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New Trifluoropropanone Sulfides as Highly Active and Selective Inhibitors of Insect Juvenile Hormone Esterase

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A series of α,α′-bis(2-oxo-3,3,3-trifluoropropylthio)alkanes was synthesized by the alkylation of the appropriate α,α′-alkanedithiol with 3-bromo-1,1,1-trifluoropropan-2-one. The compounds were tested for *in vitro* inhibition of insect juvenile hormone esterase (JHE), electric eel acetylcholinesterase (AChE), porcine carboxylesterase, and yeast lipase. Inhibition of hemolymph JHE from fifth instar larvae of the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), was in the nanomolar range for many compounds. The inhibition of the other three enzymes was only moderate in comparison to JHE. The inhibition of JHE was compared to carbon chain length, differing heteroatoms, and presence of methyl side-chains. The most effective compound was 1,1,1,16,16,16-hexafluoro-4,13-dithiahexadecane-2,15-dione (I_{50}: 8.2×10^{-10} *M*). The kinetics of inhibition were found to be time-dependent against both JHE and AChE enzymes using representative compounds of both high and low inhibitory potential. Second-order inhibition velocity constants were determined and found to be in agreement with the I_{50} values. © 1989 Academic Press, Inc.

INTRODUCTION

Juvenile hormone esterase (JHE)[2] is considered to play a critical role in the proper timing of insect metamorphosis (1). A precipitous drop in the titer of juvenile hormone (JH) is required to initiate the developmental events resulting in metamorphosis (2). In addition to a cessation in the biosynthesis of JH, the decline in the hormone titer is believed to be caused by increased metabolism. While patterns of JH metabolism vary among the species studied, ester hydrolysis by JHE appears to be the principle route of JH metabolism when the enzyme is present in lepidopteran larvae (3). Evidence in support of the importance of JHE in the regulation of the JH titer has come from varied approaches. One of these approaches is an indirect cause–effect relationship between JHE inhibition *in vivo* and a delay in the time of metamorphosis (4–7). In most of these studies *O*-ethyl *S*-phenyl phosphoramidothioate (EPPAT) was the most effective chemical probe for demonstrating this effect. However, the use of EPPAT for illustrating the involvement of JHE in regulating the JH titer has been a subject of criticism. At doses of EPPAT that inhibit most JHE activity, other esterases of possible toxicological and physiological significance are also inhibited. To reduce concern over possible side effects of chemical inhibitors, different chemical classes of compounds are needed as probes. This need for new and selective JHE inhibitors has stimulated several synthetic programs in different laboratories (4, 5, 8).

Fluoromethyl ketones have been proven to inhibit proteases and esterases with varying selectivity (9–13). In a series of al-

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[2] Abbreviations used: JHE, juvenile hormone esterase; AChE, acetylcholinesterase; JH, juvenile hormone; EPPAT, *O*-ethyl *S*-phenyl phosphoramidothioate; TFT, 1,1,1,-trifluorotetradecan-2-one; OTFP, 3-octylthio-1,1,1-trifluoropropan-2-one; BTFA, 3-bromo-1,1,1-trifluoropropan-2-one (bromotrifluoroacetone); ME, malathion esterase; LP, lipase; TEA, triethylamine; SAR, structure–activity relationship; IEF, isoelectric focusing; TMS, tetramethylsilane.

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iphatic trifluoromethyl ketones, 1,1,1-trifluorotetradecan-2-one (TFT) was found to be a highly effective inhibitor of JHE (14). The placement of a sulfur atom β to the carbonyl significantly increased the inhibitory activity, possibly by mimicking the α,β double bond of the natural JHs. 3-Octylthio-1,1,1-trifluoropropan-2-one (OTFP), was the most active JHE inhibitor of these trifluoropropanone sulfides (5). In a recent report, OTFP derivatives methylated on the carbon atoms adjacent to the sulfur showed elevated inhibitory activity against JHE (8).

In the present study we have synthesized a new group of trifluoropropanones and tested them against insect JHE from the larval hemolymph of *Trichoplusia ni* (cabbage looper), electric eel AChE, porcine carboxylesterase, and yeast lipase.

MATERIALS AND METHODS

Synthesis of inhibitors. The title compounds were synthesized by the reaction of the appropriate alkanedithiol with BTFA (5) (Fig. 1). A typical synthesis is outlined below. 1,6-Hexanedithiol (0.46 ml, 3 mmol) was dissolved in 8 ml dry CCl_4 under N_2 and BTFA (0.58 ml, 6.6 mmol) was added through a rubber septum via syringe. The stirring mixture was cooled in an ice bath and triethylamine (0.92 ml, 6.6 mmol) was added via syringe over 15 min. The reaction was monitored by TLC on silica gel plates (Whatman, 25-μm layer) using a hexane/acetone (7:1) mixture as a solvent system. The spots were detected in an iodine chamber, where the thiol compounds were readily visualized by a characteristic whitening. The starting dithiol had the highest R_f (0.93) and disappeared as two spots formed at R_f 0.45 (bis-trifluoromethyl ketone) and 0.76 (mercaptoalkyl-trifluoromethyl ketone). The latter spot usually disappeared after stirring the mixture overnight at room temperature. The precipitated triethylamine hydrobromide was filtered off and washed with ether. The combined organic phase was washed with 3 × 15 ml of water, dried over anhydrous Na_2SO_4, and evaporated to give the product with 89% isolated yield. ir (liquid film) cm^{-1}: ν OH 3370, ν CH_2,as 2933, ν CH_2,s 2859, ν CO 1747, δ CH_2,s 1365, ν CF_3 1188; NMR ($CDCl_3$) ppm: δ 1.4 (c,$(CH_2)_n$); 2.3 (t,$R\underline{CH_2}SCH_2$); 2.9 (m,$RCH_2S\underline{CH_2}CO$). Anal. Calcd for $C_{12}H_{16}F_6O_2S_2$: C, 38.91; H, 4.35. Found: C, 38.6; H, 4.4. The product can be named either 1,6-bis(2-oxo-3,3,3-trifluoropropylthio)hexane or 1,1,1,16,16,16-hexafluoro-4,13-dithiahexadecane-2,15-dione.

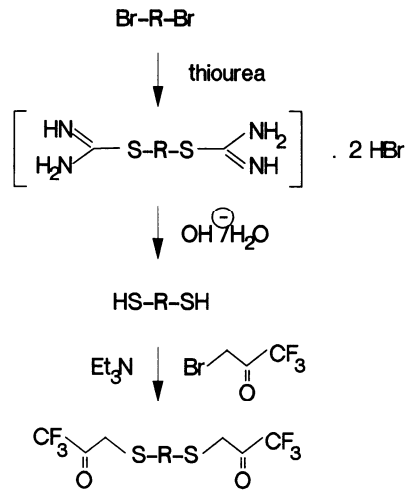

FIG. 1. *Reaction scheme for the preparation of α,α′-alkanebis(thiotrifluoropropanones).*

The ir and NMR spectra of the compounds synthesized were very similar: ir (liquid film or $CHCl_3$ solution) cm^{-1}: ν OH 3275–3425, ν CH_2,as 2920–2945, ν CH_2,s 2850–2865, ν CO 1705–1755, δ CH_2,s 1340–1365, ν CF_3 1165–1195; NMR ($CDCl_3$) ppm: δ CH_3,$(CH_2)_n$: 1.2–1.7; R–$\underline{CH_2}$–S–CH_2: 2.1–2.3; R–CH_2–S–$\underline{CH_2}$CO: 2.5–3.0; CH_2–O–CH_2: 3.6.

The starting mercaptans were purchased from Aldrich Chemicals or synthesized from the appropriate α,α′-dihaloalkane via the thiourea method (15). Thiourea (0.50 g, 6.6 mmol) was dissolved in 4 ml refluxing ethanol and 1,10-dibromodecane (0.90 g, 3 mmol) was added. The mixture was refluxed for about 4 hr and allowed to stand overnight. The white precipitate that formed was filtered off and washed twice

with ether and once with ethanol to give the appropriate bis-isothiuronium salt in a quantitative yield. The solid was suspended in 4 ml of water containing 0.36 g (9 mmol) NaOH and refluxed under N_2 for at least 5 hr. After cooling, benzene (5 ml) was added. The organic layer was collected, and the aqueous layer was acidified with 40% H_2SO_4 and extracted several times with ether. The combined organic phase was washed with water, dried over Na_2SO_4, and evaporated giving the product with 89% isolated yield. NMR ($CDCl_3$) ppm: δ 1.4 (c,$(CH_2)_n$); 2.5 (m,RCH_2S). Anal. Calcd for $C_{10}H_{22}S_2$: C, 58.19; H, 10.74. Found: C, 58.3; H, 10.7.

Analysis and purification. Infrared spectra for each compound were obtained on a Perkin–Elmer Model 337 run as a thin film or in $CHCl_3$ solution on sodium chloride plates. Proton magnetic resonance (^1H NMR) spectra were obtained on a Varian EM-390 (90 MHz) in a dilute solution of carbon tetrachloride or deuterated chloroform ($CDCl_3$), with or without deuterium oxide. Tetramethylsilane (TMS) was used as internal standard. Chemical shifts are reported in parts per million (ppm) relative to TMS. Trifluoromethyl ketone compounds were examined in the hydrated form. To obtain the elemental composition for C and H, combustion analyses were also obtained. Elemental analyses were carried out at the Berkeley Analytical Laboratory (University of California at Berkeley).

The compounds were purified by preparative thin-layer chromatography on 2000-μm silica gel plates (Analtech), or flash chromatography on silica gel (40 μm average particle size, J. T. Baker) using a hexane/acetone gradient. Analytical TLC was performed on silica gel F_{254} plates (Whatman). The compounds were barely detectable as spots which quenched the uv fluorescence of the gel at 254 nm because they lack chromophoric functionalities. However, they could be readily detected in an iodine chamber or by spraying the plate with Dräggendorff reagent (methanol/1:1 HCl solution saturated with iodine and potassium iodide). TLC plates were developed in hexane/acetone (7:1) or benzene/ethyl acetate (9:1) solvent systems (the latter system was avoided as much as possible, because of the carcinogenicity of benzene). Where possible, the purity of the products was also evaluated by GC 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.

Enzyme assays. To measure the *in vitro* inhibition of the compounds on the esterases, both endpoint 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 endpoint assay for JHE a 10-min incubation time was used. In the other three assays, esterase activity was monitored within the first 10–20 min, during which the time—activity relationships were found to be linear, and the time vs activity slopes of the inhibited samples were compared to that of the uninhibited enzyme solutions. The enzyme assays were run in triplicate.

For the determination of the juvenile hormone esterase (JHE) inhibition by the title compounds the radiometric partition method (16) was used. Hemolymph from Day 2 of fifth instar (L_5D_2) *T. ni* larvae was diluted 1:500 with 0.08 *M* phosphate buffer, pH 7.4, with 0.1% phenylthiourea to inhibit tyrosinases and assayed for JHE activity. In L_5D_2 larvae, the titer of JHE is at a maximum (17–19). A mixture of radiolabeled [10-^{3}H]JH III (New England Nuclear) and unlabeled JH III (Calbiochem) was used as substrate in ethanol to give a final concentration of 5×10^{-6} *M* JH III containing ~30,000 dpm/assay. 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 (AChE) analyses were performed using lyophilized enzyme

from electric eel (EC 3.1.1.7) (Sigma) in 0.05 *M* phosphate buffer (pH 7.4) at 20 μg/ml concentration. Acetylthiocholine iodide was used as a substrate at a final concentration of 5×10^{-4} *M*. The assay was run at 37°C. 5,5′-Dithiobis-(2-nitrobenzoic acid) at a final concentration of 3.8×10^{-2} *M* was used to monitor the released thiocholine according to a published procedure (20) with slight modifications. Acetone was used as a solvent for the inhibitors.

For measuring the inhibition of malathion esterase (malathionase, ME), general carboxylesterase from porcine liver (EC 3.1.1.1) (Sigma) was used at a final concentration of 16 μg protein/ml in 0.1 *M* Tris–HCl buffer (pH 7.5). Malathionase activity was measured by coupling the hydrolysis of malathion to the reduction of a tetrazolium dye (21). An acetone solution of malathion was used as substrate at a final concentration of 3×10^{-4} *M*.

Lipase (LP) assays were performed using enzyme from *Candida cylindracea* (EC 3.1.1.3) (Type VII; Sigma) in 0.1 *M* Tris-HCl buffer (pH 7.0) at a final enzyme concentration of 50 μg/ml. *p*-Nitrophenyl acetate in acetone was used as a substrate to give the final substrate concentration of 1.35 m*M*. The formation of *p*-nitrophenol was monitored kinetically for 10 min.

In the AChE, ME, and LP assays the rates of hydrolysis of the substrates were detected spectrophotometrically using Titertek multiscan or V_{max} 96-well microplate readers; the absorbances were detected at wavelengths of 405 nm (AChE, LP) and 492 nm (ME). Statistical variabilities among each assay test were compared to each other using the r^2 values of the linear regression equations used for the calculation of the pI_{50} values of the compounds against the four enzymes used. These values were no lower than 0.923, 0.940, 0.961, and 0.907 for JHE, AChE, ME, and LP assays, respectively.

In vivo assays. Two compounds were tested *in vivo* for their ability to delay pupation. The compounds were applied topically to L_5D_1 and L_5D_2 larvae of *T. ni*. One or two microliters of the 0.1 *M* solution of the inhibitors in ethanol was applied to each larvae at 4, 12, and 17 hr after lights on and the time of pupation was recorded. Control larvae were treated with 2 μl of ethanol. The insects were reared at 27°C under a 14 hr light:10 hr dark controlled photoperiod. The time of pupation for each treatment group was compared to that of the control group.

RESULTS AND DISCUSSION

Preparation of dithiols. The thiourea reaction proved to be successful for the synthesis of the primary thiols: yields for the formation of the isothiuronium salts were quantitative and the overall yield was always higher than 80%. In general, the intermediate salts readily crystallized. If not, the crystallization was promoted by cooling or partial evaporation. Analysis of the compounds by TLC and NMR indicated that the products were clean enough to continue the synthesis without further purification.

In the case of the secondary dithiols XIII and XIV, the reaction proceeded with significantly lower yields (32 and 43%) and required much longer reaction times (14 hr). The isolation and purification of the intermediate isothiuronium salts was more difficult because these products were viscous, tar-like semisolids instead of the crystals obtained for the primary compounds. Efforts to synthesize a number of unsaturated derivatives such as 2-butene-1,4-dithiol and 2-butyne-1,4,-dithiol via the same thiourea method were unsuccessful.

Synthesis of hexafluorodiketones. Fourteen new inhibitors of the general structures A and B were synthesized.

$CF_3C(O)CH_2S-(CH_2)_n-SCH_2C(O)CF_3$	A
n: 3–12	compounds I–X
$CF_3C(O)CH_2S-R-SCH_2C(O)CF_3$	B
R: $-(CH_2)_2-O-(CH_2)_2-$	compound XI
$-(CH_2)_2-S-(CH_2)_2-$	compound XII
$-CH(CH_3)(CH_2)_2-$	compound XIII
$-CH(CH_3)(CH_2)_3-$	compound XIV

Addition of triethylamine (TEA) to the reaction mixture in carbon tetrachloride as a solvent considerably accelerated the reaction. However, because the reaction of BTFA in the presence of TEA is highly exothermic, the rate at which the latter reactant was added was crucial to the final purity of the product. In addition, a slight excess of BTFA promoted the reaction, but in that case the reaction mixture must be washed extensively to remove BTFA. The final purity of the products was generally suitable for enzyme assays without further purification. Where necessary, preparative TLC in benzene/ethyl acetate (9:1) or hexane/acetone (7:1) was used for purification.

The overall yield was between 59 (III) and 98% (V), generally higher than 85% and greatly dependent on how fast the amine base was added. Using chloroform as a solvent increased the yield. However, because the solubility of triethylamine hydrobromide is much higher in this solvent and because $CHCl_3$ gives a very stable emulsion with water, it considerably complicated the workup.

In the case of β-dithiols (1,2-ethanedithiol, 1,2-propanedithiol), the reaction was incomplete and a very complex product mixture resulted with an apparent thiol spot remaining on TLC. These compounds were purified by preparative TLC. According to the NMR analysis, the products contained primarily 1,4-dithiane or 1,4-thioxane derivatives: cyclic (thio)hemiketals formed by the condensation of the carbonyl and thiol groups. (T. Schierling, University of California at Davis, personal communication).

Enzyme inhibition. Summarized in Table 1 are the I_{50} values for α,α′-alkanebis(thiotrifluoroketones) against JHE, AChE, malathionase, and lipase. All of the compounds tested showed much higher inhibitory activity against JHE than against the other three enzymes. In fact, the molar I_{50} values against the former enzyme were even lower than the K_m value reported for

TABLE 1
Inhibition of Esterases by α,α′-Alkanebis(thiotrifluoropropanones) and OTFP

Compound	Number of carbons	Molar I_{50} value[a] (*M*)			
		JHE[b]	AChE[c]	ME[c]	LP[c]
I	3	9.87×10^{-7}	1.14×10^{-5}	3.63×10^{-6}	—
II	4	3.06×10^{-9}	8.44×10^{-6}	3.79×10^{-6}	—
III	5	7.00×10^{-9}	4.30×10^{-6}	2.38×10^{-6}	—
IV	6	1.50×10^{-8}	2.98×10^{-6}	9.02×10^{-7}	1.58×10^{-6}
V[d]	7	1.68×10^{-8}	2.98×10^{-6}	4.89×10^{-6}	1.19×10^{-6}
VI[d]	8	8.17×10^{-10}	7.78×10^{-6}	4.62×10^{-6}	—
VII	9	2.67×10^{-9}	1.34×10^{-5}	5.00×10^{-4}	1.70×10^{-6}
VIII[d]	10	4.54×10^{-9}	4.35×10^{-6}	1.66×10^{-5}	1.29×10^{-6}
IX[d]	11	1.33×10^{-8}	5.25×10^{-6}	1.03×10^{-3}	4.42×10^{-6}
X[d]	12	8.15×10^{-9}	1.03×10^{-5}	3.02×10^{-3}	1.35×10^{-6}
XI	2-O-2	2.16×10^{-8}	6.01×10^{-6}	8.58×10^{-6}	—
XII	2-S-2	4.94×10^{-9}	7.12×10^{-6}	3.53×10^{-6}	—
XIII[d]	3′	4.68×10^{-7}	3.47×10^{-6}	4.56×10^{-6}	2.15×10^{-6}
XIV[d]	4′	8.20×10^{-9}	8.12×10^{-7}	5.22×10^{-5}	1.96×10^{-6}
OTFP[e]		1.81×10^{-9}	5.21×10^{-6}	4.31×10^{-6}	3.25×10^{-7}

[a] JH III was used as a substrate for JHE from *T. ni*, acetylthiocholine iodide for electric eel AChE, malathion for carboxylesterase from porcine liver, and 4-nitrophenyl acetate for lipase from *Candida cylindracea*.

[b] Determined in an endpoint assay after 10 min incubation time.

[c] Kinetic assays: the I_{50}'s were determined from the slopes of uninhibited and inhibited enzyme preparations within the first 10–20 min.

[d] The dithiol was prepared from alkyl-dibromide.

[e] Reported molar I_{50} values for the inhibition of JHE: 2.3×10^{-9} *M* (5), 3.1×10^{-9} *M* (8); for the inhibition of AChE: 2.3×10^{-6} *M* (5), 4.7×10^{-6} *M* (8); for the inhibition of ME: 2.5×10^{-6} *M* (13).

JH III (22), showing that the compounds in the present series bound to JHE tighter than the substrate JH III itself. This result is consistent with the theory that trifluoromethyl ketones act as transition state analogs (23) of the natural substrates of JHE (juvenile hormones). Also, this theory is supported by the finding that using JH III as a substrate, alkylthiotrifluoropropanones show a much higher and selective inhibition on JHE than on porcine carboxylesterase (Abdel-Aal, unpublished). The structure–activity relationship (SAR) for the trifluoromethyl ketone inhibition of JHE can be interpreted in light of its suspected natural substrate JH. In contrast, porcine carboxylesterase yet has no known natural substrate so the data are difficult to interpret.

Since OTFP is the most characterized member of the trifluoromethyl ketone sulfide inhibitors and is regularly used for the affinity chromatography purification of JHE (24), we have determined the molar I_{50} values of this compound for all four enzymes as shown in Table 1. The measured values were similar to the ones reported earlier in the cases of JHE and AChE (5, 8) and ME (13). Inhibition of lipase by trifluoromethyl ketones is shown for the first time in this paper. For this enzyme OTFP was found to be the most active whereas in the case of the other three esterases its I_{50} values were in the same range as α,α′-alkanebis(thiotrifluoroketones) of the same molecular size.

Although the inhibition of JHE is much higher than the other three enzymes, the slopes of the inhibition curves were lower for JHE than for AChE and ME in most cases. Malathionase inhibition had the highest slopes, except for compounds III and IV, which were higher for AChE inhibition. The slopes of the inhibition of lipase were in the same range or lower than for that of JHE. In some cases, a clear break was observed in the inhibition curves for the present series of compounds and for some aryl thiotrifluoropropanones tested against JHE from *T. ni* in an earlier study (5). This type of curve was observed for seven trifluoropropanone sulfides of the present series and some aromatic compounds. Figure 2 shows three of these cases. As seen in Fig. 2, there is a decrease in the slope of the inhibition curves above 65–70% inhibition. The lower slopes for JHE inhibition possibly can be explained by the heterogeneity of the catalytic sites that differ in their sensitivity to the inhibitor. The presence of multiple iso-forms of JHE in the hemolymph from *T ni* (25–27), from *Diatraea saccharalis* (sugar cane borer) (28), from *Manduca sexta* (tobacco hornworm) (29), and from *Heliothis zea* (corn earworm) (30) has been discussed previously. In the present study, the inflexion of the inhibition curve observed at approximately 70% inhibition (Fig. 2) is in agreement with the integrated areas of JHE activity detected in high-resolution IEF studies of *T. ni* JHE (27) and with the similar observation for alkyl derivatives of OTFP substituted α and α′ relative to the sulfur atom (8). The data in this study support the concept of multiple catalytic sites, but they do not distinguish between the sites being on the same or different proteins.

The empirical structure–activity correlations for the title compounds are different for each enzyme tested. In previous studies (5, 31) the inhibitory activities of trifluoromethyl ketones were compared to their molar refractivities. However, because the calculated molar refractivities are additive properties of the atoms of the actual molecule, in the case of homolog series of aliphatic compounds, a facile alternative approach for the qualitative SAR study is to relate the inhibitory activities (pI_{50}) simply to the number of carbon (or hetero-) atoms in the aliphatic chain. For inhibition of JHE, the data showed similar SAR (Fig. 3A) as the 3-alkylthio-1,1,1-trifluoropropan-2-ones (Fig. 3B) reported earlier (5). Optimum inhibition appears in both studies with increased size of the inhibitor molecules. In the present study, however, two peaks of inhibition were observed: a

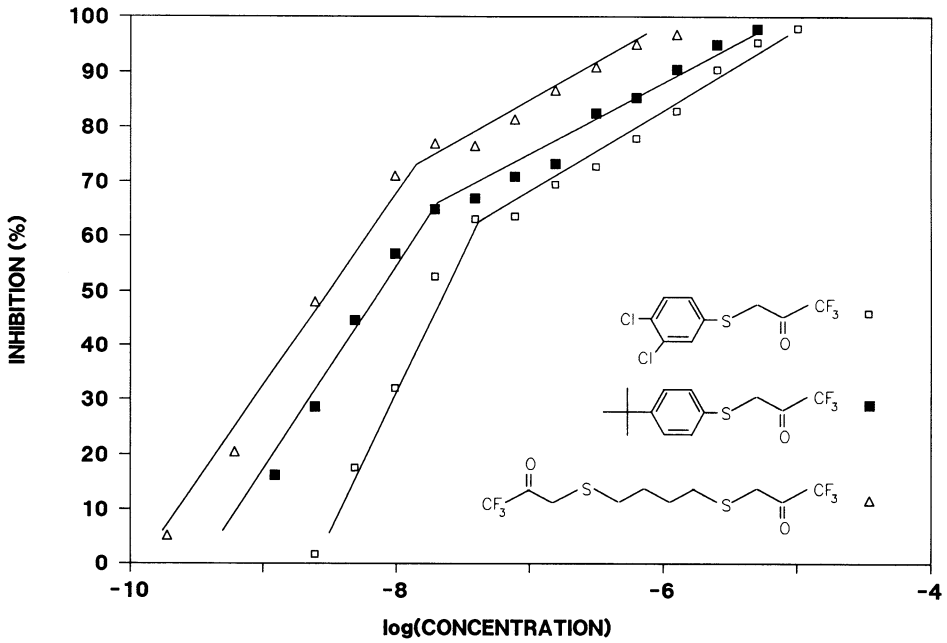

FIG. 2. *Plots of percentage inhibition of T. ni larval hemolymph JHE against log molar concentration of some aliphatic and aromatic trifluoromethylketones: 1,1,1,12,12,12-hexafluoro-4,9-dithiadodecane-2,11-dione* (△), *3-(4-(1,1-dimethylethyl)phenyl)thio-1,1,1-trifluoro-2-propanone* (■), *and 3-(3,4-dichlorophenyl)thio-1,1,1-trifluoro-2-propanone* (□). *The inhibition curves indicate the presence of at least two enzymes or two catalytic sites with different sensitivity to the inhibitors.*

local optimum and a global optimum occur at carbon numbers four and eight, respectively. (In the case of compounds **XI–XIV**, we considered the appropriate atom numbers to be 5, 5, 3 and 4, respectively.) It is worth noting that the number of carbons in the alkyl chain are not directly comparable in Figs. 3A and 3B because the title compounds, α,α′-alkanebis(thiotrifluoroketones), are four atoms "longer" (considering the extra CF_3COCH_2S– moiety). Therefore, the appropriate carbon numbers should be corrected by four. With this correction, the first peak of inhibitory activity in Fig. 3A is equivalent to the peak activity observed previously for OTFP in Fig. 3B. Although this first peak at a chain length of four carbons is not a strong one, the decrease in inhibition between chain length four and seven carbons seems to be significant for at least two reasons. First, there are four datum points observed in this interval and the standard error of the pI_{50} values is very low. Second, the pattern of inhibition from chain length three to seven carbons appears to be the same as in Fig. 3B. Therefore the curve on Fig. 3A can be interpreted as a superposition of two curves of the type in Fig. 3B. This suggests that after reaching a particular size, the title compounds might be able to interact concurrently with two catalytic sites on the same or different enzymes. Another possible explanation for the presence of a second inhibition maximum is that the second polarized carbonyl group of the inhibitor interacts with the enzyme on another highly hydrophilic part with no catalytic activity. Based on the chemical bond distances and angles reported ((32) and references therein), the approximate distance of this functionality from the active site is no more than 1.69 nm (the maximal distance between the two carbonyl groups in compound **VI**), stabilized by the primary or secondary structure of the enzyme.

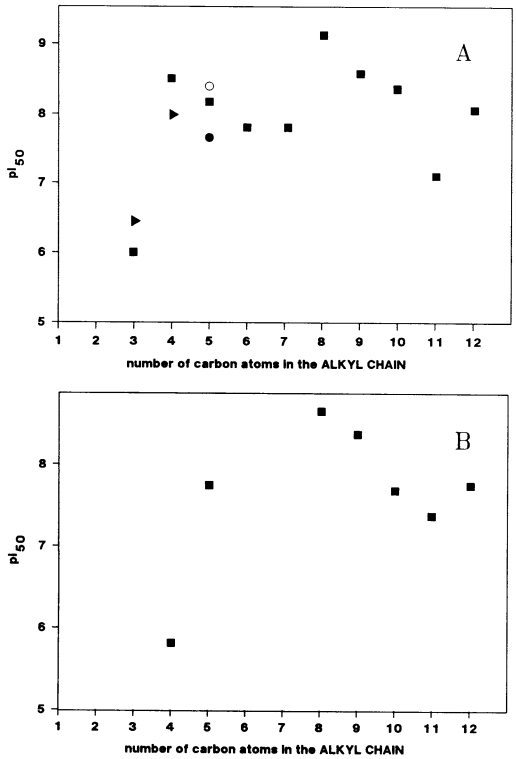

FIG. 3. *Relationship between the inhibitory activity of α,α′-alkanebis(thiotrifluoropropanones) (A) [compounds I–X (■), compound XI (●), compound XII (○), and compounds XIII and XIV (▲)] and α-alkylthiotrifluoropropanones (B) against JH esterase from Tricoplusia ni and the carbon number in the alkylene chain of these compounds.*

The presence of a heteroatom in the alkyl chain slightly modifies the inhibitory activity, with a decrease in activity for the oxygen derivative (XI) and increase for the sulfur derivative (XII) when compared to the appropriate member of the homolog series (III). In addition, methyl substitution on the carbon atom of the aliphatic chain adjacent to the sulfur led to a slight increase in activity for compound XIII and a slight decrease for compound XIV when compared to the appropriate nonmethylated compounds (I and II, respectively).

In contrast to the JHE inhibition, cholinesterase and lipase did not respond in an apparently systematic fashion to the structural variation. Malathionase showed intermediate response with a generally decreasing potency as a function of the number of carbon atoms. In some cases, whose I_{50} values are not reported in Table 1, activation of the lipase enzyme was observed at lower concentrations of the inhibitor. This activation was similar to that of carboxylesterase as reported earlier (13). The details of this type of activation of lipase enzyme will be discussed elsewhere.

Kinetics of inhibition. In order to clarify the mechanism of inhibition for esterases, the time dependence of the inhibition process was evaluated for some of the tested compounds. For this purpose, enzyme assays were performed as for the I_{50} determinations but the preincubation times were varied from 0 to 20 min.

Compounds I and VI were chosen for the kinetic analysis because of their significantly different activities on JHE and similar activities on AChE. The slopes of the inhibition curves for JHE and AChE using these compounds are similar, 56.2 and 54.8 for compound I and 48.9 and 58.0 for compound VI, respectively (Fig. 4), which means that the difference between the minimal concentration causing complete inhibition of the enzyme and the maximal concentration showing no significant effect does not exceed two orders of magnitude.

The inhibition of JHE by aliphatic trifluoromethyl ketones has been found to be reversible. However, inhibition of JHE by trifluoropropanone sulfides (OTFP), in contrast to compounds lacking the thioether moiety (TFT), showed clear time dependence within the first 4–10 min of preincubation time (17). It is not surprising then, that JHE inhibition by the α,α′-alkanebis(thiotrifluoropropanones) also was dependent on the time of preincubation. This indicates that the inhibition of JHE by these compounds follows pseudo-first-order kinetics, which can be due to slow dissociation of the enzyme–inhibitor complex as a rate limiting step for the equilibrium and/or the tight binding of the inhibitor to the enzyme proceeds in two steps: a

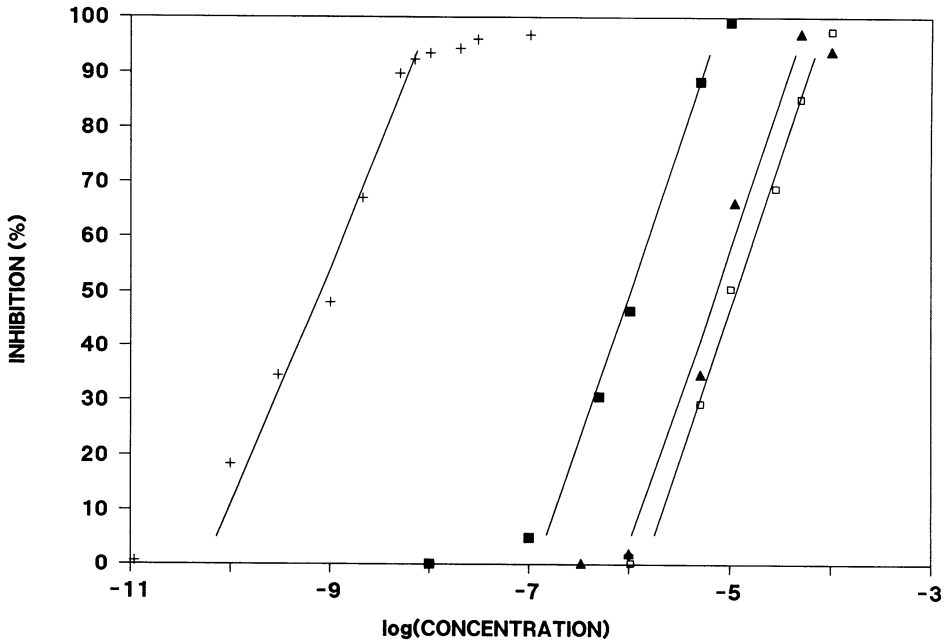

FIG. 4. *Comparison of inhibition of JHE and AChE by compounds I and VI: compound I against JHE* (■) *and AChE* (□), *compound VI against JHE* (+) *and AChE* (▲).

fast complex formation stabilized by H-bonds and a second, slow and first-order step of forming a covalent enzyme–inhibitor complex. Deprotonation of the geminal diol at the presumably active-site serine might also be involved in the stabilization of the *E–I* complex (11) and make the compound behave as a pseudo-irreversible inhibitor and follow saturation kinetics.

The inhibition velocity is a linear function of the concentration of the enzyme and the inhibitor,

$$-dE/dt = k_2 [I] [E]. \quad (1)$$

The integration of this equation results in

$$\ln(E_o/E) = k_2 [I] t. \quad (2)$$

Therefore, the logarithm of the residual activity is a linear function of time, with a slope of $-k_2 [I]$.

$$\ln(A/A_o) = -k_2 [I] t. \quad (3)$$

To illustrate this, Fig. 5 shows the time course of JHE inhibition by compounds I and VI at different concentrations, where the initial linear relationship between the logarithm of the percentage residual activity vs time is obvious. Slopes and correlation coefficients were calculated by linear regression, and k_2 values at different inhibitor concentrations were calculated from slopes below -0.1 min^{-1} and were averaged for each compound. The coefficients of determination (r^2) are between 0.969 and 0.989. The k_2 values are 1.03×10^5 M^{-1} min^{-1} for compound I (an average of data calculated at two concentrations, 5×10^{-6} M and 1×10^{-6} M) and 2.12×10^7 M^{-1} min^{-1} for compound VI (an average of data calculated at four concentrations, 1×10^{-7} M, 3×10^{-8} M, 1×10^{-8} M, and 7×10^{-9} M), respectively. Agreement was obtained between the experimental I_{50} values and those values calculated from the relationship between $[I]$ and k_2 (33) when $A_o/A = 2$ at 10 min preincubation time (Table 2):

$$I_{50} = \ln 2/(k_2 \cdot t). \quad (4)$$

It was stated by Brodbeck *et al.* (9) that

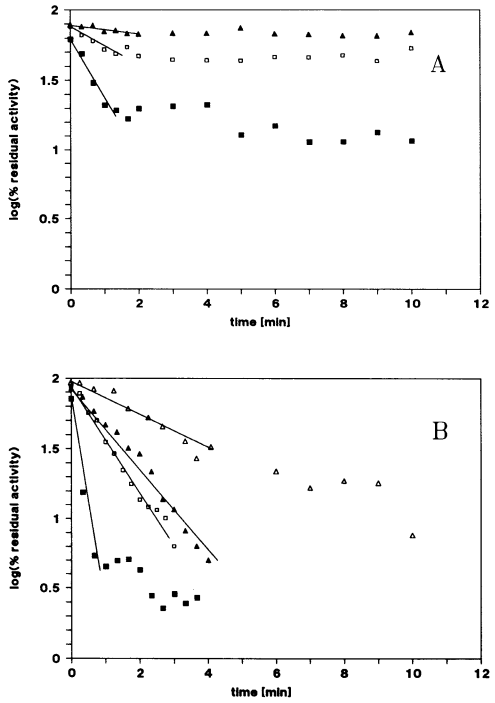

FIG. 5. *Time dependence of inhibition of JHE by compounds I (A) and VI (B) at different concentrations. (A) 5 × 10⁻⁶ M (■), 1 × 10⁻⁶ M (□), and 5 × 10⁻⁷ M (▲); (B) 1 × 10⁻⁷ M (■), 3 × 10⁻⁸ M (□), 1 × 10⁻⁸ M (▲), and 7 × 10⁻⁹ M (△).*

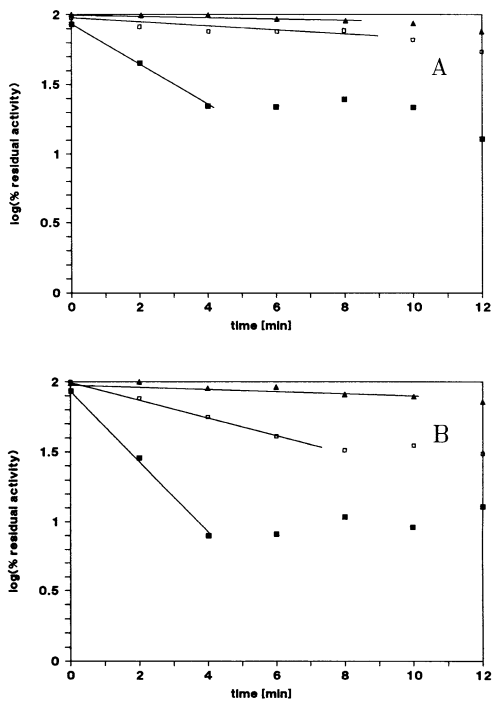

FIG. 6. *Time dependence of inhibition of AChE by compounds I (A) and VI (B) at concentrations of 5 × 10⁻⁵ M (■), 1 × 10⁻⁵ M (□), and 5 × 10⁻⁶ M (▲).*

"compounds that in aqueous solution readily form hydrates inhibit acetylcholinesterase in a time-dependent process." In the present study, inhibition of AChE by compounds I and VI was found to be time-dependent within the first 4 min (Fig. 6). The coefficients of determination are between 0.974 and 0.991. The k_2 values calculated as for the JHE inhibition are 3.82 × 10^3 M^{-1} min^{-1} for compound I (calculated from data at 5 × $10^{-5}M$ concentration) and 6.27 × 10^3 M^{-1} for compound VI (an average of data calculated at two concentrations, 5 × 10^{-5} M and 1 × 10^{-5} M). The calculated I_{50} values (Eq. (4)) are in agreement with the values measured (Table 2).

The time-dependent process of inhibition of AChE can be explained by the hypothetical mechanism of inhibition. Acetylcho-

TABLE 2

Molar I_{50} Values of Compounds I *and* VI *against Insect Juvenile Hormone Esterase and Acetylcholinesterase*

	Molar I_{50} value (M)			
	JHE		AChE	
Compound	Measured[a]	Calculated[b]	Measured[a]	Calculated[b]
I	9.87 × 10^{-7}	8.54 × 10^{-7}	1.14 × 10^{-5}	1.81 × 10^{-5}
VI	8.17 × 10^{-10}	5.60 × 10^{-9}	7.78 × 10^{-6}	1.12 × 10^{-5}

[a] Measured for the 10 min preincubation inhibition curves.

[b] Calculated from the second-order rate constant of the inhibition reaction when t = 10 min according to Eq. (4) in the text.

linesterase (acetylcholine hydrolase, EC 3.1.1.7) is a serine esterase enzyme, containing a serine residue necessary for catalytic activity in its active site. Aldehydes and ketones interact with the enzyme by forming hemiacetals and hemiketals with this serine. Since, however, in aqueous solution trifluoromethyl ketones predominantly exist in a hydrated form, we propose that the inhibition of the enzyme proceeds in two consecutive steps: H-bonded complex formation with the enzyme which results in the dehydration of the ketone hydrate in the highly hydrophobic environment of the enzyme, and carbonyl addition to the enzyme through the formation of a stable, covalent hemiketal at the active site. Inhibition of the enzyme directly by the small proportion of the carbonyl form of the inhibitor present in aqueous solution makes only a small contribution to the overall inhibition of esterases and proteases by these potent compounds. However, as we consider compounds that bind less tightly to the enzyme, enzymatic dehydration of the inhibitor from the diol to the carbonyl form becomes less significant and direct inhibition by the carbonyl form in aqueous solution becomes relatively more significant.

Although the architecture of the JHE active site is only speculative, there is evidence that supports the proposition that a serine residue is essential for catalysis in the active site unit (Abdel-Aal and Hammock, unpublished), therefore one would expect some similarities in the behavior of the title inhibitors in their final destination on the nucleophile serine hydroxyl. The fact that the characteristics of enzyme inhibition by α,α'-alkanebis(thiotrifluoropropanones) was the same for both, AChE and JHE, can contribute to the supposition that JHE is, in fact, also a serine esterase.

In vivo assays. Using the irreversible inhibitor EPPAT, Sparks and Hammock (6) demonstrated that JHE could be inhibited *in vivo* and pupation could be delayed in *T. ni,* presumably by maintaining an abnormally high level of JH and preventing release of the prothoracicotropic hormone to initiate metamorphosis. TFT failed to have this effect while OTFP was effective in inhibiting JHE and delaying pupation when repeated doses of 0.2 μmol were applied topically to larvae on L_5D_1 and L_5D_2 (4, 5).

In order to provide a comparison between the *in vivo* activity of the two most effective *in vitro* compounds from the new series (II and VI), and the activity of OTFP, the three compounds were screened for their ability to delay pupation in *T. ni* using the previously described protocol (4). In the control group, larvae treated with 2 μl of ethanol, 90–100% of larvae had pupated by L_5D_5 9 AM. Treatment with 0.2 μmol of VI on L_5D_1 and L_5D_2 was as effective as OTFP in delaying pupation—only 45–75% of the larvae pupated by L_5D_5 in each case. Treatment with compound II did not result in a significant delay in the time of pupation—70–100% pupating by L_5D_5.

The observed *in vivo* activity demonstrated by these compounds does provide an important and interesting comparison to the *in vitro* activity. However, the lipophilicity of each compound influences its penetration through the cuticle, and will influence the biological activity. Compound II is much more water soluble than VI and this may effect penetration. These data emphasize that *in vivo* activity often is difficult to predict from the *in vitro* data alone.

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