Kinetics of Solvolysis and Muscarinic Actions of an *N*-Methyl-*N*-(2-bromoethyl)amino Analogue of Oxotremorine

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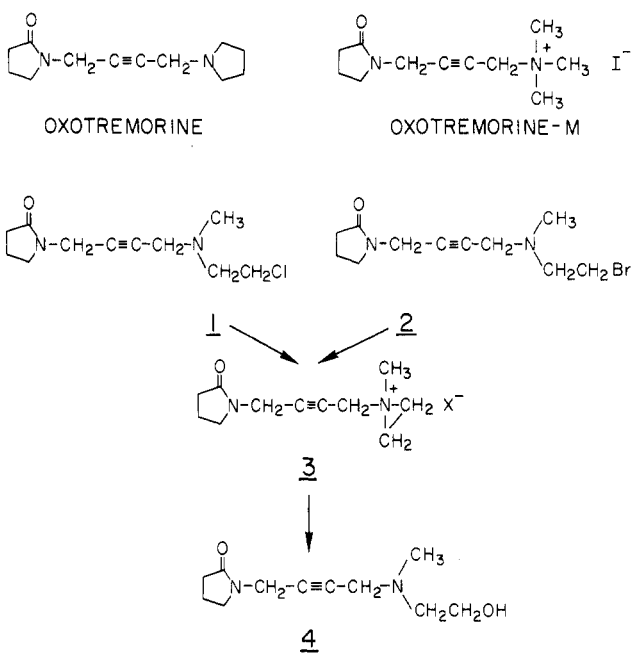
An *N*-methyl-*N*-(2-bromoethyl)amino analogue (**2**) of oxotremorine cyclized in phosphate buffer to an aziridinium ion (**3**). The first-order rate constants (k_1) for the cyclization reaction at 22 and 37 °C (pH 7.3) were 0.14 and 0.85 min^{-1}, respectively. Determination of k_1 as a function of pH gave a pK_a value of 5.6 for **2**. The rate constants (k_2) for the hydrolysis of **3** at 22 and 37 °C (pH 7.3) were 0.0083 and 0.040 min^{-1}, respectively. Compound **3** was 3-fold more active than oxotremorine as a muscarinic agonist on the guinea pig ileum, whereas its nicotinic actions, as estimated on the frog rectus, were quite weak. Because of its greater rate of cyclization and the higher peak concentrations of the aziridinium ion which ensue, **2** may offer advantages over its (2-chloroethyl)amino analogue (**1**) as an alkylating ligand for muscarinic receptors.

We have shown previously that (2-chloroalkyl)amino analogues of the muscarinic agent oxotremorine cyclize spontaneously at neutral pH to reactive aziridinium ions.[1] One such derivative, **1** (BM 123), forms an aziridinium ion that is structurally similar to the potent muscarinic agonist oxotremorine M (Scheme I).[2] Compound **1** causes an irreversible reduction in the binding of ^3H-labeled antagonists to muscarinic receptors in various neuronal and muscular tissues.[1,3,4] These observations are consistent with a covalent interaction of the aziridinium ion of **1** with muscarinic receptors.

Pharmacologically, these 2-chloroalkylamines behave as potent and selective muscarinic agonists.[1] When administered in vivo, they produce signs typical of muscarinic stimulation, e.g., tremor, salivation, and hypothermia, followed by a phase of long-lasting antimuscarinic effects.[5,6] Both the stimulatory and blocking effects are elicited by the aziridinium ion formed by in vivo cyclization of the parent 2-chloroalkylamine. Therefore, the rate of cyclization should be an important factor in determining the pharmacological properties of these 2-chloroalkylamines. Compound **1** cyclizes rather slowly and, as a consequence, gives rise to relatively low peak levels of the aziridinium ion.[1] We now have synthesized a (2-bromoethyl)amino analogue, **2** (BR 401), of **1** that cyclizes to the same aziridinium ion (**3**) as does **1** (Scheme I), but considerably faster. This paper describes the kinetics of the chemical transformations of **2** in aqueous solution and its muscarinic activity in vitro. Preliminary accounts of this work have been presented.[7,8]

Scheme I



Results

Rates of Cyclization of 2 and Rates of Hydrolysis of 3. The cyclization of **2** was studied in phosphate buffer. Table I shows the effect of pH on the apparent first-order rate constant (k_1) for bromide release from **2** at 22 °C. First-order kinetics were observed at all pH values through at least 3 half-lives. Regression analysis yielded an estimated pK_a value of 5.58 for **2** and a first-order rate constant (k_0) for cyclization of the free base of 0.146 ± 0.004 min^{-1}. At 37 °C and pH 7.3, the apparent first-order rate constant for bromide release from **2** was 0.850 ± 0.035 min^{-1}. Thus **2** cyclized about 50-fold faster than its (2-chloroethyl)amino analogue, **1**.[1]

Table I. Effect of pH on the Rate of Cyclization of **2** at 22 °C

pH	k_1,[a] min^{-1}	$t_{1/2}$, min
4.24	0.00266 ± 0.00005	261
5.46	0.0678 ± 0.0028	10.2
6.25	0.1146 ± 0.0033	6.1
7.29	0.1406 ± 0.0022	4.9
7.67	0.1512 ± 0.0049	4.6

[a] Apparent first-order rate constant for the cyclization of **2** in 50 mM phosphate buffer as measured from bromide ion release. Values are means ± SE from two to three independent estimates.

The cyclization reaction also was monitored by utilizing the quantitative reaction of the formed aziridinium ion with sodium thiosulfate. This reaction was also used to follow the decay of the aziridinium ion. At 22 °C and pH 7.3, the concentration of **3** reached a peak of 83% of the initial concentration of **2** at 20 min after dissolution in phosphate buffer. At 37 °C, the peak was 87% at 3 min. The rate constant (k_1) for the cyclization, as measured by the rate of formation of **3**, was 0.145 ± 0.008 min^{-1} at 22 °C and 0.845 ± 0.027 min^{-1} at 37 °C. These rate constants were in very good agreement with those estimated from

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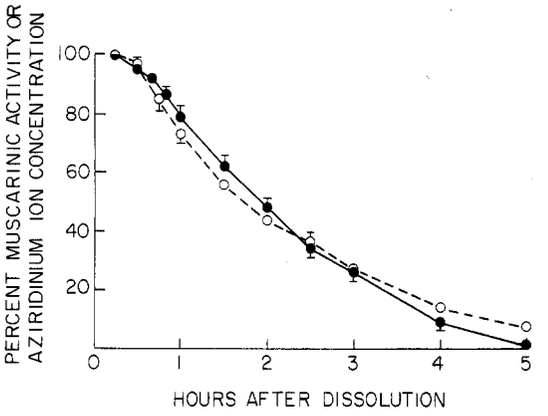

Figure 1. Correlation of muscarinic activity and aziridinium ion concentration in a solution of **2**. Compound **2** was dissolved in phosphate buffer (pH 7.3) at 22 °C. Samples were withdrawn at various times and assayed for muscarinic activity on the guinea pig ileum and for aziridinium ion. Muscarinic activity (○) and aziridinium ion concentration (●) were expressed as percentages of the muscarinic activity and the aziridinium ion concentration of a sample withdrawn at 15 min. Vertical bars are standard errors of three to five determinations. At some points standard errors were too small to be plotted.

bromide release. The rate constants (k_2) for the hydrolysis[9] of **3** at pH 7.3 were 0.008 29 ± 0.000 03 and 0.0395 ± 0.0009 min^{-1} at 22 and 37 °C, respectively. Corresponding values obtained under slightly different conditions (at pH 7.0 in 30 mM phosphate buffer) with **1** as the precursor of the aziridinium ion were 0.0093 and 0.033 min^{-1}, respectively, at 23 and 37 °C.[1]

Muscarinic Activity. Compound **2** was assayed as a muscarinic agonist on the guinea pig ileum. As expected, the potency of solutions of **2** in phosphate buffer (pH 7.3) varied greatly with time after dissolution. Maximum activity was observed with a solution kept at room temperature for 10–20 min. The muscarinic activity closely paralleled the aziridinium ion concentration as determined by thiosulfate consumption (Figure 1). At the time of peak activity, **3** was 3-fold more active than **1** and oxotremorine and 7-fold more active than carbachol. The equipotent molar ratio (relative to oxotremorine M) of **3** was 0.54 ± 0.022 (N = 8). The short exposure (about 1.5 min) of the ileum to the low concentrations of **3** required to record concentration–response curves did not alter the sensitivity of the muscle to oxotremorine M; i.e., no alkylation of muscarinic receptors was detected under these conditions. Methylatropine antagonized competitively the actions of **3**. A concentration of 10 nM increased the EC_{50} value of **3** 15.6 ± 2.5 (N = 4) times, whereas hexamethonium (0.3 mM) was without effect. These results confirm the muscarinic nature of the contractile response to **3**. Furthermore, thiosulfate treatment (0.3 mM for 15 min) of **3** completely abolished the response. In contrast, sodium thiosulfate had no effect on the muscarinic activity of solutions of oxotremorine M.

Nicotinic Activity. Compound **2**, cyclized as described in the Experimental Section, elicited slow contractions of the frog rectus abdominis muscle similar to those caused by carbachol. The equipotent molar ratio relative to carbachol was 12.3 ± 0.7 (N = 5).

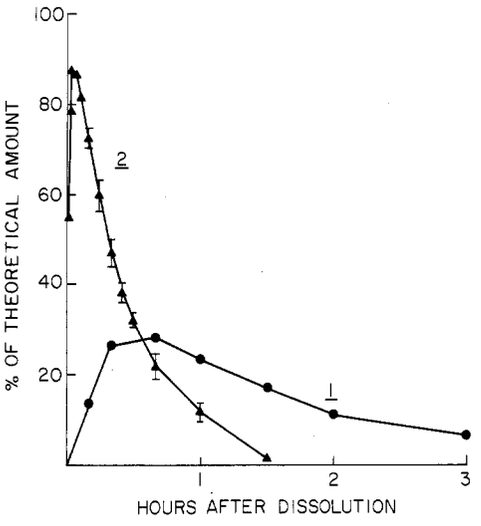

Figure 2. Aziridinium ion formation and decay at 37 °C in phosphate buffer. The data for **2** are means ± SE from three determinations. The results for **1** are from Ringdahl et al.[1]

Discussion

Although **2** cyclized 50-fold faster than **1**, its rate of cyclization was lower than those of most tertiary 2-bromoethylamines, which cyclize almost instantaneously at physiological pH.[12] This lower rate of cyclization of **2** may be attributed in part to its low basicity as evidenced by the estimated pK_a value of 5.6. The concentration of the aziridinium ion at any given time after dissolution of **1** and **2** is determined by both the rate constant for formation (k_1) and the rate constant for hydrolysis (k_2) of the ion. The time to peak aziridinium ion concentration is determined similarly by these rate constants. Thus the observation[1] that, for **1**, k_1 was smaller than k_2 readily accounts for the rather low peak level of the aziridinium ion and also for the late appearance of the peak as well as the relatively long persistence of the ion in solution (Figure 2). Compound **2**, because of its more rapid cyclization ($k_1 > k_2$), gave rise to a much higher peak concentration of the aziridinium ion, which, however, declined more rapidly. As discussed elsewhere,[13] the different rates of cyclization of **1** and **2**, and the concomitant effects on the aziridinium ion concentration, have profound influence on the pharmacokinetic and pharmacodynamic properties of these 2-haloethylamines.

Compound **3** was an extremely potent and selective muscarinic agonist. The 3-fold greater in vitro muscarinic activity of **2** as compared to **1** is in agreement with the 3-fold higher peak concentration of the aziridinium ion obtained with **2** (Figure 2). The present results leave little doubt that **3** is the molecular species responsible for the muscarinic actions of **2**. Thus, contractile activity on the guinea pig ileum closely paralleled the aziridinium ion concentration (Figure 1) and was abolished by treatment of solutions of **3** with thiosulfate. Although the inactivity of **2** as a muscarinic agonist is difficult to verify experimentally, it is virtually unprotonated at physiological pH and it is well known that a protonated nitrogen is essential for muscarinic activity of tertiary amines.[14] Finally, the alcohol **4** formed by hydrolysis of **3** (Scheme I) was a very weak muscarinic agent.[1]

(9) Throughout this report, k_2 comprises all bimolecular reactions by which the aziridinium ion decays in phosphate buffer. Except for hydrolysis to the alcohol, **4** (Scheme I), a phosphate ester may also be formed as shown for other aziridinium ions.[10,11]

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The relative merit of 1 and 2 for receptor-inactivation studies in vivo is difficult to assess since different optimal cyclization rates are expected for different sites of action, administration routes, and animal species. For example, 2 appears to have an almost optimal rate of cyclization for producing high levels of aziridinium ion in the brain after intravenous administration to mice, while after intraperitoneal administration, a large proportion of 2 will cyclize before it reaches the brain.[13] For receptor inactivation in vitro, however, where the cyclized form is desired, 2 has definite advantages over 1, as it provides high concentrations of the aziridinium ion without the need for long preincubation (Figure 2). Furthermore, with 2, it is possible to prepare solutions of the aziridinium ion that are virtually free from the parent compound, whereas in solutions of 1, the concentration of the parent compound always exceeds that of the aziridinium ion. Compound 2 should become a valuable complement to 1 as an alkylating agonist for in vivo and in vitro studies of muscarinic receptors.

Experimental Section

Melting points were determined in a heated metal block using glass capillaries and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, and agreed with theoretical values within ±0.3%. Mass spectra were recorded on a Hewlett-Packard 5981A mass spectrometer at 70 eV. ^{1}H NMR spectra were obtained at 25 °C on a JEOL FX 90-MHz spectrometer. Chemical shifts are reported in ppm (δ) downfield from internal $(CH_3)_4Si$ standard. TLC was carried out on Merck silica gel 60 F_{254} analytical plates. Visualization was done with UV and I_2.

***N*-[4-[(2-Bromoethyl)methylamino]-2-butynyl]-2-pyrrolidone (2).** Compound 4 (Scheme I) was synthesized as described previously[15] and dried before use by dissolving it in dry benzene and removing the solvent under vacuum. Ph_3P was dried similarly. A stirred solution of 4 (6.0 g, 29 mmol) and Ph_3P (8.0 g, 31 mmol) in anhydrous CH_2Cl_2 (200 mL) was cooled to −5 °C in a sealed bottle flushed with dry nitrogen. CBr_4 (12.3 g, 37 mmol) dissolved in CH_2Cl_2 (50 mL) was added dropwise through a rubber septum. The reaction mixture was allowed to warm up to room temperature and pentane (300 mL) was added to precipitate most of the $Ph_3P{=}O$. After filtration, the solvent was evaporated under reduced pressure. Compound 2 was extracted from the resulting oil by careful trituration at 0 °C with ether–pentane (1:2). The organic phase was concentrated under vacuum and the trituration was repeated until all of the $Ph_3P{=}O$ had been removed as shown by TLC on silica gel plates with CH_2Cl_2–MeOH (9:1) as solvent. The resulting clear oil was dissolved in anhydrous ether and an ethereal solution of HBr was added at 0 °C. The precipitate was triturated with acetone to give 6.0 g (48%) of 2·2HBr as a yellowish powder: mp 133–135 °C. Anal. ($C_{11}H_{19}Br_3N_2O$) C, H, N, Br. Compound 2·2HBr was dissolved in ice-cold water and the solution was neutralized with K_2CO_3. Compound 2 was rapidly extracted into ether, which was filtered through $MgSO_4$ into oxalic acid in anhydrous ether. Compound 2 oxalate was recrystallized from ethanol–ether to give white crystals: mp 113–115 °C; ^{1}H NMR (CD_3OD) δ 4.05–4.25 (m, 4 H, $CH_2C{\equiv}CCH_2$), 3.4–3.9 (m, 6 H, NCH_2CH_2Br, $CONCH_2$), 2.91 (s, 3 H, NCH_3), 1.9–2.5 (m, 4 H, CH_2CH_2CO); MS, *m/e* (%) 193 (10.7), 179 (14.2), 136 (90.0), 109 (14.9), 108 (48.8), 107 (100). Anal. ($C_{13}H_{19}BrN_2O_5$) C, H, N.

Measurements of Formation and Decomposition of Aziridinium Ion (3). The reaction mixture, kept at constant temperature (22 or 37 °C), contained 2·2HBr (1.5 mM) in 50 mM sodium phosphate buffer. Bromide ion released during the cyclization was measured at various pH values by argentometric titration according to the method of Volhard.[16] The method used to quantitate the aziridinium ion was essentially identical with that described previously,[1] except that the cyclization was stopped by the addition of glacial acetic acid.

Rate constants for the cyclization reaction (k_1) and for the hydrolysis of 3 (k_2) were estimated by fitting kinetic models to the data by an unweighted Gauss–Newton nonlinear regression routine as described previously.[1] Estimates of k_1 at various pH values (4.2–7.7) were used as input for a regression analysis[1] to provide estimates of the pK_a of 2 and of the rate constant for bromide release from the free base of 2 (k_0).

Isolated Guinea Pig Ileum. A standard guinea pig ileum preparation was set up in Tyrode's solution (pH 7.4) at 37 °C as described previously.[17] Contractions were recorded isotonically at 1 g of tension. The muscarinic activity of 2 at various times after its dissolution (as a 1.5 mM solution) at room temperature in 50 mM sodium phosphate buffer (pH 7.3) was estimated by recording cumulative concentration–response curves at intervals over a time period of 5 h. The potency at each time point was expressed as the equipotent molar ratio (±SE) relative to oxotremorine M used as a standard agonist.

Frog Rectus Abdominis. A standard frog rectus abdominis preparation was set up at 22 °C in aerated Clark–Ringer solution (pH 7.4) as described previously.[18] Contractions were recorded as described above. The preparation was exposed to each drug concentration for 5 min. Equipotent molar ratios (±SE) relative to carbachol were determined in three-point assays.[19] Compound 2 was cyclized for 15 min as a 10 mM solution in 100 mM phosphate buffer (pH 7.4) because of the high concentrations required to obtain responses in this preparation.

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Registry No. 2, 106976-61-8; 2·2HBr, 106976-62-9; 2 (oxalate), 106987-86-4; 3 (X = Br), 106976-63-0; 4, 42972-77-0.

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