6-Methyl-6-azabicyclo[3.2.1]octan-3α-ol 2,2-Diphenylpropionate (Azaprophen), a Highly Potent Antimuscarinic Agent

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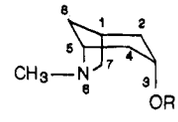
The synthesis and antimuscarinic properties of 6-methyl-6-azabicyclo[3.2.1]octan-3α-ol 2,2-diphenylpropionate (1, azaprophen) are described. Azaprophen is 50 times more potent than atropine as an antimuscarinic agent as measured by the inhibition of acetylcholine-induced contraction of guinea pig ileum and is more than 1000 times better than atropine in its ability to block α-amylase release from pancreatic acini cells induced by carbachol. In addition, azaprophen is 27 times more potent than atropine as an inhibitor of binding of [*N*-*methyl*-^{3}H]scopolamine to muscarinic receptors, with human IMR-30 neuroblastoma cells. The potencies of azaprophen and atropine in altering operant behavior were similar. The structural features of 1 are compared to the standard anticholinergic drugs atropine and quinuclidinyl benzilate by using energy calculations and molecular modelling studies. A modification of the pharmacophore model hypothesis for cholinergic agents is suggested.

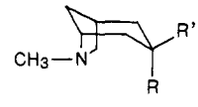
Understanding how antimuscarinic compounds interact with their receptor(s) is of great interest in drug development.[1] Despite extensive studies directed toward the identification of the active receptor-bound conformation of muscarinic antagonists, the active conformation still remains unclear. One method for probing the surface geometry of the muscarinic receptor site is the synthesis and biological evaluation of conformationally restricted analogues of antimuscarinic agents. In order to gain additional information concerning the effects of stereochemical factors on antimuscarinic potency, we have prepared the conformationally rigid 6-methyl-6-azabicyclo[3.2.1]-octan-3α-ol 2,2-diphenylpropionate (1, azaprophen). The unique features of 1 are as follows: (a) the cationic head is derived from 6-methyl-6-azabicyclo[3.2.1]octan-3α-ol (**2a**), which is isomeric with tropine, the amino alcohol component of atropine; (b) the esteratic group and cyclic substituent are derived from 2,2-diphenylpropionic acid, which makes up the acid part of aprophen, a highly potent antimuscarinic compound;[2] and (c) compound 1 has the acyloxy and amino groups in proximity different from that found in atropine.

We have determined the antimuscarinic activity of 1 in three assays and compared the results to other potent antimuscarinic compounds. In addition, effects of azaprophen on operant behavior were compared with those of atropine. Molecular modelling studies were used to compare the structural features of 1 to atropine and other anticholinergics.

Chemistry. Azaprophen was synthesized by lithium aluminum hydride reduction of 6-methyl-3-oxo-6-azabicyclo[3.2.1]octane (**2b**)[3] to a 40:60 mixture of the 3α- and 3β-alcohols **2a** and **2c**, respectively. Esterification of the



1: R = $(C_6H_5)_2C(CH_3)CO$



2a: R = OH, R' = H
b: R, R' = =O
c: R = H, R' = OH

mixture with 2,2-diphenylpropionyl chloride in tetrahydrofuran at 25 °C gave 1 plus recovered **2c**·HCl. This unexpected selectivity may be due to reversible formation of an acyl ammonium salt with **2a** and **2c**, which in the case of **2a** is followed by a facile N → O intramolecular transfer to give 1. Intramolecular N → O migration is not possible with the salt from **2c** and apparently the usual intermolecular process does not occur under the reaction conditions.[4] Compound 1 was purified as the hydrochloride salt and was characterized by elemental analysis and its ^{1}H NMR and IR spectral properties. The ^{1}H NMR spectrum of 1·HCl in $CDCl_3$ showed a multiplet centered at 5.35 ppm for the C-3 proton, a doublet at 2.13 ppm (J = 5.1 Hz) for the *N*-methyl group, and a broad NH^+ resonance at 12.1 ppm. The IR spectrum of 1·HCl showed a broad peak centered at 2500 cm^{-1} for the $^+$NH group and a peak at 1735 cm^{-1} for the ester carbonyl.

Table I. Selected Proton NMR Data

compound	C-3 hydrogen δ	C-3 hydrogen $W_{1/2}$, Hz
2a[a,b]	4.1	12.0
2c[a,c]	3.94	22.4
tropine[d]	3.97	11.6
pseudotropine[d]	3.87	21.6
1·HCl[d]	5.35	11.8
atropine[d]	5.01	10.5

[a] Measured in D_2O. [b] The resonance for **2a** was obtained by a difference NMR method from the ^{1}H NMR spectrum of the **2a** and **2c** mixture. [c] The salt **2c**·HCl showed a C-3 hydrogen resonance at 4.01 with $W_{1/2}$ = 21.7 Hz. [d] Measured in $CDCl_3$.

The stereochemical assignment of 1 was established by a direct comparison of the ^{1}H NMR spectra of **2a**, the alcohol from which 1 is derived, to the spectra of its C-3 isomer **2c**. The C-3 hydrogen resonance of interest for stereochemical assignment was evident from its chemical shift and, in the case of **2a**, the characteristic downfield shift of this resonance in the spectrum of 1. Recorded in Table I are the chemical shifts and observed half-height widths ($W_{1/2}$) for the two alcohols. For comparison, similar

† Research Triangle Institute.
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(1) Triggle, D. J. In *CRC Handbook of Stereoisomers: Drugs in Psychopharmacology*; Smith, D. F., Ed.; CRC: Boca Raton, FL, 1984; pp 31–66.
(2) Gordon, R. K.; Padilla, F. N.; Moore, E.; Doctor, B. P.; Chiang, P. K. *Biochem. Pharmacol.* **1983**, *32*, 2979.
(3) Furstoss, R.; Teissier, P.; Waegell, B. *J. Chem. Soc., Chem. Commun.* **1970**, 384.
(4) The stereoselective esterification of **2a** could also be explained by intramolecular general-base catalysis with the amino nitrogen of **2a** acting as the internal base.

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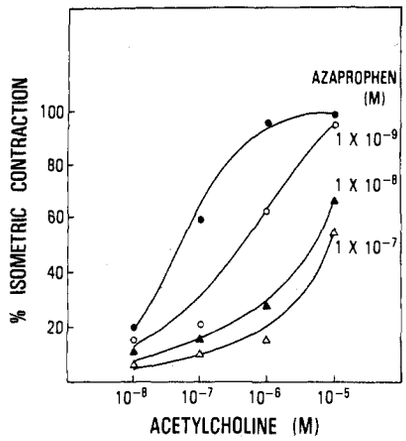

Figure 1. Inhibition of the acetylcholine-induced contraction of guinea pig ileum by azaprophen.

Table II. Antimuscarinic Activity of Azaprophen and Standard Compounds

compound	guinea pig ileum contraction		pancreatic acini α-amylase release
	pA_2	K_B, M	I_{50}, M
azaprophen	10.4 ± 0.4	3.9×10^{-11}	$6.8 \times 10^{-11} \pm 2.4$
aprophen	8.5 ± 0.1	3.1×10^{-9}	$1.1 \times 10^{-8} \pm 0.2$
adiphenine	7.1 ± 0.3	9.2×10^{-8}	$3.5 \times 10^{-7} \pm 0.3$
benzctyzine	8.2 ± 0.8	6.5×10^{-9}	$3.0 \times 10^{-8} \pm 0.5$
atropine	8.7 ± 0.1	2.0×10^{-9}	$5.1 \times 10^{-8} \pm 3.2$
QNB	8.7 ± 0.9	2.0×10^{-9}	$2.1 \times 10^{-8} \pm 0.6$
pirenzepine	4.3 ± 0.4	5.0×10^{-5}	$2.8 \times 10^{-6} \pm 1.2$

data for tropine and pseudotropine are also included. The distinction between an axial and an equatorial alcohol in an epimeric pair can usually be made on the basis of relative chemical shift and/or the $W_{1/2}$ value of the methine resonance.[5] Innumerable results obtained from the spectra of compounds containing six-membered rings establish firmly that the $W_{1/2}$ for an equatorial proton is invariably significantly smaller than $W_{1/2}$ for an axial proton. Typically, an equatorial proton exhibits a $W_{1/2}$ of 5–12 Hz and an axial one a value of 15–30 Hz.

Examination of the data in Table I shows that **2a** gives rise to a narrow resonance ($W_{1/2}$ = 12.0 Hz) characteristic of equatorial hydrogen whereas **2c** gives a relatively broading resonance ($W_{1/2}$ = 22.4 Hz) expected for a strongly coupled axial hydrogen. It follows that **2a** and **2c** are the 3α-hydroxy and 3β-hydroxy isomers, respectively. The relative positions for the chemical shifts of 4.1 and 3.90 ppm for **2a** and **2c**, respectively, are also in accord with this assignment.[5] In addition the chemical shift and $W_{1/2}$ values for **2a** and **2c** are analogous to those found for tropine and pseudotropine, respectively (see Table I). Since azaprophen is derived from **2a**, this compound is the 3α-isomer as depicted in structure **1**. Additional support for this assignment follows from a comparison of the $W_{1/2}$ (11.8 Hz) of **1**·HCl to that of atropine ($W_{1/2}$ = 10.5 Hz, see Table I).

Biological Studies. Azaprophen, along with standard compounds, was assayed for its ability (a) to block the acetylcholine-induced contraction of the guinea pig ileum,[6-8] (b) to inhibit the carbachol-induced release of α-amylase from pancreatic cells,[9,10] (c) to inhibit the binding of [*N*-methyl-^{3}H]scopolamine (^{3}H-NMS) muscarinic receptor of human IRM-30 neuroblastoma cell, and (d) to inhibit the operant behavior of rats.

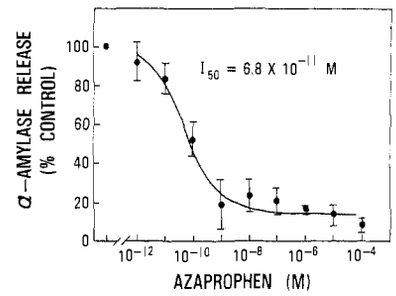

Figure 2. Inhibition of the release of α-amylase from pancreatic acini cells induced by 10^{-5} M carbachol by azaprophen.

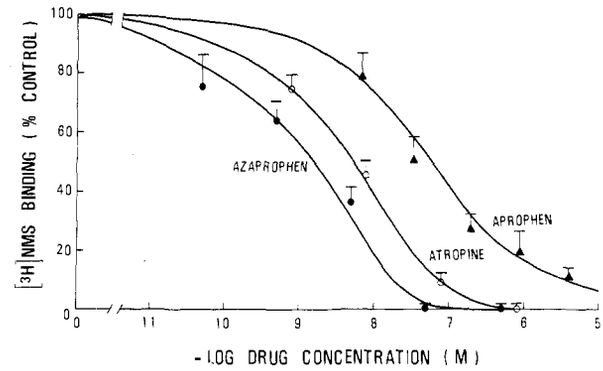

Figure 3. Inhibition of ^{3}H-NMS binding to the muscarinic receptors of IMR-32 neuroblastoma cells by atropine, aprophen, and azaprophen.

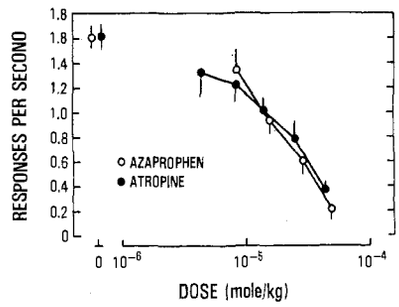

Figure 4. Inhibition of behavior of rats maintained by food delivery by azaprophen hydrochloride and atropine sulfate when given intraperitoneally 30 min prior to behavioral experiments.

Guinea Pig Ileum Studies. Figure 1 shows the inhibition of the ACh-induced contraction of guinea pig ileum by azaprophen. Azaprophen had a pA_2 of 10.4 (K_B = 3.9×10^{-11} M) (Table II) with a slope of −0.7 ± 0.2 determined by the Schild plot[11] (the three runs gave pA_2 values of 10.4, 10.6, and 10.6 and Schild slopes of −0.7 ± 0.2, −0.7 ± 0.3, and −0.7 ± 0.1, respectively. While the slopes are less than unity, the pA_2 values are presented for

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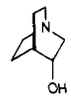
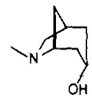
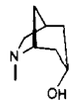
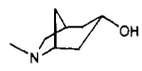
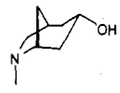
(6) Pankaskie, M. C.; Kachur, J. F.; Itoh, T.; Gordon, R. K.; Chiang, P. K. *J. Med. Chem.* **1985**, *28*, 1117.
(7) *Pharmacological Experiments on Isolated Preparations*, Department of Pharmacology, University of Edinburgh; Churchill Livingstone, Edinburgh, 1970; pp 58–87.
(8) Gordon, R. K.; Chiang, P. K. *J. Pharmacol. Exp. Ther.* **1986**, *236*, 85–89.
(9) Peikin, S. R.; Rottman, A. J.; Batzri, S.; Gardner, J. D. *Am. J. Physiol.* **1978**, *235*, E743–749.
(10) Gardner, J. D.; Jackson, M. J. *J. Physiol.* **1977**, *270*, 439–454.
(11) Tallarida, R. J.; Murray, R. B. *Manual of Pharmacological Calculations with Computer Programs*; Springer-Verlag: New York, 1981; pp 29–31.

Table III. Conformational Energies and Nitrogen–Oxygen Distances for A–G

conformer		N–O distance, Å	total energy, kcal
A		3.81	25.2
B		3.84	25.5
C		3.46	21.9
D		2.78	26.5
E		3.20	26.2
F		4.20	30.3
G		4.51	29.8

comparison purpose.[12] Thus, azaprophen is 50–2000 times more potent than atropine, benactyzine, aprophen, adiphenine, and QNB and is about 6 orders of magnitude more potent than pirenzepine (see Table II).

α-Amylase Release. Figure 2 shows the inhibition of the carbachol-induced release of α-amylase from pancreatic acini cells. The I_{50} of azaprophen (6.8×10^{-11} M) is 160 times lower than that of aprophen, 300–5000 times lower than that of benactyzine, adiphenine, and quinuclidinyl benzilate (QNB), more than 1000 times better than that of atropine, and 5 orders of magnitude better than that of pirenzepine (Table II).

Muscarinic Receptor Studies. Figure 3 shows the comparison of the inhibition of ^{3}H-NMS to IMR-32 neuroblastoma cells. The K_i values are 2.7×10^{-10} M for azaprophen, compared to 1.0×10^{-9} M for atropine and 2.3×10^{-8} M for aprophen (standard errors are less than 10%).

Operant Behavior Studies. Figure 4 shows that the decreases in the rates of lever pressing by rats were comparable between those treated with azaprophen and those treated with atropine ($I_{50} = 1.9 \times 10^{-5}$ and 2.3×10^{-5} mol/kg, respectively). Effects of saline administration are shown by the control points above 0 mol/kg. Azaprophen hydrochloride and atropine sulfate were given intraperitoneally 30 min prior to behavior experiments. Each point represents the mean ± SEM for single or duplicate determinations made in six (atropine-treated) or four (azaprophen-treated) rats.

Molecular Modelling Studies. The molecular models (A–G) in Table III were constructed by using X-ray fragments and normal computer-supplied connections available from the SYBYL computer graphics system.[13] The conformational energies were obtained by exhaustive minimization using the MM2 molecular mechanics program of Allinger,[14] which provided identical minima for each conformer from at least three different starting geometries. Table III contains a summary of the results of these studies in which the minimum energy for each compound or conformation is given as the total energy in kilocalories and the nitrogen–oxygen atom distance measured from the resultant minimum energy geometry is given in angstroms.

Discussion

Extensive structure–activity relationship (SAR) studies have been directed toward exploring the active receptor-bound conformation of acetylcholine and its antagonist.[1] Acetylcholine and most of its antagonists are flexible molecules. Even though the solid- and solution-state conformation in many cases are known, these conformations are not necessarily those of the receptor-bound species. The observed differences in biological activities of muscarinic antagonists may be due to differences in their relative abilities to adopt conformation necessary for maximum interaction at the receptor binding site. Clearly, any such transition is of paramount significance in regard to quantitative SAR studies.

It is possible to circumvent the problem of flexible molecules by the design and evaluation of conformationally rigid anticholinergic agents. In the present study, information as to the head-group binding geometry of anticholinergics is provided by the evaluation of azaprophen, which possesses a conformationally rigid azabicyclic ring (**2a**) cationic head group isosterically related to tropine, the cationic head group of atropine. For comparison, **2a** and tropine can be viewed as a cyclohexane ring containing a CH_2–N–CH_3 bridge and a *N*-methylpiperidine possessing a $-CH_2CH_2-$ bridge, respectively. Thus, with **1** the axially oriented acyloxy group is cis to the $-CH_2$–N–CH_3 bridge whereas in atropine the axially oriented acyloxy group is trans to the nitrogen bridge. As a result, the proximity of these two groups in **1** and atropine is different. Regardless of the point of view, the important structural features of **2a** and **1** are locked into rigid position to give topographical information about the anionic functional group of the receptor site.

To investigate the confromational energetics and possible pharmacophore binding modes for azaprophen (**1**), molecular modelling studies were performed with its rigid bicyclic amino alcohol head group and, for comparison, the bicyclic amino alcohols tropine and 3-quinuclidinol, corresponding to the standard anticholinergic drugs atropine and quinuclidinyl benzilate, respectively.

The energies of the exo- and endo-methyl conformations of tropine, A and B, respectively, were as expected within 0.3 kcal, with an N–O distance of ~3.8 Å. The corresponding N–O distance for 3-quinuclidinol (C) was ~3.5 Å. Thus, both tropine and quinuclidinol fit the simple distance geometry pharmacophore, constraints established previously for the acetylcholine receptor.[15] With use of

(12) Nonunity slopes indicates that other mechanisms may be involved (Tallarida, R. J.; Jacob, L. S. *The Dose–Response Relation in Pharmacology*; Springer-Verlag: New York, 1979). Or, as depicted in Figure 1, it is strictly a noncompetitive inhibitor with respect the guinea ileum assay, or a multimolecular interaction between azaprophen and the muscarinic receptors (Kenakin, T. P. *Can. J. Physiol. Pharmacol.* **1982**, *60*, 249–265). We presently are looking into the possibility of calcium blocking activity of azaprophen.

(13) Tripos Associates, Inc., St. Louis, MO 63117.

(14) Allinger, N. L. *Adv. Phys. Org. Chem.* **1976**, *13*, 1.

(15) Bebbington, A.; Brimblecombe, *Adv. Drug Res.* **1965**, *2*, 143 and references therein.

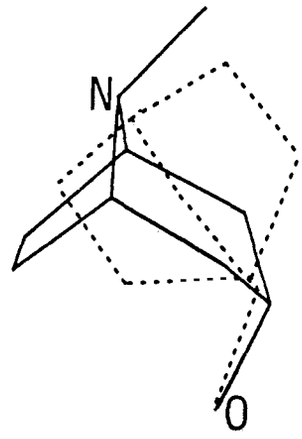


Figure 5. Graphic comparison of atropine (-) and quinuclidinol (--).

a classical three-point pharmacophore, consisting of the nitrogen and oxygen atoms and the carbon atom α to oxygen for B and C, an excellent three-dimensional fit is achieved for these structurally unique head-group ligands by simple least-squares regression analysis (FIT). The molecular overlays resulting from this procedure are presented in Figure 5. Similar results are obtained for a large variety of cholinergic ligands analyzed in this manner by ourselves and others.[16]

Four conformations of the amino alcohol portion of azaprophen were studied; the chair exo methyl (D), the chair endo methyl (E), the boat exo methyl (F), and the boat endo methyl (G). The global minimum-energy conformer computed by MM2 was the chair endo-methyl form (E) (see Table III). Exo-/endo-methyl energy differences were quite small for both the chair (0.3 kcal) and the boat (0.5 kcal) forms. The ground-state energy difference between the chair and boat forms of these conformers was 3.6–3.8 kcal, suggesting that only the chair forms would be significantly populated. The N–O distances for the chair forms were 2.8–3.2 Å and the boat forms 4.2–4.5 Å. Thus, in either the chair form or the boat form, the N–O distance geometries differ significantly from either those of tropine or quinuclidinol and presumably also from atropine and quinuclidinyl benzilate. Computer-fitting experiments by least-squares regression analysis, using the same three-point pharmacophore model described above, revealed that none of these four conformational forms produce a reasonable pharmacophore fit to the tropine–quinuclidinol model (Figure 5).

In view of the exceptionally high receptor affinity and in vitro efficacy of azaprophen and the fact that the stereochemistry of the oxygen side chain is identical to that in atropine, we are led to conclude that the cationic head group of azaprophen may bind to the cholinergic receptor anionic site in a different manner than previously analyzed cholinergic agents, i.e., isosterically with atropine. Such a binding mode would require an expansion of the present pharmacophore model hypothesis for cholinergic agents, in a manner similar to the "dual-faced" model successfully evoked to explain the activity of various phenylpiperidine ligands at the opiate receptor.[17] Since the anionic site of the cholinergic receptor is believed to be the side-chain carboxylate of either an Asp or Glu residue[18] on the protein, this group can potentially accommodate the offset in the position of the nitrogen atom that would be required

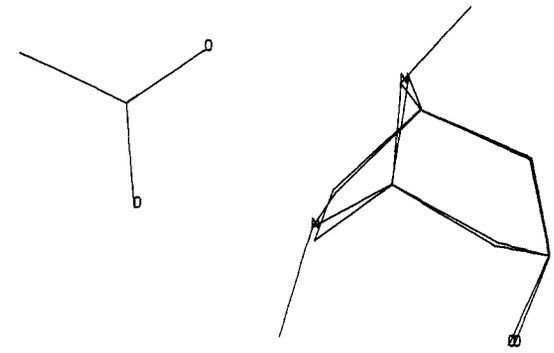


Figure 6. Computer-generated molecular overlays of tropine (conformer B) and **2** (conformer E) oriented toward the carboxylate of the receptor represented in this figure by the acetate anion.

by an "isosteric fit" hypothesis for the atropine and azaprophen head groups. Figure 6 contains the computer-generated fit of endo-methyl tropine (B) and endo-methyl azaprophenol (E) and the hypothetical position of the carboxylate anion that would be required. Interestingly, both atropine and azaprophen can relieve steric congestion to the approach of the carboxylate anion from this face of either molecule by conformational inversion of the methyl group at little or no energetic cost.

Conclusion

Azaprophen (1) is a novel antimuscarinic agent that is more potent than atropine and other anticholinergics in (a) the inhibition of the ACh-induced contraction of guinea pig ileum, (b) the inhibition of carbachol-induced release of α-amylase from pancreatic acini cells, and (c) the inhibition of binding of ^{3}H-NMS to IMR-32 neuroblastoma cells. The high potency of azaprophen combined with the observation that azaprophen is about one million times more potent than pirenzepine in blocking the contractile responses in the guinea pig ileum, and amylase secretion from pancreatic acini suggests that azaprophen is a strong anticholinergic at M2 receptors.[19] The comparable potency of azaprophen and atropine in altering operant behavior suggests that azaprophen may function in vivo as a muscarinic antagonist at doses that do not produce the deleterious behavioral side effects of classic antimuscarinics.

Molecular modelling studies reveal that 1 does not fit the simple distance geometry pharmacophore constraints normally accepted for the acetylcholine receptor, and 1 may bind to the cholinergic receptor anionic site in a manner different from previously analyzed cholinergics. Thus, azaprophen may provide the first example of an anticholinergic drug with a distinct modification of head group binding geometry. We are actively working to further test this new pharmacophore hypothesis.

Experimental Section

Melting points were determined on a Kofler hot-stage microscope with a calibrated thermometer. IR spectra were measured with a Perkin-Elmer Model 267 or 467 grating infrared spectrophotometer. NMR spectra were recorded on a Bruker WM-250 spectrometer using tetramethylsilane as an internal standard. Microanalyses were carried out by Atlantic Microlab, Inc. Molecular models were built with a molecular modelling system, which consists of an Evans and Sutherland PS330 graphics system linked to a Digital Equipment Corp. VAX work station. Software employed was the SYBYL program (version 3.3) from Tripos Associates.[13]

(16) Unpublished results.
(17) Humblet, C.; Marshall, G. R. *Drug Dev. Res.* 1981, *1*, 419.
(18) Kosower, E. M. *Biophys. J.* 1984, *45*, 13.
(19) Pirenzepine is considered to be selective for M1 receptors. Additional studies will be required to establish the selectivity of azaprophen with respect to M1 and M2 receptors.

6-Methyl-6-azabicyclo[3.2.1]octan-3-ols (2a and 2c). To a suspension of 10.0 g of $LiAlH_4$ in 500 mL of distilled THF was added a solution of 11.6 g (0.083 mol) of 6-methyl-3-oxo-6-azabicyclo[3.2.1]octane (**2b**)[3] in 150 mL of THF dropwise, and the resulting mixture was heated to reflux for 2 h. The excess of $LiAlH_4$ was destroyed by careful addition of a 20% solution of sodium potassium tartrate, and the mixture was extracted with CH_2Cl_2. The CH_2Cl_2 solution was washed with a saturated solution of NaCl, dried (Na_2SO_4), and evaporated to give 10.0 g (85%) of 6-methyl-6-azabicyclo[3.2.1]octan-3-ols (**2a** and **2c**): NMR ($CDCl_3$) δ 2.40 (d, 2 NCH_2), 2.42 (s, 3, NCH_3), 4.1 (m, 1, *H*COH).

This product was used in the next step without further purification.

6-Methyl-6-azabicyclo[3.2.1]octan-3α-ol 2,2-Diphenylpropionate (1, Azaprophen) Hydrochloride. To a stirred solution of 5.5 g (0.039 mol) of isomeric 6-methyl-6-azabicyclo-[3.2.1]octan-3-ols (**2a** and **2c**) in dry THF at 0–5 °C was added dropwise a solution of 2,2-diphenylpropionyl chloride prepared from 2,2-diphenylpropionic acid (4.64 g, 0.019 mol) and thionyl chloride. After the mixture was stirred overnight at 25 °C, the precipitate, 6-methyl-6-azabicyclo[3.2.1]octan-3β-ol (**2c**) hydrochloride, was separated by filtration and washed with dry THF. The precipitate was dried under vacuum to give 3.2 g of tan crystals, mp 230–236 °C. Recrystallization of **2c**·HCl from methanol ether with a charcoal treatment gave 2.89 g of crystals: mp 235–236 °C; ^1H NMR (CD_3OD) δ 2.88 (s, 3, NCH_3), 3.85 (m, 1, C_1-H), and 4.01 (m, 1, C_3-H, $W_{1/2}$ = 21.7 Hz). Anal. ($C_8H_{16}ClNO \cdot {}^1/_4H_2O$) C, H, N.

The residue obtained on evaporation of the solvents from the filtrate above was dissolved in CH_2Cl_2 (50 mL), washed with 5% $NaHCO_3$ solution and saturated sodium chloride solution, and dried (Na_2SO_4) The residue on evaporation was chromatographed on silica gel (200 g) with $CHCl_3$/MeOH/NH_4OH (80:18:2) as eluant to give 4.51 g (66% based on **2a**) of pure free base as a waxy solid: ^1H NMR ($CDCl_3$) δ 1.96 (s, 3, CH_3), 2.11 (s, 3, NCH_3), 5.22 (t, 1, >CHO), 7.12–7.38 (aromatics).

The hydrogen chloride salt was prepared by treating the above free base with a solution of 3% HCl in dry MeOH. Recrystallization of the solid obtained from EtOAc/Et_2O gave 3.60 g (48%) of azaprophen: mp 187–191 °C; ^1H NMR ($CDCl_3$) δ 1.97 (s, 3, CH_3), 2.12 (d, 3, NCH_3), 2.47 (d, 2, NCH_2), 5.35 (m, 1, CHO), 7.12–7.63 (Ar H). Anal. ($C_{23}H_{28}ClNO_2$) C, H, N.

Guinea Pig Ileum Assay. The ability of azaprophen to block the acetylcholine-induced contraction of the guinea pig ileum was assayed by reported procedure.[6-8] Distal ileum was obtained from male albino guinea pigs (200–500 g). A segment of distal ileum about 20 cm in length was excised 5 cm above the ileocaecal junction and immediately placed in oxygenated Krebs–Ringer solution containing 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 0.93 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 11 mM glucose. Segments 2.5 cm in length were suspended in a 10-mL organ bath, which was aerated with 5% CO_2 and 95% O_2 and maintained at 37 °C. Isometric contractions were recorded by a transducer (Harvard Apparatus, Natick, MA) at 1-g tension. The concentration–response curve for acetylcholine (ACh) was obtained with a series of ACh doses of increasing contraction; the maximal contractile response was designated 100%. The antimuscarinic activity of azaprophen and standard compounds (ability to block the ACh-induced contraction) was expressed as K_B or pA_2 values, which were calculated by using computer programs for the Schild plot.[11]

α-Amylase Release from Pancreatic Acini Cells. Pancreatic acini cells were prepared from a male Sprague–Dawley rat (150 g) according to reported procedures.[8-10] Dispersed pancreatic acini were prepared by three successive incubations with collagenase (0.8 mg/mL, Sigma Chemical Co.) and resuspended in 16 mL of Dulbecco's minimum essential medium containing 0.2% albumin, 0.01% trypsin inhibitor, and 0.09% theophylline, aerated with 100% O_2, and diluted 1:5 before use. Viability test by trypan blue exclusion was greater than 99%. Dispersed acini were incubated with varied doses of azaprophen and 10^{-5} M carbachol in 0.5 mL of incubation medium. α-Amylase secreted from the acini was determined as described[9,10] with the Phadebas kit (Pharmacia) diluted 1:2. The I_{50}, the concentration of azaprophen or standard compound required for 50% inhibition, was calculated by the computer program ALLFIT.[20]

Binding of [*N-methyl*-^{3}H]Scopolamine (^{3}H-NMS) to Muscarinic Receptors. Human neuroblastoma cells, IMR-32, were grown in RPMI 1640 medium, supplemented with 15% fetal calf serum, mycostatin (20 units/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were harvested by washing the monolayers with phosphate-buffered saline without calcium and magnesium. Cells were suspended in Eagle's basal medium with Hanks' balanced salts and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.3). Viable cells were determined by the trypan blue exclusion test.

To assay the inhibition of ^{3}H-NMS (74 Ci/mol, Amersham) binding to IMR-32 cells, triplicate samples of 0.4×10^6 cells in 0.25 mL of medium containing 0.6 nM ^{3}H-NMS with or without azaprophen, aprophen, or atropine were used. Nonspecific binding was carried out in 96-well flat-bottom microtest tissue culture plates with lids (Falcon, #3072). After 30 min at 25 °C, the reaction was terminated by harvesting the cells with 0.9% NaCl (PHD cell harvester) onto MASH III glass fiber filter strips (MA Bioproducts). The filter strips were dried prior to the determination of radioactivity in scintillation fluid. The K_i values for the inhibition of binding were determined by first determining the I_{50} values by ALLFIT[19] and then calculated by the equation of Cheng and Prusoff.[21]

Operant-Behavior Determination. Lever-press responses of male Sprague–Dawley strain rats (350 g) were maintained by a schedule that required 10 responses for food presentation;[22] session duration was 30 min or 50 food presentations, which ever occurred first.

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