Heterocyclic Derivatives of 3-Substituted-1,1,1-trifluoro-2-propanones as Inhibitors of Esterolytic Enzymes

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Received December 4, 1989

A series of (alkylthio)trifluoropropanones containing a heterocyclic moiety was synthesized. The compounds were tested for in vitro inhibition of four hydrolytic enzymes including insect juvenile hormone esterase (JHE), eel acetylcholinesterase (AChE), yeast lipase (LP), and bovine α-chymotrypsin. The I_{50} values ranged from 10^{-3} to 10^{-7} M. 3-(2-Pyridylthio)-1,1,1-trifluoro-2-propanone was found to be the most potent inhibitor as compared to the other tested heterocyclic analogues with an I_{50} value of 98 nM against JHE from the fifth-instar larvae of *Trichoplusia ni*. Results from X-ray crystallography showed that the compound exists in a tetrahedral *gem*-diol form stabilized by an intramolecular hydrogen bond in the solid state. X-ray crystallography of a less potent inhibitor, 3-(4-pyridylthio)-1,1,1-trifluoro-2-propanone, showed that it also exists in the hydrated form, but it lacks an intramolecular hydrogen bond. These results provide indirect support that trifluoromethyl ketones are transition-state mimic inhibitors of esterases, and the bearing of the results on the transition-state mimic theory is discussed. The I_{50} values against AChE were in the micromolar range. Compounds containing a imidazolyl, triazolyl, and pyrimidyl moiety showed the highest inhibition of this enzyme. Differential selectivity of inhibition was associated with the bond distances between the nitrogen and the carbonyl group as in the natural substrate, when measured in the molecules in their minimal energy conformations. Inhibition of LP was moderate to weak, when compared to JHE and AChE. None of the tested compounds showed significant inhibition of α-chymotrypsin. In vivo toxicity assays with the fruit fly, *Drosophila melanogaster*, indicate considerable variation among the compounds, with differential toxicity toward different life stages of the insect, but all were significantly less toxic than malathion.

Introduction

Esterolytic enzymes play an important physiological role in the phase I metabolism of toxicants and xenobiotics (*1*), as well as in the hydrolysis of endogenous substrates. Enzymatic esterolysis is often associated with amidase activity; thus, these two means of hydrolysis are now regarded as different manifestations of the same activity. A wide range of hydrolytic enzymes of toxicological importance are distributed in the body in both vertebrates and invertebrates, occurring in many tissues and in both microsomal and cytosolic fractions.

Polarized carbonyls, such as trifluoromethyl ketones (TFK's),[1] are putative isosteric analogues of tetrahedral intermediates formed during the hydrolysis of esters and amides. Since these tetrahedral intermediates are thought to be similar to the transition state along the reaction coordinate, some TFK's are strong inhibitors of hydrolytic enzymes having varying specificity toward insect juvenile hormone esterase (JHE) (*2–4*) and other hydrolases (*5–8*). Results in the quest of finding more potent inhibitors of JHE have shown that a sulfur atom β to the carbonyl moiety greatly increases JHE inhibition and specificity (*9–12*). Subsequently, TFK's have been used to purify JHE to homogeneity by affinity chromatography (*13*). A possible toxicological interest in the research on the TFK inhibitors is that these compounds seem to distinguish between esterases and amidases: I_{50} values against esterases range from millimolar to nanomolar range, while amidase inhibition is often weak to moderate. However, to date no heterocyclic derivatives have been studied in these series, although some of these might have significant activity against JHE and other enzymes as well as increased water solubility.

JHE is one of the key enzymes responsible for regulating the titer of juvenile hormones (JH) which play a basic role in the metamorphosis of insects (*14*). Inhibition of this enzyme partially protects JH from degradation and, thus, disturbs the normal development of the insect (*15*, *16*).

Besides JHE, other esterolytic enzymes such as acetylcholinesterase (EC 3.1.1.7, AChE) are also inhibited by TFK's (*2*, *10–12*, *17*, *18*). AChE has long been a target site for cholinergic blocking agent nerve gases, drugs, and insecticides (*19*), and therefore selective inhibitors of the

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[1] Abbreviations: JHE, juvenile hormone esterase; AChE, acetylcholinesterase; LP, lipase; TFK, trifluoromethyl ketone; JH or JH-III, juvenile hormone; ACh, acetylcholine; BTFA, 3-bromo-1,1,1-trifluoro-2-propanone; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; DFP, diisopropyl fluorophosphate; PhSTFP, 3-(phenylthio)-1,1,1-trifluoro-2-propanone; OTFP, 3-(octylthio)-1,1,1-trifluoro-2-propanone.

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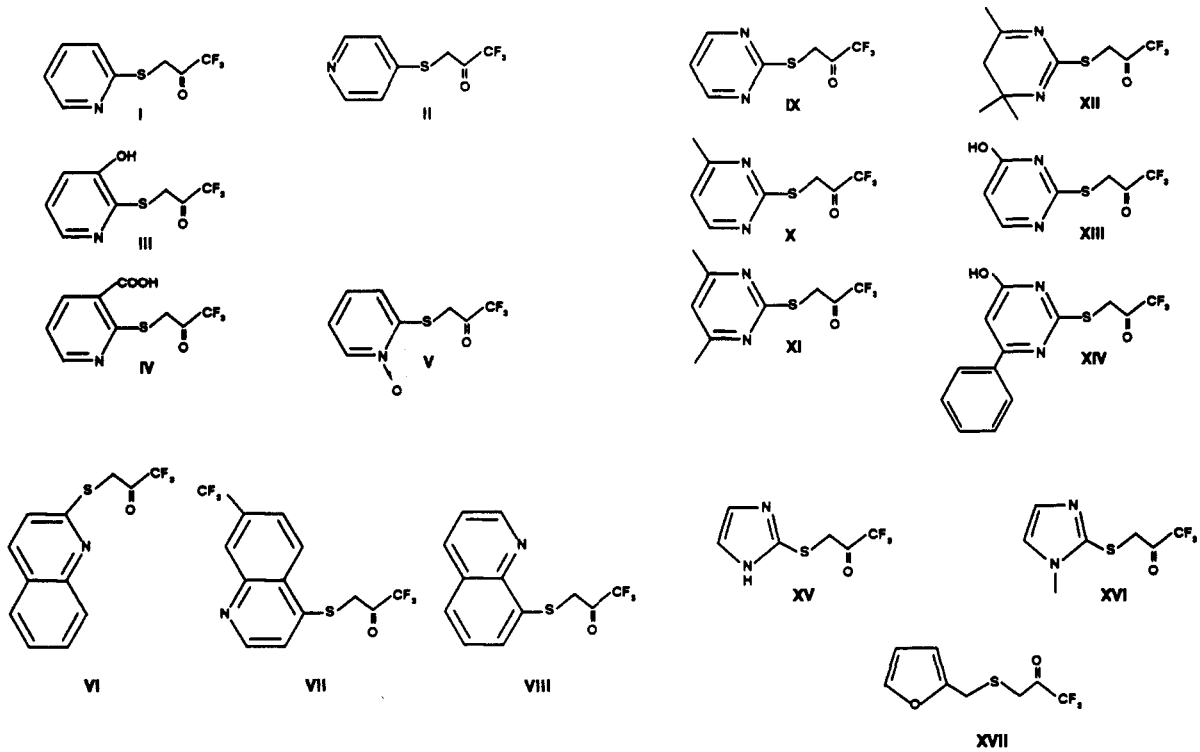

Figure 1. Chemical structures of the 17 novel 3-(heteroarylthio)-1,1,1-trifluoro-2-propanone compounds.

enzyme have been thoroughly investigated and efforts have been directed to this enzyme in clinical and environmental toxicology (for reviews, see refs 20 and 21). The enzyme regained attention recently, when the aging of the muscarinic ACh receptor in the brain was proven to be related to Alzheimer's disease (22). Structure-based descriptors have been found important in QSAR studies of AChE inhibition, suggesting an optimal shape/configuration that likely mimics the AChE receptor site (23).

Lipases also fall into the class of esterolytic enzymes, although they appear evolutionarily unrelated or distant from some esterases and they differ from general esterases on the basis of their natural substrates (B-esterases). Lipases preferentially act on triglycerides of long-chain fatty acids in an emulsified form. Some lipases, e.g., porcine pancreatic lipase, are irreversibly inhibited by diethyl *p*-nitrophenyl phosphate (paraoxon) with the modification of a single active center serine hydroxyl group and, thus, may come under the class of serine hydrolases (24). In our previous study (11) with alkylthio trifluoromethyl ketones we showed inhibition of yeast lipase with I_{50} values in the micromolar range. Like lipases, chymotrypsins are also serine hydrolases mainly acting on proteins.

In this study, 17 novel heterocyclic TFK's were synthesized, and their inhibitory potency was measured against four esterolytic enzymes, insect JHE, eel AChE, yeast LP, and a protease, α-chymotrypsin. The inhibitory activities were shown to be related to their three dimensional chemical structure obtained by X-ray crystallography and computer modeling.

Experimental Procedures

Materials. 3-Bromo-1,1,1-trifluoro-2-propanone (BTFA) was obtained from PCR (Gainesville, FL), and 8-quinolinethiol was from Sigma (St. Louis, MO); all other chemicals were from Aldrich (Milwaukee, WI) unless stated otherwise.

Instrumentation. Proton magnetic resonance (^{1}H NMR) spectra were obtained on a Varian EM-390 (90 MHz) in a deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6). Tetramethylsilane was used as internal standard. Chemical shifts are reported in parts per million (ppm). Elemental analyses for all TFK compounds were carried out at the Berkeley Analytical Laboratory (UC Berkeley, California). Positive and negative ion fast atom bombardment (FAB) and electron ionization (EI) mass spectra (MS) were obtained on a ZAB-HS-2F spectrometer (VG Analytical, Wythenshawe, U.K.) with 1-mA, 8-keV xenon FAB or 70-eV electron ionization. Samples were introduced as solid probes (EI) or FAB probes using 3-nitrobenzyl alcohol for low resolution or a mixture of poly(ethylene glycol) 300 and 3-nitrobenzyl alcohol for high resolution (FAB). The purity of the products was demonstrated by TLC in multiple solvent systems, as well as by gas chromatography for the volatile compounds, using a Varian Series 1400 with a flame ionization detector and a glass column (2 m × 2 mm) packed with 5% SE 30 on GAS Chrom Q.

Synthesis. The title compounds, a series of substituted pyridyl, *N*-oxopyridyl, pyrimidyl, quinolinyl, imidazolyl, and triazolyl compounds (Figure 1), were synthesized by the reaction of BTFA with the appropriate heterocyclic thiol under nitrogen atmosphere.

3-(2-Pyridylthio)-1,1,1-trifluoro-2-propanone (I). 2-Pyridinethiol (0.22 g; 2 mmol) was dissolved in 3 mL of dry acetone, stirred magnetically under nitrogen, and cooled in an ice bath, and BTFA (0.18 mL; 2 mmol) was added dropwise, via syringe, within about 15 min. The mixture was stirred overnight at room temperature, the solvent was evaporated, and the product was recrystallized from cyclohexane/acetone (1:1) in a pyridine hydrobromide form (79% yield). The salt was dissolved in water and the solution was made alkaline to pH 8 with 6 N NH_4OH. The resulting solid was filtered off and air-dried to give the pure product in 71% yield: Anal. Found: C, 41.43; H, 2.85; N, 6.01; S, 14.04. Calcd for $C_8H_6F_3NOS$: C, 43.44; H, 2.73; N, 6.33; S, 14.50. ^{1}H NMR ($CDCl_3$) δ 3.45 (s, 2 H, CH_2), 6.95–7.65, 8.30 (m, 4 H, pyridyl); FAB-MS, *m/z* 222.02 $[M + H]^+$; EI-MS, *m/z* 221 $[M]^+$.

The basicity of the ring nitrogens in the pyrimidine (IV–XIV) and in some pyridine (IV, V) derivatives is too low to autocatalyze the S-alkylation reaction. The synthesis of these compounds was carried out in a similar fashion to the procedure of Schickaneder et al. (25), with the modification that 10% molar excess of triethylamine as a base (110% excess in the case of IV) in a polar aprotic solvent (DMSO) was used. Since in these cases the products could not be separated from solution in their hydrobromide salt form, the solvent was evaporated to dryness under vacuum (1–2 Torr) and the residue was taken up in 10 mL of ether.

Table I. Synthesis and Characteristic Physical Properties of Heterocyclic Trifluoromethyl Ketones

compd	solvent	yield, %	mp,[a] °C	R_f[b]	NMR[c] SCH_2CO
I	acetone	71[d]	82–3	0.74	3.40
II	acetone	89[d]	104–5	0.31	3.30
III	acetone	69[d]	oil	0.64	3.35
IV	acetone	69[d]	oil	0.60	3.15
IV	DMSO/TEA	14[e]	oil	0.60	3.15
V	acetone/TEA	44[e]	oil	0.48	3.05
VI	acetone	73[d]	104–5	0.73	3.30
VII	acetone	89[d]	126–7	0.64	3.20
VIII	acetonitrile	64[d]	112–3	0.66	3.05
IX	DMSO/TEA	45[e]	65–8	0.27	2.95
X	DMSO/TEA	51	oil	0.29	3.00
XI	DMSO/TEA	37[e]	oil	0.30	3.05
XII	DMSO/TEA	39[e]	oil	0.53	3.15
XIII	DMSO/TEA	32	60–5 dec	0.21	3.05
XIV	DMSO/TEA	35	oil	0.66	3.00
XV	THF	83[d]	103–4	0.56	3.25
XVI	THF	82[d]	129	0.17	3.20
XVII	CCl_4/TEA	74	oil	0.73	2.90

[a] Melting points are uncorrected. [b] Solvent system: hexane/acetone (3:2). [c] Characteristic chemical shift of the methylene protons adjacent to the sulfur atom. Reported in ppm relative to TMS. [d] Precipitated from water with 1 N NaOH. [e] Purified by preparative TLC.

Table II. Table of Crystal Data for Compounds I and II

	compound I	compound II
formula	$C_8H_8F_3NO_2S$	$C_8H_8F_3NO_2S$
fw	239.22	239.22
crystal system	monoclinic	monoclinic
space group	Pc	$P2_1/c$
a, Å	10.723 (6)	11.263 (3)
b, Å	10.747 (7)	9.616 (3)
c, Å	9.376 (4)	8.940 (3)
β, deg	113.95 (4)	101.63 (2)
V, Å^3	987.4 (9)	948.4 (4)
T, K	130	130
Z	4	4
d_{calcd}, g cm^{-3}	1.61	1.68
radiation (Å)	Mo Kα (λ = 0.71069)	
μ(Mo Kα), cm^{-1}	3.4	3.5
R	0.059	0.039
R_w [$w = \sigma^2(F_0)^{-1}$]	0.058	0.041

The organic phase was washed with water and dried over sodium sulfate. Details of the syntheses and physical properties of the products are summarized in Table I. 3-Furfuryl-1,1,1-trifluoro-2-propanone (XVII) was prepared similarly to the previously published procedure (*11*).

When it was necessary, the compounds were purified by preparative TLC on 1000-μm silica gel plates (Analtech, Newark, DE), or by flash chromatography on silica gel (40 μm average particle size, J. T. Baker, Phillipsburg, NJ) using a hexane/acetone (3:2) solvent system. Analytical TLC was performed on silica gel F_{254} plates (Whatman, Clifton, NJ); TLC plates were developed in hexane/acetone (3:2) or benzene/ethyl acetate (9:1) solvent systems. The compounds were detected as spots that quenched the UV fluorescence of the gel at 254 nm, in an iodine chamber or by spraying the plate with a reagent of methanol/aqueous HCl solution (1:1) saturated with iodine and potassium iodide.

X-ray crystallography for compounds I and II was carried out by using a Syntex $P2_1$ X-ray diffractometer equipped with a modified LT-1 low-temperature apparatus. The radiation used was graphite-monochromated Mo Kα (λ = 0.71069 Å). Crystallographic computer programs were from SHELXTL, Revision 5.1, installed on a Data General Eclipse computer. Neutral atom scattering factors and corrections for anomalous dispersion were from common sources (*26*). The absorption correction was performed by using a local program, XABS (*27*). Crystal data for the two compounds are reported in Table II. Additional data collection and refinement parameters are given in the supplementary material. The structures were solved by direct methods. Hydrogen atoms bonded to the carbon atoms were included at calculated positions according to a riding model, with C–H of 0.96 Å and $U_H = 1.2U_C$. The hydrogens on the hydroxyl groups were located in a difference map and refined with the additional constraint that the O–H distance be 1.00 (3) Å. One of the hydrogen atoms [H(1)] in compound I was not located in the difference map but is illustrated in Figure 2 at a probable location. An absorption correction was applied. For compound I, sulfur atoms and one of the fluorine atomms which showed large thermal motion were refined with anisotropic thermal parameters. For compound II, all non-hydrogen atoms were refined with anisotropic thermal parameters. The largest feature on a final difference map was 0.46 e Å^{-3} in height for compound I and 0.30 e Å^{-3} for compound II. Tables of atomic positional parameters, bond distances and angles, and anisotropic thermal parameters have been deposited as supplementary material. Drawings of the molecular structures of the two compounds are depicted in Figure 2. Crystals for X-ray crystallography were grown from hexane/ether (1:1) solution.

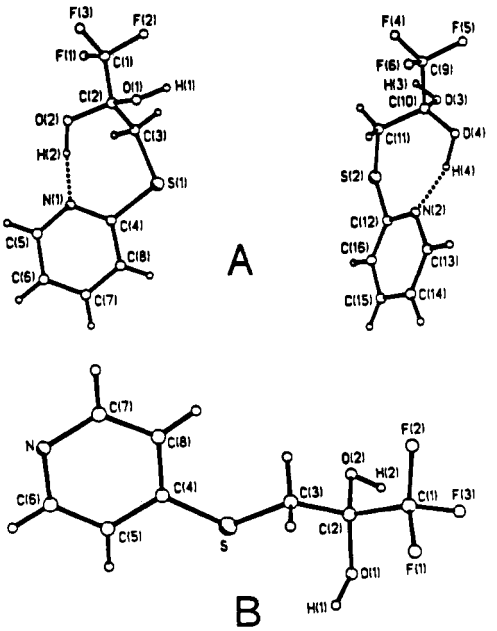

Figure 2. Molecular structures of 3-(2-pyridylthio)-1,1,1-trifluoro-2-propanone (I) and 3-(4-pyridylthio)-1,1,1-trifluoro-2-propanone (II) obtained by X-ray crystallography. (A) View of the two stereoisomers of I. The dotted lines in the structures indicate intramolecular H-bonds. (B) View of the structure of II. Intermolecular H-bonding interactions appear in this compound.

Enzyme Assays. To measure the in vitro inhibition of the compounds, both end-point and kinetic assays were used. The inhibitors were dissolved in ethanol or acetone so that less than 1% organic cosolvent was added to the 100-μL enzyme reaction, preincubated with the enzymes for 10 min, and then incubated with the substrates. In the end-point assay for JHE a 10-min incubation time was used. For the other three assays, enzyme activity was monitored within the first 10 min, during which the time–activity relationships were found to be linear, and the rates of the inhibited samples were compared to those of the uninhibited enzyme solutions. All enzyme assays were run in triplicate. Molar I_{50} values were calculated by least-squares regression from the linear portion of a semilogarithmic plot of activity versus inhibitor concentration. At least four inhibitor concentrations were used to determine the I_{50}'s, two above and two below 50% inhibition. Inhibitory potencies were not measured for concentrations higher than 10^{-3} M.

Juvenile Hormone Esterase. The radiometric partition method (*28, 29*) was used for assaying JHE activity. Hemolymph from 2-day-old fifth-instar larvae of *Trichoplusia ni* was the source of JHE used in the present studies. It was diluted 1:500 with 0.08 M phosphate buffer [pH = 7.4, 0.08 M with 0.01% (w/v) phenylthiourea to inhibit tyrosinases]. [10-^{3}H]JH-III (New England Nuclear, Boston, MA) and unlabeled JH-III (Calbiochem, San Diego, CA) were used as substrate at a final concentration of 5 × 10^{-6} M JH-III (containing ~30 000 dpm/assay) in absolute

ethanol as a solvent. The assays were performed at 30 °C. The rate of hydrolysis was detected by measuring the amount of JH acid in the aqueous phase after the incubation time, using an LKB Wallac liquid scintillation counter.

Acetylcholinesterase. Assays were carried out by modifying the procedure of Ellman et al. (*30*). Lyophilized enzyme (EC 3.1.1.7) from electric eel (Sigma) was dissolved at a final concentration of 80 ng/mL in 0.05 M phosphate buffer (pH = 7.4) containing 0.015% of DTNB. Acetylcholine iodide was used as a substrate at a final concentration of 5×10^{-4} M in buffer. The assays were run at 30 °C. The release of thiocholine iodide was monitored kinetically at 405 nm for 10 min in a V_{max} reader using a 96-well plate.

Lipase. Lipase assays were performed similarly as published previously (*11*). Lipase (glycerol-ester hydrolase, EC 3.1.1.3) from *Candida cylindracea* (type VII, Sigma) was used at a final enzyme concentration of 50 μg/mL. Assays were carried out in 0.1 M Tris-HCl buffer (pH 7.0) containing 0.1 mM of DFP to inhibit nonspecific esterases. *p*-Nitrophenyl acetate in acetone was used as a substrate to give the final substrate concentration of 1.35 mM. The formation of *p*-nitrophenol was monitored kinetically at 405 nm for 10 min on a V_{max} reader using a 96-well plate.

α-Chymotrypsin. α-Chymotrypsin (EC 3.4.21.1) from bovine pancreas (type I-S, Sigma) was dissolved in 0.01 M acetate buffer (pH 4.0) just prior to performing assays at the concentration of 5 mg/mL. For the assays, 10 μL of this enzyme solution was mixed with 280 μL of 0.01 M phosphate buffer (pH 8.0). *p*-Nitrophenyl acetate in acetone was used as a substrate at the final concentration of 9.4×10^{-4} M. The formation of *p*-nitrophenol was monitored kinetically at 405 nm for 10 min on a V_{max} reader using a 96-well plate. Inhibitors were applied at 3.4×10^{-4} M final concentration.

Molecular Modeling. Computer-assisted molecular modeling analysis was performed by using Alchemy (Tripos Associates) (*31*) to determine the minimum-energy conformation of each molecule in both the hydrated and the carbonyl form. The resultant three-dimensional optimized conformations were used to determine bond distances and angles in the molecules and molecular fit to different substrate structures.

In Vivo Toxicity. The *CS* strain of *Drosophila melanogaster* was used for the in vivo toxicity assay. Inhibitors were added to 8-dram shell vials in a 100-μL volume in ethanol, and the solvent was evaporated under nitrogen. Adult flies (3–5 days old) were exposed to a residue of the different TFK's and malathion, as described previously (*32*). Desiccation of the flies was prevented by adding 2 mL of water on the cotton the vials were plugged with. Triplicate doses of 4 nmol/vial–12.5 μmol/vial (in 5-fold increments) of the inhibitors were employed, and mortality counts were tabulated at 1, 3, 5, 7, 11, and 20 h after exposure and compared to that of untreated vials and ones similarly treated with 100 μL of ethanol.

Larval mortality was determined by adding 0.1–12.5 μmol/vial (in 5-fold increments) of the inhibitors in 100 μL of ethanol to the surface of standard cornmeal fly food in 8-dram shell vials. The vials were allowed to dry for 24 h, and 40 eggs were added to each. The number of larvae surviving to pupae and eclosing as adults was tabulated in the triplicate vials for each dose and controls. LD_{50} values, 95% confidence intervals for LD_{50}'s, and slopes for both larval and adult assays were calculated from the mortality rates by Finney's probit analysis.

Results and Discussion

Synthesis. Seventeen new heterocyclic derivatives of 3-substituted-1,1,1-trifluoro-2-propanones were synthesized. The structures of the new inhibitors are shown in Figure 1. Although the syntheses were similar to those described earlier (*10*, *11*), several variations were employed. The use of an inert atmosphere during synthesis seems critical due to the instability of some of the products and starting mercaptans, especially the 2- and 4-pyridinethiols. The heterocyclic trifluoro ketones formed can also undergo a cyclization between the carbonyl group and the aromatic nitrogen, giving ionic hemiaminals as was reported for the reaction of haloketones with 2-mercaptobenzimidazole (*33*). Since the carbonyl is polarized by the fluorines, it might be supposed that these compounds would have a high tendency to cyclize. The tertiary hemiaminals are, however, quite unstable, and the equilibrium of their formation is pushed to the carbonyl form. No significant hemiaminal formation was found in the case of *N*-methylimidazole (XV) and *N*-methyltriazole (XVI). Moreover, since the intramolecular hemiaminal formation is favored in nonaqueous solvent systems and since in aqueous solutions the ketones undergo hydration, such cyclization does not interfere with the observed biological activities. Similarly to other TFK's containing no heterocyclic moiety (*34*), the X-ray crystal structures showed that compounds I and II exist in the hydrated, *gem*-diol form (Figure 2). Molecular ion masses belonging to the *gem*-diols in FAB-MS spectra $[M + 19]^+$ also confirmed that several of the heterocyclic TFK's exist partially hydrated in the organic solvent used for MS (3-nitrobenzyl alcohol).

The chemical behavior of the 2-pyrimidine derivatives is very similar to that of 2-[(carboxymethyl)thio]pyrimidines (*35*) and -uracils (*36*): the resulting trifluoro ketones are very sensitive to hydrolysis, forming 2-hydroxypyrimidines and trifluoroacetonyl sulfide and/or disulfide. This is in agreement with the experience of Alper and Lipshutz (*37*), who reported C–S cleavage in the case of the similar 2-mercaptopyrimidine derivative. The trifluoro ketones could be isolated, however, with poor yields, if the excess of BTFA was avoided and the reaction mixture was always alkaline. Because of their poor solubility in organic solvents, DMSO was used for these reactions.

Addition of triethylamine to the reaction mixtures considerably accelerated the reactions. Since it reacts with BTFA very exothermically, the rate of addition of this reactant was crucial to the final purity of the product. The yields for all compounds were between 14% (IV) and 89% (II). Very poor yield occurred in the alkylation of 2-mercaptonicotinic acid (IV). Since this compound exists in a zwitterionic form (*38*), a polar aprotic organic solvent, DMSO, was used.

The resulting heteroarylthio trifluoro ketones were readily identified by the NMR signals in the aromatic zone (6.5–8.5 ppm) and by the characteristic two-proton signal of the methylene group, adjacent to the sulfur atom, at 2.9–3.4 ppm (Table I) and in some cases by X-ray crystallography (Figure 2). In addition to molecular masses, EI-MS spectra also showed molecular fragment ions of $[CF_3]^+$ (*69*) or loss of the trifluoromethyl group $[M - 69]^+$, as well as molecular fragment ions of $[CF_3CO]^+$ (*97*) or loss of the (trifluoromethyl)carbonyl group $[M - 97]^+$.

The X-ray structure showed that molecules of compound I exist in two slightly different conformations in the asymmetric unit. Within the molecules the H···N distances are 1.68 (4) and 1.66 (4) Å, and the O–H···N angles are 155 (4)°. There are several short intermolecular contacts indicating hydrogen bonding between the molecule also, particularly O(4)···O(1)′ of 2.76 (2) Å and O(3)···O(2)″ of 2.75 (2) Å. (Symmetry code: ′ = x, $-y$, $0.5 + z$; ″ = x, $y - 1$, $1 + z$.) Compound II exhibits only intermolecular hydrogen bonding. There is a hydrogen bond between O(2) and H(1)′ 1.76 (2) Å in length, and between N and H(2)″ 1.63 Å in length. (Symmetry code: ′ = x, $0.5 - y$, $z - 0.5$; ″ = x, $1 + y$, z.)

Enzyme Assays. The heterocyclic TFK derivatives (compounds I–XVII) were tested as potential inhibitors of three esterolytic enzymes and a protease, i.e., insect JHE from *T. ni*, eel acetylcholinesterase, yeast lipase, and bovine α-chymotrypsin. The enzyme inhibition by the title compounds is summarized in Table III. The I_{50} values for

Table III. Inhibition of Three Esterolytic Enzymes, Insect Juvenile Hormone Esterase, Eel Acetylcholinesterase, and Yeast Lipase, by Compounds I–XIX

compound	molar I_{50} value,[a] M		
	JHE	AChE	LP
I	9.78×10^{-8}	8.01×10^{-6}	1.06×10^{-4}
II	1.12×10^{-5}	1.09×10^{-5}	$>1 \times 10^{-3}$
III	5.93×10^{-5}	5.49×10^{-5}	2.58×10^{-5}
IV	1.01×10^{-3}	$>1 \times 10^{-3}$	$>1 \times 10^{-3}$
V	2.39×10^{-5}	1.52×10^{-4}	6.33×10^{-5}
VI	6.31×10^{-7}	1.48×10^{-5}	1.75×10^{-4}
VII	6.16×10^{-5}	5.34×10^{-5}	$>1 \times 10^{-3}$
VIII	3.48×10^{-6}	1.34×10^{-6}	8.03×10^{-5}
IX	1.61×10^{-4}	2.89×10^{-6}	5.79×10^{-4}
X	1.41×10^{-4}	4.40×10^{-6}	1.05×10^{-4}
XI	3.85×10^{-4}	1.38×10^{-5}	1.35×10^{-4}
XII	1.00×10^{-3}	5.52×10^{-5}	1.03×10^{-4}
XIII	$>1 \times 10^{-3}$	4.37×10^{-6}	$>1 \times 10^{-3}$
XIV	$>1 \times 10^{-3}$	2.52×10^{-6}	$>1 \times 10^{-3}$
XV	3.24×10^{-4}	6.27×10^{-7}	$>1 \times 10^{-3}$
XVI	1.35×10^{-4}	1.44×10^{-6}	$>1 \times 10^{-3}$
XVII	7.58×10^{-5}	1.56×10^{-6}	1.31×10^{-4}
OTFP[b]	1.81×10^{-9}	5.21×10^{-6}	3.25×10^{-7}
PhSTFP[c]	9.13×10^{-6}	6.06×10^{-6}	5.20×10^{-4}

[a] JH-III was used as a substrate for JHE from *T. ni*, acetylthiocholine iodide for AChE from electric eel, and *p*-nitrophenyl acetate for LP from *Candida cylindracea*. [b] As reported earlier (*11*). Other reported molar I_{50} values (*10*, *12*) are 2.3×10^{-9} and 3.1×10^{-9} M against JHE and 2.3×10^{-6} and 4.7×10^{-6} M against AChE. [c] Reported molar I_{50} values (*10*, *36*) are 8.2×10^{-6} M against JHE and 3.7×10^{-6} M against AChE.

most of the compounds against JHE were around 10 μM or higher, which is somewhat higher than that of the formerly synthesized 3-(phenylthio)-1,1,1-trifluoro-2-propanone (8–9 μM) (*10*, *39*). These compounds were obviously synthesized as AChE inhibitors. Therefore, it was surprising that 3-(2-pyridylthio)-1,1,1-trifluoro-2-propanone (I) was far more potent against JHE (I_{50} = 98 nM) than expected.

Both of the most active compounds against JHE (I, VI) contain a nitrogen atom in the aromatic ring in the ortho position relative to the sulfur atom, which allows H bonds between the hydrated carbonyl and the nitrogen and/or makes it possible to form a five-membered cyclic hemiaminal. Structural isomers containing the nitrogen in a further position of the ring (II, VII, VIII) show significantly lower activity. Hydrophilic substitution on the ring in all cases seems to lower the inhibitory potency.

It is rather interesting that the activity of compound I against JHE is orders of magnitude higher than that of compound II or 3-(phenylthio)-1,1,1-trifluoro-2-propanone (PhSTFP), reflecting the difference in pyridyl ring position that allows intramolecular hydrogen bonding. The results of the X-ray crystallography show that compound I exists in two conformations (Figure 2A). Strong intramolecular H bonding occurs in both conformations between one of the hydroxyl hydrogens and the pyridyl nitrogen. In contrast, no intramolecular H bonding can occur in PhSTFP or in compound II (Figure 2B), since the nitrogen is too distant in the latter compound. Although both I and II show intermolecular OH···N and OH···O hydrogen bonds, these bonds are much weaker and are expected to be of less importance in solution.

A comparison of inhibition of JHE by these other TFK compounds supports the hypothesis that a single ortho pyridyl nitrogen is important for strong inhibitory activity. Just as compound I is far more potent than PhSTFP, compound VI is far more potent than its naphthyl analogue. The binding pocket of JHE is known to be very hydrophobic; thus it is not surprising that increasing the number of polar groups on candidate inhibitors dramatically reduces the I_{50} value. Although slightly less active on JHE than compounds in the aliphatic TFK series, i.e., 3-(octylthio)-1,1,1-trifluoro-2-propanone (OTFP), compound I may be very promising in affinity chromatography since it is more water soluble while having fewer detergent properties than OTFP.

The outstanding difference in inhibitory potency against JHE between compounds I and II can be added to our understanding of the mechanism of inhibition of serine esterases. For many years scientists have assumed that the catalytic triad shown for serine proteases (Asp-His-Ser) also applies to serine esterases. Recently it has been demonstrated by sequence analysis (*40*) either that such a triad of proteases does not exist in all esterases or that these enzymes show a radically different folding pattern. Thus, only the serine residue, which has been proven to be catalytically active, is included in this hypothesis.

As discussed earlier (*4*), there are two obvious routes by which polarized ketones could inhibit esterases (Figure 3). The most simplistic (Figure 3A–C) assumes that the low-abundance carbonyl form mimics the substrate and proceeds along the reaction coordinate to mimic a tetrahedral intermediate similar to several possible transition states (Figure 3, structure 3) which could be stabilized by hydrogen bonding to an appropriate heterocyclic nitrogen (Figure 3, structure 4). However, the carbonyl form is certain to be a very low abundance form, and if the hydrogen bonding shown in the solid state for compound I is significant in aqueous solution, the equilibrium reaction A will be shifted even further in favor of the hydrated state (Figure 3, structure 5). A second possibility is that the more abundant *gem*-diol (Figure 3, structure 5) could associate directly with the catalytic site due to a partial resemblance to the natural substrate of the enzyme. A difficult problem in this case is how such a form actually inhibits the enzyme. We have previously advanced the hypothesis (*4*) that, in the hydrophobic environment of the catalytic site, the *gem*-diol (Figure 3, structure 6) slowly reverts to the carbonyl form (Figure 3, structure 2). Possibly the equilibrium H is shifted slightly toward the carbonyl form, and certainly, once the carbonyl form is generated, reaction with the serine residue will be very rapid to yield the tetrahedral adduct (Figure 3, structure 3). The time needed for reactions H and C to occur may account for the slow tight binding phenomenon observed for the interaction of TFK's with some serine hydrolases.

An exciting possibility raised by the current study is that the enzyme accelerates the direct reaction of the serine with the *gem*-diol (Figure 3, structure 7). This could be catalyzed by a general-acid–base catalyst often hypothesized to be present in the catalytic site of esterases or in the case of compound I by an intramolecular reaction (Figure 3G).

The I_{50} values against acetylcholinesterase were often in the same range as against JHE. Compared to many previously synthesized transition-state mimics of AChE, the compounds reported here are very potent and are promising for the extension of the affinity purification system developed for JHE (*13*) or AChE. In an earlier attempt to inhibit JHE, a thioether was placed β to the carbonyl in order to mimic the α double bond of JHE. It was found that these compounds were more potent inhibitors not only of JHE but also of AChE (*10*). This observation was explained in part by X-ray crystallographic studies showing that a hydrogen bond from the *gem*-diol to the thioether might stabilize the serine adduct (*34*). The formation of a tetrahedral hemiacetal as a

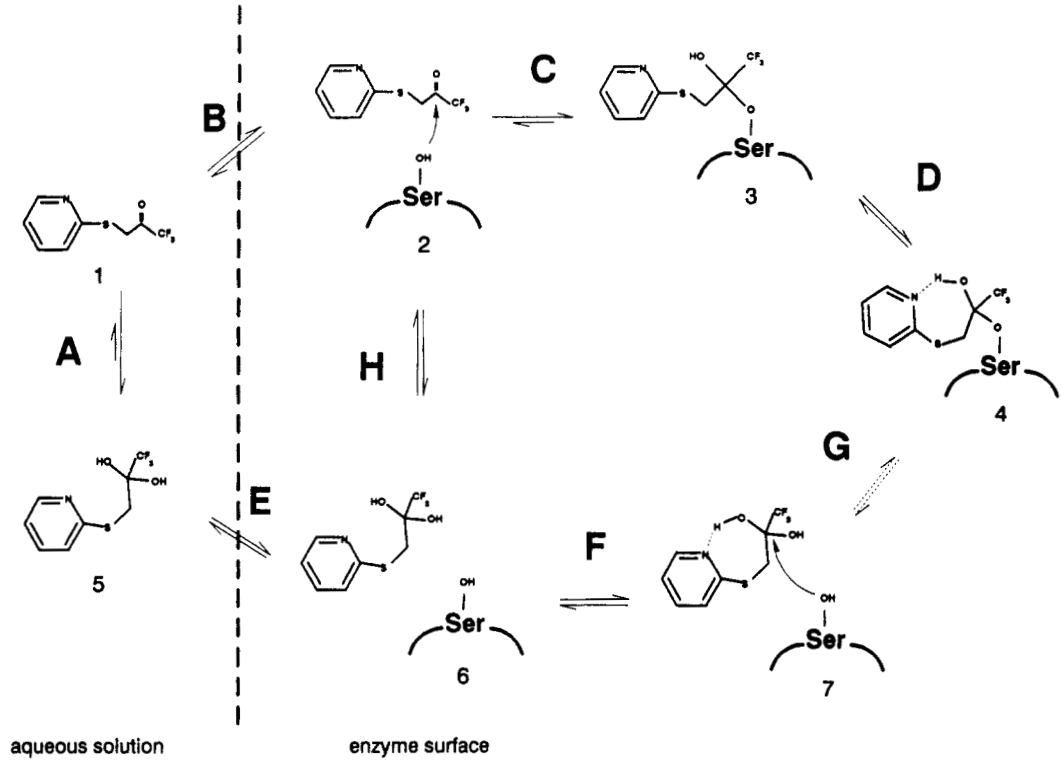

Figure 3. Scheme for proposed reaction pathways for inhibition of serine esterases by heterocyclic trifluoromethyl ketones.

transient intermediate in the interaction between AChE and a TFK inhibitor has recently been shown by ^{19}F NMR analysis (*41*). The title compounds were prepared as candidate inhibitors of AChE based on the idea that the heterocyclic nitrogen could mimic that of ACh. If the nitrogen also could act to stabilize the serine adduct, one would expect compound IX with two ortho nitrogens to be a more potent inhibitor of AChE and a less potent inhibitor of JHE than compound I with a single nitrogen. Indeed, these trends are observed. Both hydrophobic and hydrophilic substitution on the pyrimidine ring lowered the inhibitory potency. One also would expect the *N*-methylimidazole (XVI) derivative to be a potent inhibitor of AChE, and it is the most potent compound of this series on AChE.

To test this hypothetical involvement of the heterocyclic nitrogens, distances between the aromatic nitrogen atom and the carbonyl group in the molecules were compared to that measured in ACh and correlated to the inhibitory activities. The distances were determined in the minimal-energy conformation of the compounds in both the carbonyl and hydrated carbonyl forms. Using the Alchemy molecular modeling software, the compounds, in their carbonyl and hydrated forms, were also fit to the structure of ACh. [Three matching pairs of atoms were used for the least-squares fitting routine in the carbonyl forms (N, carbonyl C, carbonyl O atoms), and four pairs were used in the hydrated forms (N, *gem*-diol C, both *gem*-diol O atoms).]

The nitrogen–carbonyl distance in ACh was found to be 4.9 Å in the carboxyl form (ground state) and 5.0 Å in the hydrated form (tetrahedral transition state). The slight difference between the two distances is due to the elongation of the C–O bonds in the hydrated form. These values are in agreement with earlier calculations [5.2 Å (*42*)] and with the distance of the esteratic and anionic loci at the AChE active site [≥4.7 Å (*21*)].

The N–CO distances in the title compounds are in good correlation with the inhibitory potencies: the closer they are to 4.9–5.0 Å, the better the AChE inhibition. The distances between the nitrogen and the carbonyl are 3.1 Å (I, VI), 4.3 Å (IX–XI), and 3.2 Å (XV, XVI), respectively. Compounds XV and XVI are probably more important than anticipated from these calculations because one of the ring nitrogens is methylated. These results also show why some pyrimidine derivatives can better mimic the substrate, ACh: one of the aromatic nitrogen remains in H-bonding interaction with the (hydrated) carbonyl while the second might mimic the nitrogen in ACh. In addition, the fact that all compounds showed better fit in their hydrated forms to ACh hydrate (transition states) than in their carbonyl forms to ACh (ground states) also supports the theory that these TFX compounds are transition-state mimic inhibitors of AChE.

In order to increase the inhibitory potency of compound I against AChE, we attempted an N-methylation reaction. This modification followed by a partial reduction of the pyridine ring might also enhance in vivo effects by improving the ability to penetrate through biological membranes (*43*). In our case, however, the alkylation was unsuccessful, which was possibly also due to the intramolecular hemiaminal formation.

Similarly to arylthio trifluoromethyl ketones, such as 3-(phenylthio)-1,1,1-trifluoro-2-propanone, the I_{50} values for most heterocyclic TFK's against yeast lipase mainly fall into the 10^{-4} M range. This is about 2 orders of magnitude lower inhibitory potency than that of OTFP and alkylthio trifluoromethyl ketones (*11*), which is probably due to their poorer resemblance to the natural substrate, glycerol esters of fatty acids. In an earlier study, alkane and arene boronic acids were tested as inhibitors of porcine pancreatic lipase (*44*). The boronic acid binding site on lipase was shown to be at or near the active center serine since modification of this residue by diethyl *p*-nitrophenyl phosphate was prevented by boronic acid. Like TFK's, boronic acids are believed to be analogues of the tetrahedral transition state. Although we used a yeast lipase, the results suggest that inhibition by TFK's was

Table IV. Comparative Toxicity of Selected Heterocyclic Trifluoromethyl Ketones against Larvae and Adults of *D. melanogaster*

	adult test[a]			larval test		
compd	LD_{50}, μmol/vial	95% CI[d] for LD_{50}, μmol/vial	slope	LD_{50}, μmol/vial	95% CI[d] for LD_{50}, μmol/vial	slope
I	2.10	1.45–2.99	1.09	>50		
II	0.99	0.57–2.12	0.81	0.73[b]	0.38–1.71	1.95
IX	>50			>50		
XV	1.07	0.66–1.68	0.97	9.55[c]		
XVI	0.40	0.25–0.60	1.26	2.82[c]		
OTFP	20.9	16.5–32.8	3.24	19.3[c]	14.2–25.1	5.13

[a] Mortality counts were taken after 20 h. [b] Mortality occurred in the pupal adult transition. [c] 90% of the mortality appeared in larval stages. [d] CI = confidence interval.

also due to the formation of a tetrahedral intermediate.

In contrast to esterases, a protease α-chymotrypsin was not inhibited by any of the title compounds at the concentration of 3.4×10^{-4} M. These results were similar to previous studies (*9*, *10*) with aliphatic and aromatic TFK's where no inhibition was observed at the concentration of 1×10^{-4} M.

A related compound, a trifluoro ketone derivative of furfuryl mercaptan (XVII), was prepared in the series, too. The inhibitory potency of this compound was in the same range as that of the N-heterocyclic compounds against all enzymes tested, showing greater AChE inhibition.

In Vivo Assays. Five heterocyclic TFK's as well as OTFP and malathion were tested as toxicological agents on larvae and adults of *D.melanogaster*. The results are summarized in Table IV.

With the exception of compound IX that was found to be inactive, LD_{50}'s for heterocyclic TFK's against adult flies were in the same range and at least 1 order of magnitude more potent than OTFP. Confidence intervals for the LD_{50}'s indicate that the difference between compound XVI and the other heterocyclic TFK's is statistically significant. In contrast to TFK's, the applied dose range of malathion killed flies largely within an hour. LD_{50} for malathion toxicity after 1-h exposure was found to be 33 ± 5 nmol/vial. In a previous study, a lower dose range of malathion gave a 24-h LD_{50} of 3.2 ± 2 nmol/vial (*32*). There is only a weak or no correlation between adult toxicity and in vitro AChE inhibition of the tested compounds.

To detect mortality at the larval and pupal stages, the number of emerged pupae and eclosing adults were counted to describe larvae-to-pupae and pupae-to-adult transitions. For the purposes of statistical analysis, the entire larvae-to-adult transitions were used. As in the adult assay, compound IX was inactive, but compound I, which was active on adults, is inert on larvae. Compounds XV and XVI were significantly more toxic than OTFP on the larval stage. In contrast, the most active compound on juveniles (II) produced mortality in the pupal stage.

In general, it appears there are developmental changes in the sensitivity to the toxicological effects of the inhibitors. Consequently, affinity columns based on these inhibitors could be used to isolate endogenous factors, perhaps enzymes, critical for growth and development in *D. melanogaster* and other organisms.

Summary

The synthesis and biological evaluation of 17 novel heterocyclic TFK's have led to several conclusions. Results from X-ray crystallography have shown that, in solid phase, the compounds exist in their hydrated form, as tetrahedral *gem*-diols. This observation adds further indirect evidence to the assumption that TFK's act as transition-state mimic inhibitors of esterolytic enzymes. A strong intramolecular H-bonding interaction between the aromatic nitrogen and the *gem*-diol was found in the most active inhibitor of JHE (compound I). This shows that it is the chemical reactivity of the hydrated carbonyl and its ability to form covalent hemiaminals and/or hemiacetals which enables it to act as a slow, tight binding inhibitor. Good correlation was found between the inhibitory potencies against AChE and 3D molecular geometry parameters obtained by computer-assisted molecular modeling. The fact that the compounds showed better fit to ACh in the hydrated from than in the carbonyl form also supports the transition-state mimic theory.

Acknowledgment. We express our sincere appreciation to Lawrence G. Harshman for his help in performing the in vivo toxicity tests and to Istvân Ujvâry and Angel Messeguer for their valuable comments and suggestions. This work was supported in part by Grants ES02710-07, DCB-8518697, and 85-crcr-1-1715 from NIEHS, NSF, and USDA, respectively. A.S. is a Fulbright Scholar (Fulbright Program 33917) supported by the Institute of International Education. B.D.H. is a Burroughs Wellcome Scholar in Toxicology.

Registry No. I, 127183-36-2; I·HBr, 127183-54-4; II, 127183-37-3; III, 127183-38-4; IV, 127183-39-5; V, 127183-40-8; VI, 127183-41-9; VII, 127183-42-0; VIII, 127183-43-1; IX, 127183-44-2; X, 127183-45-3; XI, 127183-46-4; XII, 127183-47-5; XIII, 127183-48-6; XIV, 127183-49-7; XV, 127183-50-0; XVI, 127183-51-1; XVII, 127183-52-2; BTFA, 431-35-6; 2-pyridinethiol, 2637-34-5; juvenile hormone esterase, 50812-15-2; acetylcholinesterase, 9000-81-1; lipase, 9001-62-1; α-chymotrypsin, 9004-07-3; esterase, 9013-79-0.

Supplementary Material Available: A table giving the chemical shift values from ^1H NMR and molecular mass values from FAB-MS and EI-MS for all compounds and 12 tables giving X-ray crystallography data (crystal data, refinement parameters, atomic coordinates, isotropic and anisotropic thermal parameters, bond lengths and angles) for compounds I and II (10 pages). Ordering information is given on any current masthead page.

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