toxins, is not advantageous for the high-affinity choline transport inhibition but that branching or cyclization of the alkyl moiety produces similar results as those found for AF64A. The observation of a high affinity by the cyclopropyl aziridinium analogue for the postsynaptic muscarinic brain receptors was quite surprising. The need for further structural analogues of the cyclopropyl derivative precludes structure–activity speculations at this time. It is hoped that this and future studies will produce compounds with a greater selectivity, at all dose ranges.

Experimental Section

Proton magnetic resonance (NMR) spectra were recorded on a 90-MHz JEOL FX90Q spectrometer and are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). Low-resolution mass spectra were recorded on a Finnigan-3200 spectrometer. Chromatography was performed with EM reagent silica gel 60 with reported solvent systems. All

reactions were monitored with TLC (EM reagent aluminum oxide 60F, 0.20-mm thickness) and visualized after incubation in an I_2 chamber. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed on unknown compounds by Galbraith Laboratories, Inc., Knoxville, TN, and unless otherwise stated, they are within 0.4% of calculated values. Yields for most compounds were not optimized. THF and diethyl ether were dried over $LiAlH_4$ and stored over Na wires. Ac_2O and $SOCl_2$ were distilled prior to a reaction. The 2,2′-(*n*-butylimino)bisethanol was purchased from Aldrich Chemical Co.

Biological Test Procedures. 1. **High-Affinity Choline Transport Assay.** The procedure used to measure the transport of choline into synaptosomes was an adaptation of the method described by Yamamura and Snyder[39] and later modified by Bader et al.[40]

Mice were killed by decapitation, and the whole brain was quickly removed from the cranium. The cerebellum was then removed from the whole brain and synaptosomes were prepared devoid of cerebellum, as described by Whittaker.[41] The brain was homogenized in 20 volumes of 0.32 M sucrose. Synaptosomes were incubated with choline both in incubation medium containing isotonic sodium and in incubation medium with no sodium.

Eight sets of culture tubes were prepared, each in triplicate, for both: (+Na) and (−Na). The first and last sets were both controls (in which no toxin was added). Increasing concentrations of toxin were added to sets 2–7. Sample tubes were then incubated in a thermostatically controlled water bath at 30 ± 0.2 °C for 8 min and then returned to an ice–water bath to stop active transport. After cooling for 2 min, 3 mL of cold incubation buffer was added to each tube. The tubes were then immediately poured over GF/F filters enclosed in a Millipore sampling manifold under a low vacuum. Each filter was rinsed with cold incubation buffer, and individual filters were subsequently transferred to a 5-mL high-density polyethylene vial and filled with 3 mL of aqueous scintillation fluid (RIA Solv-II, Research Products International, Park Grove, IL). Radioactivity of the samples was next counted by liquid scintillation spectroscopy for 2 min.

The inhibitory activity was measured as follows:

% [^{3}H]CHCl transport by the high-affinity system,
at a particular concentration of the toxin tested =
{[CPM(Tris/sodium) −
CPM(Tris/sodium free)]/[CPM(Tris/sodium) of control −
CPM(Tris/sodium free) of control]} × 100

% [^{3}H]ChCl inhibited = 100 − % [^{3}H]ChCl transported

2. **QNB Receptor Binding.** The assay procedure used to measure the affinity of cholinotoxins toward brain muscarinic cholinergic receptors was an adaptation of the procedure developed by Yamamura and Snyder.[38]

Synaptosomes were prepared as mentioned in the procedure for the high-affinity choline transport assay. Muscarinic radioligand receptor binding requires incubation of brain synaptosomal membranes with radioactive QNB, in the absence and presence of an *excess* amount of a competitive unlabelled antagonist or agonist. In the present studies, oxotremorine was used as the unlabeled competitive agonist. Incubations were conducted for 60 min at 37 °C.

After the incubations were completed, each tube was quickly inverted over GF/C filters, which had previously been soaked in buffer, and mounted onto the vacuum sampling manifold. A moderate vacuum (10–15 mmHg) was used to filter the binding mixture. The filters were then immediately washed three times with 5-mL portions of cold incubation buffer. The filters were finally placed into 5-mL plastic minivials, 3 mL of counting cocktail was added, and the collected tissue was left to be extracted from the filter overnight. The vials were vortexed and trapped radioactivity measured by liquid scintillation spectroscopy.

Percent specific binding of [^{3}H]QNB at a particular concentration of toxin studied was measured from the following relationship:

{[total binding(CPM) −
nonspecific binding(CPM)]/[total binding(CPM) of control
− nonspecific binding(CPM) of control]} × 100

% [^{3}H]QNB displaced = 100 − % specific [^{3}H]QNB binding

Preparation of 2,2′-(*n*-Propylimino)bisethanol (2) (Route b). In a three-necked flask, equipped with a condenser and $CaCl_2$ drying tube, were placed 25.61 g (2.44 × 10^{-1} mol) of diethanolamine, 25 mL of absolute EtOH, and 8.42 g (6.1 × 10^{-2} mol) of anhydrous K_2CO_3, and the mixture was refluxed with stirring. To this, 15 g (1.22 × 10^{-1} mol) of *n*-propyl bromide was added slowly through a dropping funnel. After the addition was complete, the mixture was refluxed with stirring for 4.5 h. The solution was then filtered to remove KBr. The filtrate was evaporated under reduced pressure to give 17.0 g (94.8% yield) of crude oil. The crude oil was dissolved in a minimum amount of H_2O and first washed gently with (2 × 5 mL) of C_6H_6 and then extracted with (3 × 35 mL) of $CHCl_3$. The combined $CHCl_3$ extract was dried over anhydrous Na_2SO_4 and $CHCl_3$ was then evaporated to give 13.4 g of the pure product **2** as a colorless oil. This method of purification was found to be better than column chromatography: bp 217 °C (150 mmHg); TLC (EtOAc + 5% MeOH), single spot (R_f 0.66); NMR ($CDCl_3$) δ 3.60 (t, 4 H), 3.22 (s, 2 H), 2.60 (m, 6 H), 1.50 (m, 2 H), 0.90 (t, 3 H).

Preparation of 2,2′-(Isopropylimino)bisethanol (3). (General Procedure: Route a). Isopropylamine (10.0 g, 1.70 × 10^{-1} mol) was mixed with 40.0 mL of distilled H_2O and cooled with stirring to 4 °C. Liquid ethylene oxide (16.7 g, 3.80 × 10^{-1} mol) was then added dropwise with stirring, at 4 °C. The reaction mixture was then stirred for 15 h at 4 °C. Traces of starting materials were removed on a rotary evaporator at room temperature, and H_2O was removed under high vacuum to give 24.16 g (95.6% yield) of yellow oil. The product was distilled [bp 120 °C (5 mmHg)] to give 23.25 g of pure **3** (93% yield) as colorless oil: TLC (EtOAc + 5% MeOH), single spot (R_f = 0.65); NMR ($CDCl_3$) δ 3.60 (t, 4 H), 3.18 (br s, 2 H), 3.00 (m, 1 H), 2.62 (t, 4 H), 1.03 (d, 6 H).

In the same manner, other 2,2′-(alkylimino)bisethanols were prepared from the corresponding alkylamines by route (a).

2,2′-(Cyclopropylimino)bisethanol (4): bp 115 °C (0.25 mmHg); yield 97.1%; TLC (EtOAc + 5% MeOH), single spot (R_f 0.62); NMR ($CDCl_3$) δ 3.67 (t, 4 H), 3.15 (br s, 2 H), 2.80 (t, 4 H), 1.86 (m, 1 H), 0.50 (m, 4 H).

2,2′-(Isobutylimino)bisethanol (5): bp 128 °C (4.0 mmHg); yield 94.3%; TLC (EtOAc + 5% MeOH), single spot (R_f 0.69); NMR ($CDCl_3$) δ 3.60 (t, 4 H), 2.63 (t, 4 H), 2.27 (d, 2 H), 1.78 (m, 1 H), 0.90 (d, 6 H).

Preparation of 2-[*n*-Propyl(2-hydroxyethyl)amino]ethyl Acetate (6) (General Procedure: Step c). To a magnetically stirred and ice-cooled solution of 10.0 g (6.8 × 10^{-2} mol) of **2** in 15 mL of EtOAc was added 3.6 g (3.5 × 10^{-2} mol) of Ac_2O dropwise during 15 min, and the solution was stirred for further 0.5 h. TLC indicates at this time the beginning of formation of the unwanted diacetate. The reaction mixture was then taken up in 50 mL of 5% aqueous K_2CO_3 and the more *nonpolar* diacetate was removed by gently washing the aqueous solution with (2 × 10 mL) of $CHCl_3$. The pure monoacetate **6** was then obtained by extracting the aqueous solution with 3 × 25 mL of $CHCl_3$, drying over anhydrous Na_2SO_4, and evaporating the $CHCl_3$ as 1.2 g (18.1% yield based on Ac_2O) of oil: TLC (EtOAc + 5% MeOH), single spot (R_f 0.83); NMR ($CDCl_3$) δ 4.13 (t, 2 H), 3.53 (t, 2 H), 2.90–2.30 (m, 7 H), 2.10 (s, 3 H), 1.46 (m, 2 H), 0.88 (t, 3 H).

In the same manner, other 2-[alkyl(2-hydroxyethyl)amino]ethyl acetates were prepared from the corresponding bisethanols by using step (c).

2-[Isopropyl(2-hydroxyethyl)amino]ethyl acetate (7): yield 14.6%; TLC (EtOAc + 5% MeOH), single spot (R_f 0.82); NMR ($CDCl_3$) δ 4.12 (t, 2 H), 3.53 (t, 2 H), 2.79 (m, 1 H), 2.68 (m, 5 H), 2.10 (s, 3 H), 1.03 (d, 6 H).

2-[Cyclopropyl(2-hydroxyethyl)amino]ethyl acetate (8): yield 12.1%; TLC (EtOAc + 5% MeOH), single spot (R_f 0.78); NMR ($CDCl_3$) δ 4.23 (t, 2 H), 3.60 (t, 2 H), 2.90 (m, 5 H), 2.15

(39) Yamamura, H.; I.; Snyder, S. H. *J. Neurochem.* **1973**, *21*, 1355–1374.
(40) Bader, C. R.; Baughman, R. W.; Moore, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2525–2529.
(41) Whittaker, V. P. *Prog. Biophys. Mol. Biol.* **1965**, *15*, 39–88.

(s, 3 H), 1.93 (m, 1 H), 0.50 (m, 4 H).

2-[*n*-Butyl(2-hydroxyethyl)amino]ethyl acetate (9): yield 10.4%; TLC (EtOAc + 5% MeOH), single spot (R_f 0.89); NMR ($CDCl_3$) δ 4.14 (t, 2 H), 3.55 (t, 2 H), 2.92–2.30 (m, 7 H), 2.10 (s, 3 H), 1.37 (m, 4 H), 0.93 (m, 3 H).

2-[Isobutyl(2-hydroxyethyl)amino]ethyl acetate (10): yield 8.2%; TLC (EtOAc + 5% MeOH), single spot (R_f 0.87); NMR ($CDCl_3$) δ 4.12 (t, 2 H), 3.53 (t, 2 H), 3.10–2.40 (m, 5 H), 2.24 (d, 2 H), 2.06 (s, 3 H), 1.74 (m, 1 H), 0.88 (d, 6 H).

Preparation of 2-[*n*-Propyl(2-chloroethyl)amino]ethyl Acetate Hydrochloride (11) (General Procedure: Step d). To a magnetically stirred and ice-cooled solution of 460 mg (2.4×10^{-3} mol) of **6** in 2.0 mL of $CHCl_3$ was added 382 mg (3.2×10^{-3} mol) of $SOCl_2$ dropwise with a hypodermic syringe, under an N_2 atmosphere. After the completion of addition of $SOCl_2$ (10 min), the ice bath was removed and the reaction mixture was stirred for 2.5 h at room temperature under an N_2 atmosphere to complete the reaction. Removal of $CHCl_3$ and $SOCl_2$ under high vacuum gave a thick yellow oil. The oil was dissolved in 3.0 mL of dry THF and cooled with stirring. Addition of 5.0 mL of dry diethyl ether to the stirred solution under N_2 resulted in the precipitation of a white solid, which was filtered, washed with dry diethyl ether, and then dried in an Abderhalden pistol (CH_2Cl_2 as solvent and 0.5 mmHg pressure) to give 460 mg of a white solid (78.6% yield) as the hydrochloride salt **11**: mp 96–98 °C; NMR ($CDCl_3$) δ 4.62 (t, 2 H), 4.10 (t, 2 H), 3.70–2.90 (m, 6 H), 2.60 (s, 3 H), 1.93 (m, 2 H), 1.05 (t, 3 H); MS, m/e (relative intensity), 207 (0.31, $M^+ - HCl$), 136 (34.82, $M^+ - HCl - C_3H_5O_2$), 134 (91.23, $M^+ - HCl - C_3H_5O_2$), 87 (100.00, $C_4H_7O_2$). Anal. ($C_9H_{19}NO_2Cl_2$) C, H, N, Cl.

In the same manner, other 2-[alkyl(2-chloroethyl)amino]ethyl acetate hydrochlorides were prepared from compounds **7–10** by route (d).

2-[Isopropyl(2-chloroethyl)amino]ethyl acetate hydrochloride (12): mp 72–74 °C; yield 71.9%; NMR ($CDCl_3$) δ 4.45 (t, 2 H), 3.94 (m, 3 H), 3.66 (m, 4 H), 2.15 (s, 3 H), 1.37 (d, 6 H); MS, m/e (relative intensity) 209 (0.29, $M^+ - HCl$), 207 (1.28, $M^+ - HCl$), 136 (31.13, $M^+ - HCl - C_3H_5O_2$), 134 (85.62, $M^+ - HCl - C_3H_5O_2$), 94 (32.87, C_3H_7NCl), 92 (100.00, C_3H_7NCl). Anal. ($C_9H_{19}NO_2Cl_2$) C, H, N, Cl; C: calcd, 44.44; found, 43.95.

2-[Cyclopropyl(2-chloroethyl)amino]ethyl acetate hydrochloride (13): mp 67–69 °C; yield 62.47%; NMR ($CDCl_3$) δ 4.64 (t, 2 H), 4.22 (t, 2 H), 3.64 (m, 4 H), 2.72 (m, 1 H), 2.16 (s, 3 H), 1.61 (m, 2 H), 1.00 (m, 2 H); MS, m/e (relative intensity) 205 (1.72, $M^+ - HCl$), 132 (47.65, $M^+ - HCl - C_3H_5O_2$, 87 (100.00, $C_4H_7O_2$). Anal. ($C_9H_{17}NO_2Cl_2$) C, H, N, Cl.

2-[*n*-Butyl(2-chloroethyl)amino]ethyl acetate hydrochloride (14): mp 110–112 °C; yield 85.5%; NMR ($CDCl_3$) δ 4.62 (t, 2 H), 4.12 (t, 2 H), 3.75–2.95 (m, 6 H), 2.14 (s, 3 H), 2.10–1.15 (m, 4 H), 1.00 (t, 3 H); MS, m/e (relative intensity) 223 (0.43, $M^+ - HCl$), 221 (1.29, $M^+ - HCl$), 180 (6.30, $M^+ - HCl - C_3H_7$), 178 (18.78, $M^+ - HCl - C_3H_7$), 150 (36.57, $M^+ - HCl - C_3H_5O_2$, 148 (100.00, $M^+ - HCl - C_3H_5O_2$), 108 (24.67, C_4H_9NCl), 106 (74.67, C_4H_9NCl), 87 (78.75, $C_4H_7O_2$). Anal. ($C_{10}H_{21}NO_2Cl_2$) C, H, N, Cl.

2-[Isobutyl(2-chloroethyl)amino]ethyl acetate hydrochloride (15): mp 94–96 °C; yield 88.3%; NMR ($CDCl_3$) δ 4.63 (t, 2 H), 4.15 (t, 2 H), 3.57 (t, 4 H), 3.12 (d, 2 H), 2.28 (m, 1 H), 2.15 (s, 3 H), 1.16 (d, 6 H); MS, m/e (relative intensity) 221 (0.49, $M^+ - HCl$), 180 (2.87, $M^+ - HCl - C_3H_7$), 178 (8.52, $M^+ - HCl - C_3H_7$), 87 (100.00, $C_4H_7O_2$). Anal. ($C_{10}H_{21}NO_2Cl_2$) C, H, N, Cl.

Preparation of 10 mM Solution of 1-Alkyl-1-(2-hydroxyethyl)aziridinium Chloride for Biological Assays. The hydrochloride salt **11–15** was accurately weighed and stirred in sufficient distilled H_2O to dissolve the salt. At this point, the pH was usually between 3.5 and 4.0. A sufficient amount of 5 N NaOH was added to bring the pH to 11.3–11.6. The solution was then stirred for another 30 min at room temperature while the pH was maintained between 11.3 and 11.6. Next, the pH was adjusted to 7.4 by first adding concentrated HCl to bring the pH to slightly acidic (about 6) and then adding the required amount of $NaHCO_3$. Finally, the required amount of distilled H_2O was added to make up a final solution of 10 mM concentration for use in biological assays.

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Benzo[*a*]carbazole Derivatives. Synthesis, Estrogen Receptor Binding Affinities, and Mammary Tumor Inhibiting Activity

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A number of 11-alkylbenzo[*a*]carbazoles and their 5,6-dihydro derivatives with one or two hydroxy groups in the aromatic rings were synthesized and studied for their binding affinities for the estrogen receptor. Best conditions for the receptor binding are provided by one hydroxy group at C-3 and a second one at position 8 or 9. The binding affinities of the benzo[*a*]carbazoles are somewhat lower than those of the dihydro derivatives but still high regarding the planar structure of these molecules. The highest relative binding affinity (RBA) values (e.g., 30 for **13b**, 13 for **16b**, 20 for **25a**; estradiol = 100) are close to those of the corresponding 2-phenylindole derivatives. Depending on the positions of the oxygen functions, the benzo[*a*]carbazoles behaved as strong estrogens (**13c**, **25a**) or impeded estrogens (**16c**, **28a**) in the immature mouse. Derivative **16c** inhibited the growth of dimethylbenzanthracene-induced hormone-dependent mammary tumors of the rat at a dose of 6 × 1 mg/kg per week. In vitro, **16b** and **28b** showed inhibitory activity on estrogen receptor positive MCF-7 breast cancer cells. A mode of action involving the estrogen receptor system is assumed.

Pharmacotherapy with antiestrogens has become an established method in the management of advanced breast cancer.[1-3] The advantages of this treatment are because of the greatly reduced toxicity compared to cytostatic

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