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ACETYLCHOLINESTERASE INHIBITION BY THE KETONE TRANSITION STATE ANALOGS PHENOXYACETONE AND 1-HALO-3-PHENOXY-2-PROPANONES.

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1-Phenoxy-2-propanone, 1-chloro-3-phenoxy-2-propanone, and 1-fluoro-3-phenoxy-2-propanone are competitive acetylcholinesterase inhibitors with K_I values of 30, 0.85, and 2.2 μM, respectively, compared to 2 mM for 4-phenyl-2-butanone. The substituent effect on inhibition suggests that these compounds bind by formation of a tetrahedral adduct and are transition state analogs.

Other evidence supports this conclusion: N-benzyl-2-chloroacetamide and 1-phenoxy-2-propanol are poor inhibitors (K_I = 11 and >10 mM); 1-phenoxy-2-propanone and 1-chloro-3-phenoxy-2-propanone have K_I values 330 and 140 times smaller than K_m for corresponding substrates; and 1-chloro-3-phenoxy-2-propanone protects the enzyme against irreversible inhibition by CH_3SO_2F.

Transition state analog theory (1) suggests that compounds capable of forming a tetrahedral covalent adduct with the active serine hydroxyl group of acetylcholinesterase should be potent inhibitors, since this adduct should resemble the tetrahedral intermediate formed in ester hydrolysis by the enzyme (2). In earlier work, 4-oxo-N,N,N-trimethylpentanaminium chloride (the ketone analog of acetylcholine) was shown to be a good transition state analog inhibitor (3), presumably by formation of a hemiketal with the enzyme. 4-Phenyl-2-butanone was shown to be a marginal transition state analog (4), and aliphatic ketones were found to be inhibitors but not transition state analogs (5).

The effect of substituents on binding of ketones should provide a useful test for hemiketal formation. Since electron-withdrawing substituents stabilize tetrahedral adducts formed from ketones (6), such substituents should favor hemiketal formation with the enzyme. Substituent effects on binding of

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4-phenyl-2-butanone derivatives can be compared with effects on benzyl acetate hydrolysis, and are reported here.

Experimental Procedures

General: NMR spectra were recorded on a Varian CFT-20 or A-60. 1-Phenoxy-2-propanone was purchased from Aldrich and 1-phenoxy-2-propanol from Fisher. All other chemicals were of reagent grade. Acetylcholinesterase from Electrophorus Electricus (E.C. 3.1.1.7) was a chromatographically purified preparation obtained from Sigma Chemical Company (activity >1000 units/mg).

Enzyme assays were run on a Radiometer pH Stat system; temperature was maintained at 25° by a Haake FS circulating water bath.

Syntheses: 1-Chlorophenoxypropanone 4 was obtained essentially according to the procedure of Stevenson and Smillie (7). 1-Fluoro-3-phenoxypropanone 6 was prepared by the general route of Bergmann et al. (8); the analytically pure material is a solid with m.p. 49-50°. N-benzyl-2-chloroacetamide (9) was prepared from benzylamine and chloroacetyl chloride. Identity and purity were confirmed by NMR. Structures and compound numbers are given in Table I.

Kinetic Procedures: Competitive inhibition studies were done at pH 7.5 with acetylcholine chloride as substrate in solutions containing 0.1 M NaCl and 0.04 M $MgCl_2$ in glass-distilled water. Methanol was generally added to reaction mixtures to solubilize inhibitors - volume percentages used are given in Table I. Inactivation by methanesulfonyl fluoride was followed by incubating mixtures of enzyme and inhibitors at 25° in 0.1 M NaCl, 0.04 M $MgCl_2$, and 1 mM phosphate buffer, pH 7.0. Aliquots (0.1 ml) were removed and assayed with 1.5 mM acetylcholine chloride. Details of procedures and data treatment have been described previously (4).

Hydrolysis of benzyl chloroacetate by acetylcholinesterase was followed by pH Stat at pH 7.5, 25°, in 0.1 M NaCl, 0.04 M $MgCl_2$, and 5% methanol. Initial velocities were determined over the range 0.2 - 2 mM substrate and kinetic parameters were obtained using a double reciprocal plot.

Hydration of 1-chlorophenoxypropanone 4 was measured in D_2O solution, with the HDO proton decoupled, using a Varian CFT-20 NMR. Chemical shifts assigned by varying D_2O concentration in D_2O/acetone-d_6 mixtures were 4.67 and 5.07 δ(hydrate) and 3.80 and 4.12 δ (ketone) relative to 3-trimethylsilylpropanesulfonate. Integration showed the ketone to be 80% hydrated.

Possible irreversible inhibition by 1-chlorophenoxypropanone 4 was examined by mixing stock solutions of enzyme and inhibitor in a test tube immersed in a 25° water bath. The resulting solution contained 0.1 M NaCl, 0.04 M $MgCl_2$, 1 mM phosphate, pH 7.0, and 25 - 50 units/ml of enzyme. A control contained enzyme and buffer. Aliquots (0.1 ml) were removed from both tubes at various times and added to 25 ml of assay medium (1 mM acetylcholine chloride, 0.1 M NaCl, 0.04 M $MgCl_2$) in the pH Stat. Remaining enzyme activity was measured by the rate of addition of 6 mM NaOH solution at pH 7.0.

Results

Competitive inhibition of acetylcholinesterase by these electrophilic ketones and model compounds is summarized in Table I and shown in Figure 1. Inhibition by 1-phenoxy-2-propanol 3 was barely detectable so only a lower limit for K_I is given. 1-Chlorophenoxypropanone 4 was found to be 80% hydrated

Table I

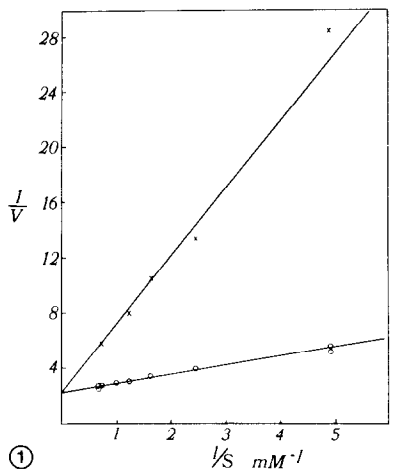
Reversible Inhibition of Acetylcholinesterase.

Number	Structure	K_I (mM)	%CH_3OH
1	$C_6H_5CH_2CH_2\overset{O}{\overset{\|}{C}}CH_3$*	2	1
2	$C_6H_5OCH_2\overset{O}{\overset{\|}{C}}CH_3$	0.030	1
3	$C_6H_5OCH_2\overset{OH}{\overset{\|}{C}}HCH_3$	>10	2
4	$C_6H_5OCH_2\overset{O}{\overset{\|}{C}}CH_2Cl$	8.5×10^{-4}	0
5	$C_6H_5CH_2NH\overset{O}{\overset{\|}{C}}CH_2Cl$	11	5
6	$C_6H_5OCH_2\overset{O}{\overset{\|}{C}}CH_2F$	2.2×10^{-3}	0

Dissociation constants for competitive inhibition (K_I) were determined in solutions containing 0.1 M NaCl, 0.04 M $MgCl_2$, and the indicated percentages of CH_3OH (v/v) at 25°, pH 7.5. All values are averages of 2 or 3 experiments. *From Ref. 4

in water by NMR. This ketone is not an irreversible acetylcholinesterase inhibitor even at 1 mM concentration (>1000 times its K_I).

Benzyl chloroacetate was shown to be a substrate for the enzyme, with K_m = 0.11 mM and V_m = 18% of V_m for acetylcholine hydrolysis in 5% methanol.



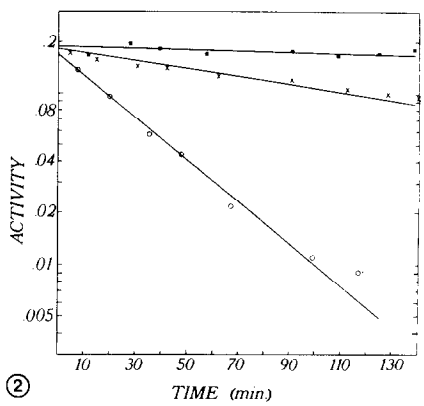


Figure 1. Reversible inhibition of acetylcholinesterase by 1-Chloro-3-phenoxy-2-propanone. Reciprocal velocity vs. reciprocal acetylcholine concentration in the absence (O) and presence (X) of 5.95 μM ketone.

Figure 2. Effect of 1-chloro-3-phenoxy-2-propanone on rate of acetylcholinesterase inactivation by CH_3SO_2F. Remaining enzyme activity is shown as a function of time after mixing for enzyme alone (■), enzyme plus 0.193 mM CH_3SO_2F (O), and enzyme plus 0.193 mM CH_3SO_2F plus 5 μM ketone (X).

Methanol is an extremely weak competitive inhibitor, with K_I = 1.6 M.

1-Chlorophenoxypropanone 4 blocks irreversible inactivation of acetylcholinesterase by CH_3SO_2F, as shown in Figure 2. The dissociation constant for the ketone is 8.2×10^{-7} M, as calculated from its ability to block this irreversible inactivation.

Discussion

The 1-halophenoxypropanones 4 and 6 are potent competitive inhibitors of acetylcholinesterase, although they do not contain the quaternary ammonium group normally introduced to interact with the anionic site of the enzyme. These compounds are roughly comparable as inhibitors to N-methyl-5-hydroxyquinolinium ion and 3-hydroxy-N,N,N-trimethylanilinium ion (10).

The 10^3 enhancement of binding caused by electron-withdrawing substituents (compared to phenylbutanone) is consistent with inductive stabilization of a hemiketal adduct with the enzyme. A quantitatively similar effect is observed in hydration of 1-chlorophenoxypropanone 4. The equilibrium constant for hydration of this ketone is more favorable than that for acetone (6) by a factor of 2.8×10^3, and ketone 4 binds to the enzyme 2.3×10^3 more tightly than phenylbutanone 1. Though data for phenoxyacetone hydration are not available, the intermediate value of its K_I is consistent with the effect of a single electron-withdrawing substituent.

The low K_m observed for hydrolysis of benzyl chloroacetate compared to benzyl acetate (0.11 mM compared to 10 mM) (4) raises the possibility that -Cl may enhance binding to the enzyme by direct hydrophobic or other interactions. However, the similar inhibition by chloro and fluoroketones makes this possibility rather unlikely; these substituents have similar inductive effects but are different in hydrophobicity (11). In addition, the substrate analog N-benzylchloroacetamide 5 inhibits with a K_I comparable to that of N-benzylacetamide-11 and 13 mM, respectively (4). These similarities imply that the -Cl substituent does not contribute to binding, but may lower the K_m for hydrolysis of benzyl chloroacetate (relative to benzyl acetate) by an inductive effect on a kinetic term.

Other criteria developed earlier were used to determine whether these ketones may be considered transition state analogs:

a. A transition state analog should bind more tightly to the enzyme than does the corresponding substrate. The dissociation constant for phenoxyacetone 2 is 330 times smaller than K_m for benzyl acetate hydrolysis. The dissociation constant for 1-chlorophenoxypropanone 4 is 140 times smaller than K_m for hydrolysis of benzyl chloroacetate and 1.3×10^4 smaller than K_I for the substrate analog benzyl chloroacetamide 5. Enhanced binding by factors of 10^2 - 10^4 is typical of transition state analogs (1).

b. Transition state analogs for acetylcholinesterase should block methanesulfonylation of the enzyme. CH_3SO_2F reacts irreversibly with the nucleophilic serine OH group of acetylcholinesterase; compounds which form reversible adducts with this OH group should protect it from CH_3SO_2F. 1-Chlorophenoxypropanone 4 provides this protection (Figure 2); the dissociation constant for the ketone is the same whether calculated from its ability to protect against CH_3SO_2F or from its competitive inhibition of acetylcholine hydrolysis. In earlier work with acetylcholinesterase, those ketones which failed to protect the enzyme against CH_3SO_2F were also not transition state analogs by other criteria (5).

c. Ketone transition state analogs for serine hydrolases should bind more tightly than the corresponding alcohols, since the alcohols cannot form hemiketals with serine. Phenoxyacetone 2 binds more than 330 times more tightly than 1-phenoxy-2-propanol 3.

The observation that 1-chlorophenoxypropanone 4 is not an irreversible inhibitor is somewhat surprising, particularly since it is a good irreversible inhibitor of chymotrypsin (7). This low activity may simply reflect differences in geometry of the active sites.

The observed inductive effects of substituents and the other evidence summarized above suggest one of two plausible mechanisms for inhibition. Either the ketones form tetrahedral adducts with a nucleophile in the active site, or the enzyme binds only the tetrahedral hydrate in a very specific

fashion. The latter interpretation is less likely for several reasons. First, tight binding of the hydrate would suggest that the enzyme operates by direct attack of water on the substrate, but all available evidence indicates instead a nucleophilic attack by the serine -OH group (2). Second, alcohols analogous to these ketones are not tightly bound. Third, highly hydrated trifluoroketones are potent but time-dependent inhibitors of acetylcholinesterase, probably because of the necessity for dehydration before binding (12).[1] Although examination of a broad range of substituents will be required to separate inductive and other effects, the available data are most easily rationalized as a consequence of hemiketal formation between these ketones and the nucleophilic serine hydroxyl group of acetylcholinesterase.

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1. In preliminary work, we have observed very similar time-dependent inhibition by 1,1,1-trifluoro-3-phenoxy-2-propanone.