m-(N,N,N-Trimethylammonio)trifluoroacetophenone: A Femtomolar Inhibitor of Acetylcholinesterase

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Received November 4, 1992®

Abstract: m-(N,N,N-Trimethylammonio)trifluoroacetophenone is a potent, reversible, time-dependent inhibitor of acetylcholinesterases. The respective second-order rate constants (k_{on}) for binding of the ketone to enzymes from *Electrophorus electricus* and *Torpedo californica* are $(1.2 \pm 0.2) \times 10^5$ and $(1.0 + 0.2) \times 10^5$ M^{-1} s^{-1}, while the corresponding dissociation rate constants (k_{off}) are $(1.0 \pm 0.2) \times 10^{-5}$ and $(1.0 \pm 0.3) \times 10^{-4}$ s^{-1}. Therefore, the apparent dissociation constants of the enzyme–inhibitor complexes ($K_i^{app} = k_{off}/k_{on}$) are 80 ± 20 pM and 1.0 ± 0.3 nM, respectively. Because the free ketone is the form that inhibits acetylcholinesterases, intrinsic dissociation constants were calculated by correcting the K_i^{app} values for the extent of hydration of the inhibitor. The resulting K_i values are 1.3 and 15 fM for inhibitions of *E. electricus* and *T. californica* acetylcholinesterases, respectively.

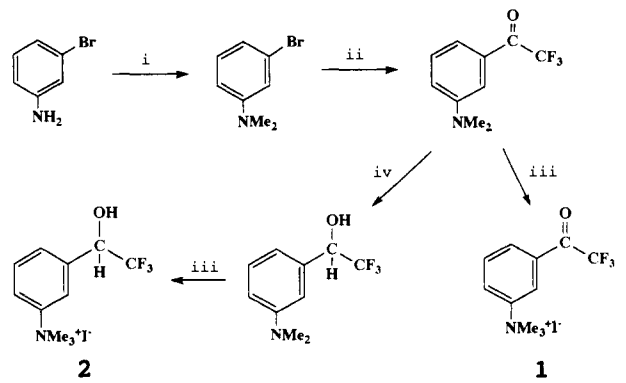
Acetylcholinesterase (AChE)[1] is an enzyme whose physiological function is hydrolysis of the neurotransmitter acetylcholine.[2-5] The enzyme effects this task with great efficiency, as reflected by the fact that the bimolecular rate constant k_{cat}/K_m is near the diffusion-controlled limit.[2,6] Unfortunately, the structural origins of this highly refined catalysis are largely undefined. The general nature of the catalytic mechanism is known, however. AChE utilizes a serine hydrolase mechanism that is closely akin to that of the serine proteases.[7] Hence, trifluoro ketone transition state analog inhibitors should prove useful in developing a structural rationale for AChE catalytic power.

Brodbeck et al.[8] reported that m-(N,N,N-trimethylammonio)-trifluoroacetophenone (**1** in Scheme I below) was a time-dependent inhibitor of *Electrophorus electricus* acetylcholinesterase (EE-AChE). They estimated an I_{50} value, a rough measure of the enzyme–inhibitor dissociation constant, of 13 nM by measuring residual AChE activities after 30 min of incubation with various concentrations of **1**. We report herein that this measure underestimates the affinity of **1** for EE-AChE by 7 orders of magnitude and describe the inhibition by **1** of *Torpedo californica* AChE (TC-AChE). For inhibition of both enzymes by **1**, the K_i values are in the femtomolar range.

Experimental Section

Materials. EE-AChE (EC 3.1.1.7), grade V-S lyophilized powder, was purchased from Sigma Chemical Co. and prior to use was dissolved in 0.05 M sodium phosphate buffer (ionic strength = 0.2 with added NaCl), pH 7.3. TC-AChE was provided *gratis* by Professor Israel Silman of the Weizmann Institute, Rehovot, Israel.[9] The active-site concentrations of AChE preparations were determined by fluorescent titration with diethyl umbelliferyl phosphate.[10] ATCh chloride, DTNB, $NaH_2PO_4 \cdot H_2O$, and $Na_2HPO_4 \cdot 7H_2O$ were used as received from Sigma Chemical Co. NaCl was used as received from EM Science. Water was distilled and then deionized by passage through a Barnstead D8922 mixed-bed ion-exchange column (Sybron Corp.). 3-Bromoaniline, dimethyl sulfate, *t*-BuLi, CF_3CO_2Et, and iodomethane were used as received from Aldrich Chemical Co. Acetone was dried over K_2CO_3, and THF and Et_2O were distilled from sodium–benzophenone ketyl prior to use.

Scheme I[a]



[a] (i) $(CH_3O)_2SO_2$, H_2O, KOH; (ii) (a) *t*-BuLi, THF, −78 °C; (b) CF_3CO_2Et, Et_2O, −78 °C to room temperature; (iii) excess CH_3I, acetone; (iv) $NaBH_4$, MeOH.

Synthesis of m-(N,N,N-Trimethylammonio)trifluoroacetophenone (1). The synthesis of trifluoro ketone inhibitor **1** is outlined in Scheme I. m-Bromo-N,N-dimethylaniline was prepared by a modification of the procedure of Gilman and Banner.[11] 3-Bromoaniline (10 g, 58 mmol) and 10 mL of water were added to a 100-mL RBF, the flask was placed in a cold water bath, and 1 equiv of dimethyl sulfate (58 mmol) was added dropwise by syringe to the stirred reaction mixture. After 1 h of stirring, the solution was neutralized (pH = 7) by careful addition of 25% aqueous KOH. A second equivalent of dimethyl sulfate was added and the mixture stirred for 1 h, after which the solution was rendered basic (pH ~ 8) by again adding 25% aqueous KOH. A final addition of 0.5 equiv of dimethyl sulfate was made, the mixture was stirred for 20 min, and the solution was made alkaline with 25% aqueous KOH. The reaction

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® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

(1) Abbreviations used: AChE, acetylcholinesterase; ATCh, (acetylthio)-choline; *t*-BuLi, *tert*-butyllithium; DMS, dimethyl sulfate; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EE-AChE, *Electrophorus electricus* AChE; RBF, round bottom flask; THF, tetrahydrofuran; TC-AChE, *Torpedo californica* AChE; TMS, tetramethylsilane.

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0002-7863/93/1515-9939$04.00/0

mixture was extracted with 200 mL of Et_2O, the extract was washed with 3 × 20 mL of water and dried over K_2CO_3, and crude product was obtained on rotary evaporation. 3-Bromo-*N*,*N*-dimethylaniline (5.6 g, 48% yield) was obtained on fractional distillation (72 °C at 0.4 mmHg) in a short-path distillation apparatus.

A 25-mL RBF was equipped with a septum, an argon tee, and a magnetic stirrer and was purged with and maintained under an argon atmosphere. To this flask was added, via syringe and with stirring, 3-bromo-*N*,*N*-dimethylaniline (10 mmol) and 10 mL of dry THF, the flask was cooled to −78 °C, and 10 mL of 1.0 M *t*-BuLi in hexane was added dropwise to the solution. After being stirred at −70 to −78 °C for 30 min, the reaction mixture was transferred by syringe to another RBF that contained 10 mmol of CF_3CO_2Et in 10 mL of dry Et_2O at −78 °C and stirred for 10 min. The reaction mixture was warmed to room temperature and extracted with 100 mL of Et_2O, and the extract was washed with 3 × 10 mL of water, dried over $MgSO_4$, and concentrated on a rotary evaporator. The crude product was purified by successive silica gel column chromatographies, with mobile phases hexanes/$CHCl_3$/MeOH (90:10:5 v/v) and CH_2Cl_2/hexanes (1:1 v/v), to afford 3-*N*,*N*-dimethylanilinyl trifluoromethyl ketone as a pale-yellow liquid; bp 76–78 °C at 1 mmHg (lit.[12] bp 74–75 °C at 1 mmHg).

Excess CH_3I (0.71 g, 5 mmol) was added to 0.20 g (0.92 mmol) of 3-*N*,*N*-dimethylanilinyl trifluoromethyl ketone in 2 mL of dry acetone in a 5-mL RBF. The flask was sealed with a rubber septum and stirred at room temperature for ~24 h. The precipitated product was collected by filtration, washed with dry Et_2O, and dried at room temperature *in vacuo* to afford **1** as a white powder.[13] ^{19}F NMR spectra in DMSO-d_6 and D_2O showed singlets at −70.4 and −82.1 ppm, respectively. On addition of 1 drop of H_2O to the DMSO-d_6 solution of **1**, the free ketone signal at −70.4 ppm disappeared and was replaced by that of the hydrate at −82.1 ppm. ^1H NMR spectra in DMSO-d_6 showed resonances at 3.96 (s, 9H, CH_3–N^+) and 7.96–8.46 (m, 4H, aromatic protons); corresponding spectra in D_2O showed trimethylammonio and aromatic resonances at 3.69 and 7.80–8.05 ppm, respectively. The high-resolution mass spectrum gave a mass for **1**–I^- of 232.0971 (calcd 232.0949); FTIR (KBr pellet) showed the expected carbonyl stretch at 1718 cm^{-1}. Because **1** is very hygroscopic, accurate elemental analysis could not be obtained.

Synthesis of 1-((*m*-*N*,*N*,*N*-Trimethylammonio)phenyl)-2,2,2-trifluoroethanol (2). 3-*N*,*N*-Dimethylanilinyl trifluoromethyl ketone (0.55 mmol) and 4 mL of MeOH were added to a 25-mL RBF that was equipped with a nitrogen tee, a solid addition tube, and a magnetic stirrer. $NaBH_4$ (0.55 mmol) was added over a 3-min period, and the mixture was stirred for an additional 30 min. The reaction mixture was extracted with 100 mL of Et_2O, washed with 2 × 10 mL of water, and concentrated on a rotary evaporator. The product alcohol (0.11 g, 91% yield) was obtained as a colorless, viscous liquid. This compound was quantitatively converted to **2** by N-methylation with CH_3I, as described above for the synthesis of **1**. The ^{19}F NMR spectrum of **2** contained a doublet at −76.1 ppm, J_{HF} = 7 Hz. The ^1H NMR spectrum in D_2O showed resonances at 3.54 (s, 9H), 5.24 (q, 1H, J_{HF} = 7 Hz), 7.61 (m, 2H), and 7.79 (m, 2H) ppm. FTIR (KBr pellet) contained a broad peak at 3297 arising from the OH stretch. Anal. Calcd for $C_{11}H_{15}ONF_3I$: C, 36.56; H, 4.18; N, 3.87. Found: C, 36.45; H, 4.23; N, 3.68.

Enzyme Kinetics. Rates for AChE-catalyzed hydrolysis of (acetylthio)-choline (ATCh) were followed at 412 nm by the coupled spectrophotometric assay developed by Ellman et al.[14] Particular reaction conditions are given in figure legends. In the presence of **1**, AChE activity drops in a time-dependent manner that is well described by first-order kinetics. Therefore, first-order rate constants of inhibition were determined by periodically measuring the initial rate of aliquots of a mixture of AChE and **1** and by subsequent nonlinear least squares fitting[15] to eq 1 of the dependence of residual activity on time:

$$\%V = (\%V_0 - \%V_{inf})e^{-kt} + \%V_{inf} \quad (1)$$

In this equation, $\%V$, $\%V_0$, and $\%V_{inf}$ are AChE activities, as percents of control activities (i.e. activity in the absence of **1**), at times t, 0, and infinity, respectively, and k is the first-order rate constant. Disposable parameters of the nonlinear least squares fit are $\%V_0$ − $\%V_{inf}$, k, and $\%V_{inf}$.

Analytical Instrumentation. ^{19}F and ^1H NMR spectra were recorded on a Bruker AC-300 spectrometer; ^{19}F and ^1H chemical shifts are reported in ppm downfield of $CFCl_3$ and TMS, respectively. FTIR spectra were recorded on a Mattson Cygnus 25 spectrometer. High-resolution mass spectra were recorded on a ZAB HF spectrometer operated in the FAB mode. Time courses for ATCh hydrolysis were recorded on an HP8452A diode array UV-visible spectrophotometer. Reaction temperature was controlled by using a water-jacketted cell holder and a VWR 1140 refrigerated, circulating water bath. The pH values of reaction buffers were measured on a Corning Model 125 pH meter equipped with a glass combination electrode.

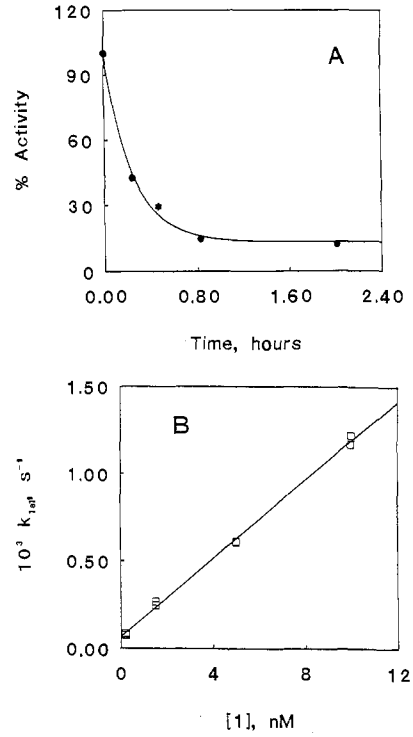

Figure 1. Slow-binding inhibition of *T. californica* AChE by **1**. Reactions were run at 25.0 ± 0.2 °C in 0.05 M sodium phosphate buffer, pH 7.26, that contained 0.125 N NaCl, 35 pM AChE, and the indicated concentrations of **1**. (A) Inhibition time course in the presence of 10 nM **1**. At the indicated times residual AChE activity was measured by determining initial rates of hydrolysis of 0.08 mM (acetylthio)choline in the presence of 0.3 mM 5,5′-dithiobis(2-nitrobenzoic acid). The nonlinear line is a least squares fit[15] to eq 1, from which k_{1st} = (1.2 ± 0.1) × 10^{-3} s^{-1} was calculated. (B) Dependence of observed first-order inhibition rate constant on inhibitor concentration. The rate constants k_{on} and k_{off} were calculated from the slope and intercept, respectively, of the displayed linear fit.

Results

Figure 1A shows a first-order time course for inhibition of TC-AChE by **1**. Residual activity is observed at long reaction times because the concentration of **1** in the assay is comparable to the apparent dissociation constant of the E·**1** complex. This observation and additional experiments discussed below are consistent with the following inhibition scheme:

$$E + \mathbf{1} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} E\cdot\mathbf{1}$$

In accord with this scheme, the pseudo-first-order rate constant determined from the fit in Figure 1A is described by eq 2:

$$k_{1st} = k_{on}[\mathbf{1}] + k_{off} \quad (2)$$

Figure 1B shows that, as predicted by this equation, the dependence of k_{1st} on [**1**] is linear, from which k_{on} = (1.12 ± 0.02) × 10^5 M^{-1} s^{-1} and k_{off} = (7 ± 1) × 10^{-5} s^{-1}. The dissociation half-life calculated from k_{off} is 2.8 h, and the apparent dissociation constant of the E·**1** complex is $K_i^{app} = k_{off}/k_{on}$ = 0.6 ± 0.1 nM.

At high concentrations of **1** eq 2 reduces to $k_{1st} = k_{on}[\mathbf{1}]$. For inhibitor concentrations that span the range 100–700 nM, the

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dependence of k_{1st} on [1] for inhibition of TC-AChE is again linear (plot not shown). The value $k_{on} = (8 \pm 1) \times 10^4$ M^{-1} s^{-1} calculated from the slope of this plot is in good agreement with the value determined from Figure 1B. The value of k_{off} was also determined by adding sufficient **1** to inhibit TC-AChE by ~90%, followed by 100-fold dilution of the sample. Consequent return of activity was monitored with time, as shown in Figure 2, and is well described by first-order kinetics. The rate constant derived from the fit is $k_{off} = (1.2 \pm 0.2) \times 10^{-4}$ s^{-1}, in reasonable agreement with the value determined from Figure 1B. Therefore, K_i^{app} = 1.5 ± 0.3 nM.

Experiments like those discussed in the preceding paragraph were also conducted for inhibition of EE-AChE by **1**. The corresponding rate constants are $k_{on} = (1.2 \pm 0.2) \times 10^5$ M^{-1} s^{-1} and $k_{off} = (1.0 \pm 0.2) \times 10^{-5}$ s^{-1} (both are averages of duplicate determinations). Therefore, the dissociation half-life of the E·**1** complex is 19 h, and K_i^{app} = 80 ± 20 pM.

The inhibitor, **1**, is extensively hydrated in aqueous solution. The extent of hydration of **1** was determined by ^{19}F NMR spectroscopy. In DMSO-d_6 the chemical shift of the ketone is −70.4 ppm. In 4:1 H_2O/D_2O (v/v) that contained 0.04 M sodium phosphate buffer, pH 7.3, 0.08 N NaCl, and 0.01 M **1** or 0.1 M **1**, the ratio of the intensities of the resonances at −83.1 ppm (ketone hydrate) and at −71 ppm (free ketone) gives $K_{hyd} = 6.2 \times 10^4$. Since the free ketone is the form of the inhibitor that blocks AChE activity,[8,16] K_i^{app} values underestimate the intrinsic affinity of AChE for **1**. The intrinsic dissociation constants are calculated according to the following equation:

$$K_i = K_i^{app}/(1 + K_{hyd}) \quad (3)$$

When this is done, the respective K_i values for inhibition of EE-AChE and TC-AChE are 1.3 and 15 fM.

The analog of **1** in which the ketone has been reduced to the racemic secondary alcohol, compound **2** in Scheme I, has also been evaluated as an AChE inhibitor. However, 1 mM **2** inhibits TC-AChE by only ~25%. Thus, as an AChE inhibitor **2** is about 10^{11}-fold less effective than is the ketone form of **1** and about 10^6-fold less effective than an equilibrium mixture of **1** and its hydrate.

Discussion

Judiciously designed trifluoromethyl ketones are effective inhibitors of serine enzymes, such as serine proteases.[17] Such compounds manifest their inhibition potency by reversible covalent interaction with the active-site serine to form a hemiketal, a tetrahedral adduct that structurally resembles transition states in the catalytic mechanism of the enzyme. X-ray crystallographic[17b,18] and NMR[17a,19] experiments demonstrate the formation of tetrahedral hemiketal adducts of trifluoro ketones at the active sites of serine proteases. Linderman et al.[20] have recently shown by ^{19}F NMR that 3-(octylthio)-1,1,1-trifluoropropan-2-one binds to EE-AChE as a hemiketal adduct. Therefore, there is considerable precedent for the operation of the tetrahedral hemiketal inhibition mechanism when **1** binds to AChE.

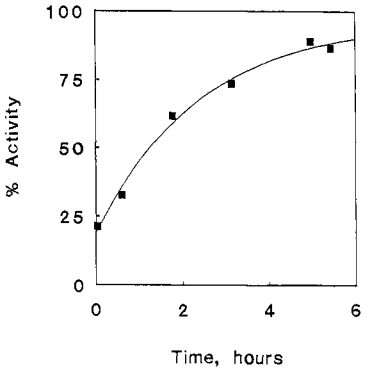

Figure 2. Time course for dissociation of **1** from its complex with *T. californica* AChE. AChE (350 pM) was incubated with 10 nM **1** until inhibition was complete, as described in Figure 1. The sample was then diluted 100-fold into 0.05 M sodium phosphate buffer, pH 7.26, that contained 2 mM Triton X100 and 0.125 N NaCl. At the indicated times the activity of the enzyme was assayed as described in Figure 1A. The nonlinear line is a least squares fit[15] to a first-order kinetics equation, from which $k_{off} = (1.2 \pm 0.2) \times 10^{-4}$ s^{-1} was calculated.

The time-dependent inhibition of AChEs by **1** gives $k_{on} \sim 10^5$ M^{-1} s^{-1}, a strikingly slow rate constant for binding of an aromatic quaternary ammonio ligand. In contrast, Nolte et al.[21] measured k_{on} values as large as 6.4×10^9 M^{-1} s^{-1} for *N*-methylacridinium binding to EE-AChE. This large discrepancy between k_{on} for **1** and other aromatic cations is resolved if only the free ketone form of **1** binds to the AChEs studied herein. In this case k_{on} for the free ketone is the observed k_{on} times the factor $1 + K_{hyd}$, which gives values in the range $5–7 \times 10^9$ M^{-1} s^{-1}, in substantial agreement with the experiments of Nolte et al.[21] That the hydrate of **1** does not inhibit is supported by the following observations: (1) The reduced analog of **1**, compound **2** in Scheme I, is not an effective inhibitor of TC-AChE. (2) Allen and Abeles[16] found that 1,1,1-trifluoro-6,6-dimethylheptan-2-one is a potent, time-dependent inhibitor of EE-AChE (K_i = 12 nM), but the corresponding racemic alcohol inhibits weakly (K_i = 0.17 mM). (3) Allen and Abeles demonstrated that the free ketone form of 1,1,1-trifluoro-6,6-dimethyheptan-2-one is the inhibiting species by *in situ* reduction of the trifluoro ketone with alcohol dehydrogenase, a manipulation that blocks inhibitor potency. (4) Brodbeck et al.[8] found that trifluoroacetone, which is in equilibrium with its hydrate, is an AChE inhibitor, while hexafluoroacetone, which is irreversibly hydrated, is not.

1 is by far the most potent reversible AChE inhibitor known. The K_m values for hydrolysis of (acetylthio)choline catalyzed by EE-AChE and TC-AChE are ~10^{11}-fold and 10^9-fold, respectively, larger than the K_i values reported herein. These binding affinity ratios are comparable to the catalytic acceleration of acetylcholine turnover that AChE effects.[2,22] Therefore, a more detailed structural picture of the interaction of **1** with the active site should provide valuable insights into the origins of the catalytic power of AChE. Work toward this end has been initiated.

Acknowledgment. We thank Professor Israel Silman for the kind gift of *T. californica* AChE and his co-worker, Lilly Toker, for skillful preparation of the enzyme. Mass spectra were obtained at the University of Iowa High Resolution MS Facility; NMR spectra were obtained at the University of Iowa High Field NMR Facility. Support of this work was received from NIH Grant NS21334.

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