Rational Design and Synthesis of Polarized Ketones as Inhibitors of Juvenile Hormone Esterase: Importance of Juvenile Hormone Mimicry

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A series of β,β-disubstituted α,β-unsaturated and β-substituted α-acetylenic trifluoromethyl ketones were prepared and assayed as inhibitors of juvenile hormone (JH) esterase and α-naphthyl acetate esterase from the cabbage looper, *Trichoplusia ni*, and of electric eel acetylcholinesterase (AChE). The most potent inhibitor from each series had a molar refractivity similar to that of the natural JH esterase substrate. The unsaturated fluoro ketones were less active than the analogous and structurally similar α-alkylthio-substituted trifluoropropanones, suggesting that mimicry of the α,β-unsaturation of JH was not a critical feature in inhibition. The structurally dissimilar acetylenic compounds were more potent inhibitors than the unsaturated fluoro ketones but had even greater activity toward AChE. Studies with several α- and α′-substituted derivatives of 3-(octylthio)-1,1,1-trifluoropropan-2-one (OTFP) demonstrated the importance of mimicry of the C-3 methyl substituent of JH in inhibition and α′-Me-OTFP emerged as the most potent inhibitor of JH esterase available. The role of the S atom in OTFP inhibition of JH esterase is discussed.

The insect juvenile hormones are methyl esters of farnesoic acid 10,11-epoxide (JH III); 11-ethyl (JH II), 7,11-diethyl (JH I), or 3,7,11-triethyl (JH 0) methyl farnesoate 10,11-epoxide; and 4-methyl JH I (iso JH 0) (Figure 1). These hormones are of interest because of their regulatory function in insect embryogenesis, larval growth, metamorphosis, reproduction, hibernation, migration, and metabolism. The specific hormone present varies among species and stages of development. Correlated with a decrease in JH levels in insects is an increase in the rate of JH degradation, and it is believed that degradation by specific, hemolymph JH esterases along with changes in the rate of JH biosynthesis is responsible for the regulation of JH titer and insect development. In last-instar cabbage loopers, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), hemolymph ester hydrolysis of JH is attributed to at least two closely related enzymes (Jones et al., 1986) that likely differ by their degree of glycosylation (Hanzlik and Hammock, 1987). Although there are differences in the substrate affinity between these forms (Jones et al., 1986), these differences are rarely detectable in the analysis of esteratic inhibition.

Interest in the selective inhibition of JH esterase to more accurately define its biological role in insect development has resulted in several structure/activity studies to define the critical interactions between polarized ketones and the active site of this esterase. Hammock et al. (1982) were the first to report that aliphatic trifluoromethyl ketones, like that of TFT (1,1,1-trifluorotetradecan-2-one; Figure 1), were potent inhibitors of JH esterase and hypothesized that the electrophilic carbonyl moiety formed a tetrahedral adduct with the theoretical serine hydroxyl of JH esterase, thus mimicking the transition state in JH metabolism. It was interesting that the most effective inhibitor of those studied (TFT) closely mimicked the backbone of JH as illustrated in Figure 1. Surprisingly, the substitution of a sulfur atom for the C(4) methylene of TFT ultimately led to a second series of JH esterase inhibitors of even greater potency (Hammock et al., 1984). OTFP (3-(octylthio)-1,1,1-trifluoropropan-2-one; Figure 1) emerged as the best inhibitor of this series. The most potent inhibitor of both the TFT and OTFP series (compare Figures 1 and 2) demonstrated a single, optimum molar refractivity similar to that for JH II, the natural substrate for the larval stage. Attempts by Prestwich et al. (1984) to increase the inhibitor potency of OTFP by mimicking the alkyl substitutions of JH at C-7 and C-11 and the C-6 unsaturation (DNTFP or 3-[((*E*)-4,8-dimethyl-3,7-nonadienyl)thio]-1,1,1-trifluoropropan-2-one; Figure 1) did not lead to any further improvements. Linderman et al. (1988) have shown by high-field ^{19}F NMR that the polarized ketone of OTFP in water is almost exclusively hydrated and in the active site of a model esterase, like that of electric eel acetylcholinesterase (AChE), is in a tetrahedral form. These data support the hypothesis of Hammock et al. (1982) that these compounds were transition-state analogue inhibitors. Székács et al. (1989) have synthesized a series of α,α′-bis[(2-oxo-3,3,3-trifluoropropyl)thio]alkanes 2.8 times more active than OTFP. Trifluoromethyl ketones are for the most part potent inhibitors of JH esterase but are also reasonably selective when their activity is compared with that of insect α-naphthyl acetate (α-NA) esterase and electric eel acetylcholinesterase.

The addition of sulfur in the OTFP series increased the potency of these inhibitors over that of the TFT series for a given molar refractivity value, by approximately 2 orders of magnitude (Figure 2). Hammock et al. (1984) hypothesized that the increased potency of the OTFP series was the result of the sulfur mimicking the α,β-unsaturation of JH. The function of the sulfur moiety is further examined here to consider steric constraints about the sulfur atom (Linderman et al., 1987) and the effect of introducing an α,β-unsaturation (alkene or alkyne) in the inhibitor as a mimic of the JH enoate structure (Lin-

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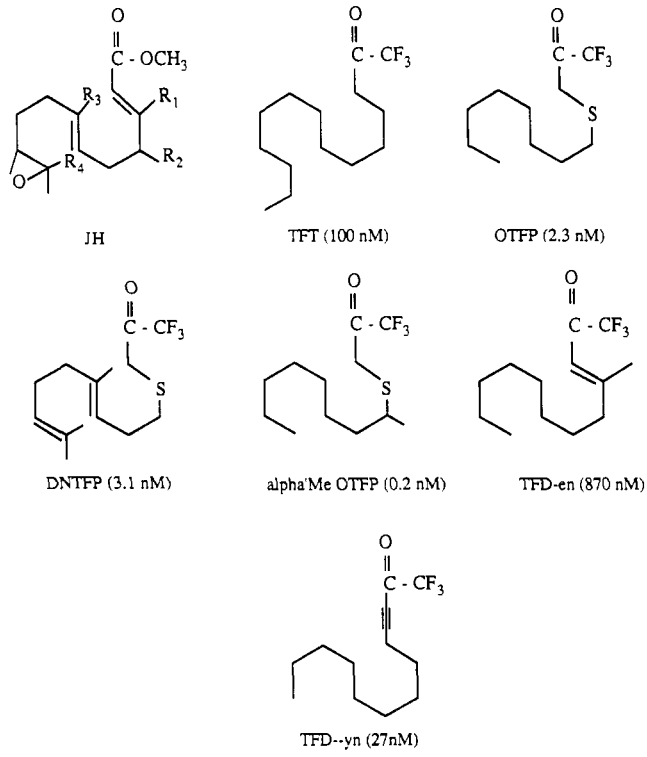


Figure 1. Juvenile hormone (JH 0, $R_1 = R_3 = R_4 = C_2H_5$, $R_2 = H$; iso JH 0, $R_1 = R_2 = CH_3$, $R_3 = R_4 = C_2H_5$; JH I, $R_1 = CH_3$, $R_2 = H$, $R_3 = R_4 = C_2H_5$; JH II, $R_1 = R_3 = CH_3$, $R_2 = H$, $R_4 = C_2H_5$; JH III, $R_1 = R_3 = R_4 = CH_3$, $R_2 = H$) and inhibitors of hemolymph juvenile hormone esterase from the cabbage looper, *T. ni*: TFT, 1,1,1-trifluorotetradecan-2-one; OTFP, 3-(octylthio)-1,1,1-trifluoropropan-2-one; DNTFP, 3-[((*E*)-4,8-dimethyl-3,7-nonadienyl)thio]-1,1,1-trifluoropropan-2-one; α'-Me-OTFP, 3-(2-nonylthio)-1,1,1-trifluoropropan-2-one; TFD-en, 4-methyl-1,1,1-trifluorododec-3(*Z*)-en-2-one; TFD-yn, 1,1,1-trifluorododec-3-yn-2-one. The concentration in parenthesis is the I_{50}, the inhibitor molar concentration necessary to inhibit 50% of the juvenile hormone esterase activity. The I_{50}'s for TFT, OTFP, and DNTFP were obtained from Hammock et al. (1982, 1984) and Prestwich et al. (1984), respectively. TFT, OTFP, TFD-en, and TFD-yn were the most potent inhibitors of the series identified by their names in Figure 2.

derman et al., 1989). In addition, structure/activity data are presented describing the importance of mimicry of the C-3 methyl substitution of JH (Linderman et al., 1987) and mimicry of the JH backbone in inhibitory potency. The function of the S atom in OTFP inhibition of JH esterase is discussed in light of this information.

MATERIALS AND METHODS

Insect Rearing and Hemolymph Collection. Larvae of *T. ni* were reared on an artificial diet (diet 1 of Roe et al. (1982)) at 27 ± 1 °C on a 14-h light–10-h darkness photoperiod. Prepupal hemolymph was collected from clipped thoracic legs into 6 × 50 mm culture tubes at 4 °C containing a few crystals of phenylthiourea (PTU) and centrifuged for 5 min at 1000*g*. The plasma was diluted (v/v) in a standard sodium phosphate buffer (0.2 M, pH 7.4, 10% sucrose, 0.01% PTU) and stored at −85 °C until used for enzyme assays. No loss of enzyme activity was observed due to freezing.

Enzyme Assays and I_{50}. Plasma JH esterase activity was assayed by the method of Hammock and Roe (1985). The substrate [C10-^{3}H]-JH III (New England Nuclear) was mixed with unlabeled JH III (Calbiochem) to obtain a final concentration of 5 μM when 1 μL of substrate in ethanol was mixed with 100 μL of plasma diluted 1/400 in standard buffer. α-NA esterase activity was measured according to the procedure of Sparks et al. (1979). A 1-mL portion of α-NA in standard buffer was mixed with 100 μL of 1/20-diluted plasma to obtain a final substrate

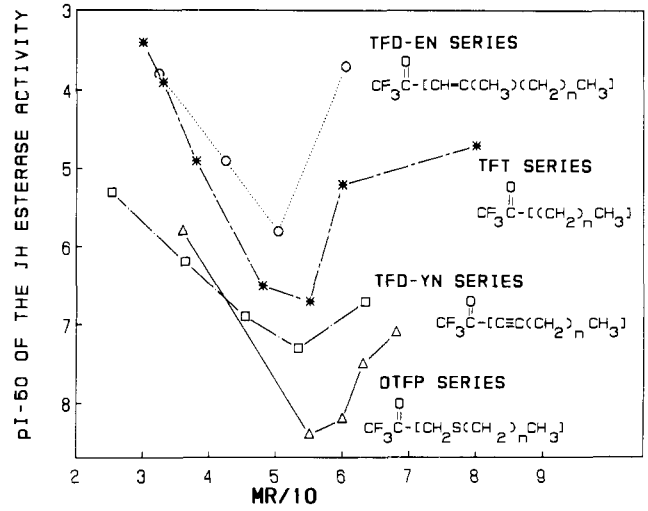


Figure 2. Relationship between molar refractivity (MR/10) and the pI_{50} of the hemolymph juvenile hormone esterase activity from the cabbage looper, *T. ni*, for four series of trifluoromethyl ketones. The MR was calculated for the R groups in the general structure RC(O)CF_3 for the TFT series (n = 5–7, 9, 11, 12, 16), for the OTFP series (n = 3, 7–10), for the TFD-en series (*Z* isomers; n = 3, 5, 7, 9), and for the TFD-yn series (n = 3, 5, 7, 9, 11). The I_{50} for the most potent juvenile hormone esterase inhibitor from each of these series is also given in Figure 1 along with its chemical name from which the series abbreviation was derived. The TFT and OTFP data are provided for comparison purposes from Hammock et al. (1982, 1984), respectively.

concentration of 0.23 mM. The incubation temperature was 30 °C in these assays. AChE activity was assayed at 37 °C according to the procedure of Boehringer (1973). A 20-μL portion of the substrate acetylthiocholine iodide (Sigma) in sodium phosphate buffer (0.05 M, pH 7.2) was added to 3 mL of 50 ng/mL of Type VI-S electric eel AChE (Sigma) in the same buffer to give a final substrate concentration of 0.25 mM. Enzyme concentrations and incubation times were then chosen for all of the above assays that resulted in a linear hydrolysis rate with time. Inhibitors were preincubated at the assay temperature for 10 min with the enzyme prior to the addition of substrate. All inhibitors were dissolved in ethanol with 1 μL/assay for JH esterase and α-NA esterase and 3 μL for AChE. Enzyme assays were always run at least in duplicate and averaged. The I_{50} values were determined from at least two independent analyses of at least five different inhibitor concentrations that bracketed the I_{50} and fell within the linear region immediate to the I_{50} and calculated by least-squares regression analysis. The I_{50} is the inhibitor molar concentration needed to inhibit half of the enzyme activity as compared to an ethanol control.

Synthesis of α-Acetylenic and α,β-Unsaturated Trifluoromethyl Ketones. The α-acetylenic trifluoromethyl ketones were synthesized from the appropriate lithium acetylide (RC≡CLi, where R = [$CH_3(CH_2)_n$–] as described for the TFD-yn series in Figure 2) and 1,1,1-trifluoroethyl acetate (in THF). Analytically pure samples of these acetylenic compounds were obtained by Kugelrohr distillation under vacuum or by column chromatography. β-Methyl β-alkyl disubstituted α,β-unsaturated trifluoromethyl ketones were then obtained by regiospecific addition of lithium dimethylcyanocuprate to the appropriate acetylenic trifluoromethyl ketone synthesized above (in THF) to produce the specific compounds described in Figure 2. Stereochemically pure *E* or *Z* isomers of the unsaturated compounds were obtained by flash chromatography on silica gel. All of the above compounds were >98% pure by chromatography. For more details and the physical data for each compound see Linderman and Lonikar (1988).

Synthesis of α- and α'-Substituted Derivatives of OTFP. The general structures for these derivatives and the α- and α'-substitutions are shown in Figure 3. For the α-substituted inhibitors, the trifluoromethyl ketone was synthesized from the Grignard reagent (RCH_2MgBr, where R represents the substituents in Figure 3) and trifluoroacetic acid in dibutyl ether

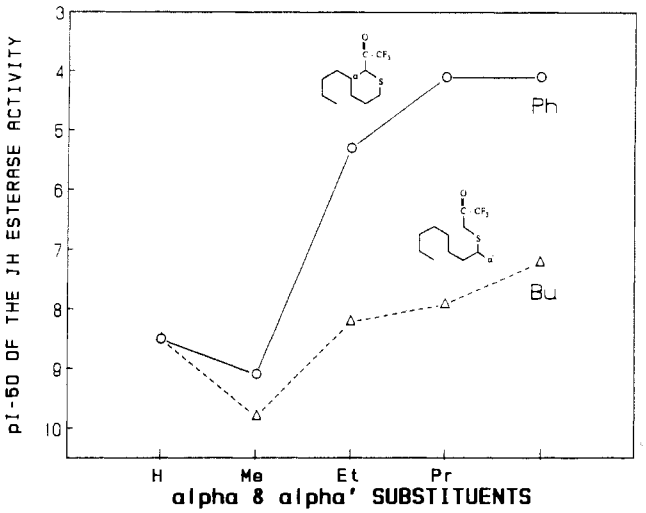


Figure 3. Relationship between R groups (H, methyl, ethyl, propyl, butyl and phenyl) for α- or α'-substitutions and the pI_{50} of the hemolymph juvenile hormone esterase activity of the cabbage looper, *T. ni*.

at −10 °C. The trifluoromethyl ketone was then brominated in sulfuric acid. The resulting α-brominated trifluoromethyl ketone was finally alkylated with octyl mercaptan (in THF/$NaHCO_3$) to produce the α-substituted inhibitors shown in Figure 3 in >98% chemical purity after flash chromatography and distillation. The α'-substituted derivatives of OTFP were synthesized first from Grignard reagent addition (RMgBr, again where R represents the substituents in Figure 3) to octyl aldehyde. The resulting alcohol was then converted to the bromide with phosphorous tribromide. The thiols were then obtained from the bromides via the isothiouronium salt and then combined with bromotrifluoroacetone (in THF/$NaHCO_3$) as before for the α-derivatives, to produce the α'-substituted trifluoromethyl ketones in Figure 3. For more details and physical data, see Linderman et al. (1987) and references therein.

Chemical Analyses. Analyses of the above reagents and products were made by ^1H NMR on either a Varian EM360A or a Varian EM390 spectrometer at 60 or 90 MHz, respectively, with tetramethylsilane as a reference (δ 0.0). ^{19}F NMR spectra were obtained on a Varian EM390 and chemical shifts recorded relative to trifluoroacetic acid. Infrared spectra were obtained on either a Beckman Acculab I spectrophotometer or a Perkin-Elmer 1430 ratio recording spectrophotometer calibrated with polystyrene film (1601 cm^{-1}). Gas chromatographic analyses were performed on a Hewlett-Packard 5890 gas chromatograph using a 30-m SE-30 fused silica capillary column and a flame ionization detector. Organic reagents were of the highest quality available and were purified just prior to use by recrystallization or distillation. Solvents were also of the highest purity available and were redistilled before use. Inorganic reagents were used without purification. Elemental analyses were preformed by Atlantic Microlab, Atlanta, GA.

Calculation of Molar Refractivity. Molar refractivity is a measure of the molecular volume by the following relationship: $MR = [(n^2 - 1)/(n^2 + 2)](M_r/d)$ where n = refractive index, M_r = molecular weight, and d = density. All of the compounds are liquids and should have a similar refractive index. MR values were calculated from the additivity principle in which known MR values for molecular fragments were combined to provide the final MR value for R in the general formula $RC(O)CF_3$. MR values for molecular fragments were obtained from Hansch and Leo (1979). The MR value for the alkylthio fragment was from Hammock et al. (1984).

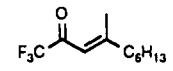
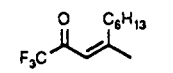
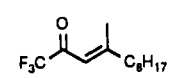
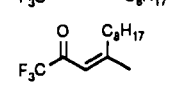
RESULTS AND DISCUSSION

Importance of the JH Backbone in Inhibition. The inhibitory potencies of four different series of trifluoromethyl ketones are presented in Figure 2 with the name of each derived from the most potent inhibitor of the series illustrated in Figure 1. The molar refractivities (MR/10) for the best inhibitor of each series (TFT, OTFP, TFD-en or 4-methyl-1,1,1-trifluorododec-3(*Z*)-en-2-one, and TFD-yn or 1,1,1-trifluorododec-3-yn-2-one) are in reasonable agreement in the range 5.03–5.50. Some variability would be expected because of the incremental nature of MR, which is dependent on the moieties added, and in some cases the differences in the I_{50} are relatively small between homologues as optimum inhibition is approached. This variability between series can also be placed in perspective by considering that a change in MR/10 of a single methylene moiety is equivalent to 0.46 unit, which is the difference between JH I and JH III. The MR/10 for JH II is 5.5. A parabolic relationship similar to those illustrated in Figure 2 was found in analogous experiments with *O*-*p*-nitrophenyl ethylphosphonates (Linderman, Tshering, Venkatesh, and Roe, unpublished data), demonstrating that the structure/activity relationship between MR and inhibitor potency and the importance of mimicry of the JH backbone in JH esterase inhibition are not strictly limited to trifluoromethyl ketones. This parabolic relationship of MR/10 to inhibitor I_{50} values also occurs for JH esterase from different insect species (Abdel-Aal, unpublished data). In contrast to the importance of the JH backbone, additional JH mimicry like the incorporation of alkyl substitutions to mimic that found at C-7 and C-11 of JH and also the addition of an unsaturation to mimic the C-6 unsaturation of the natural substrate (see DNTFP and compare to JH in Figure 1) had no significant effect on inhibitory potency (Prestwich et al., 1984).

Importance of the S Atom in Mimicking the α,β-Unsaturation of JH and Increasing Inhibitory Potency. Hammock et al. (1984) demonstrated that the addition of the S atom in compounds like OTFP increased the potency of these inhibitors over simple aliphatic trifluoromethyl ketones like TFT approximately 40-fold (compare I_{50}'s in Figure 1). This is further illustrated in Figure 2, where the addition of the sulfur increased the inhibitory potency (reduced the I_{50}) of the OTFP series over that of the TFT series at any given molar refractivity. Studies in our laboratories have demonstrated that complete oxidation of the sulfur does not reduce this effect, and structure/activity analyses of *O*-*p*-nitrophenyl and *S*-phenyl phosphonate mimics of TFT and OTFP also suggest that the importance of the S atom in JH esterase inhibition is not restricted to trifluoromethyl ketones (unpublished data).

It was originally hypothesized that the increased potency of sulfur-containing trifluoromethyl ketones resulted from the sulfur mimicking the α,β-unsaturation of the natural substrate for JH esterase. To test this hypothesis, a series of β,β-disubstituted α,β-unsaturated trifluoromethyl ketones (TFD-en series, Figure 2) were synthesized and examined as inhibitors. These were designed to mimic exactly the α,β-unsaturation, the β-methyl substituent, and the *E* isomeric configuration of JH. The importance of the β-methyl substitution will be discussed later. Interestingly, the most potent inhibitor of the unsaturated series (TFD-en) had an I_{50} approximately 10 times greater than that of TFT and 400 times greater than OTFP (compare I_{50}'s in Figure 1). This is also illustrated for both series in Figure 2 at different molar refractivities. Clearly the S atom of the OTFP series does more than simply mimic the site of unsaturation of JH. An alternative explanation is that the sulfur is involved in intramolecular hydrogen bonding and stabilizes the hydrate and/or the tetrahedral adduct formed with the presumed active site serine hydroxyl of JH esterase. Theoretical, spec-

Table I. Comparison of *E* versus *Z* Isomers from the TFN-en Series as Inhibitors of Cabbage Looper Juvenile Hormone Esterase (JHE) and α-Naphthyl Acetate Esterase (α-NAE) Activity and of Electric Eel Acetylcholinesterase Activity (AChE)

compound	isomer	I_{50}, M		
		JHE	α-NAE	AChE
	*E*	7.3×10^{-6}	8.0×10^{-6}	2.5×10^{-7}
	*Z*	8.0×10^{-5}	2.7×10^{-5}	1.3×10^{-6}
	*E*	8.7×10^{-7}	1.4×10^{-5}	1.1×10^{-6}
	*Z*	$>1.0 \times 10^{-4}$	$>1.0 \times 10^{-4}$	1.7×10^{-4}

tral, and kinetic studies to probe this possibility are currently under way. Interestingly, the *E* isomers of these unsaturated trifluoromethyl ketones reproduce the geometry of the double bond found in the natural substrate for JH esterase and, in comparison with the *Z* isomers (Table I), were more potent inhibitors. However, geometric isomer dependence was also observed for AChE and α-NA esterase inhibition (Table I).

Surprisingly, the acetylenic trifluoromethyl ketones (TFD-yn series, Figure 2) were more potent inhibitors than the corresponding unsaturated compounds. For example, TFD-yn was approximately 30 times more active than TFD-en (compare I_{50}'s in Figure 1). This result is especially surprising considering that the structure of TFD-yn is unique compared to that for JH, OTFP, and TFD-en. This provides further evidence that structural mimicry of the α,β-unsaturation of JH is not a critical feature in inhibitor/enzyme interaction and other factors like hydrogen bonding, steric constraints, hydrophobicity, and/or electronic effects are more important in this region of the inhibitor molecule. Recall that the incorporation of an unsaturation further from the carbonyl also had no effect on inhibition (Prestwich et al., 1984). In contrast to what was observed for most fluoro ketones, the acetylenic trifluoromethyl ketones (TFD-yn series, Figure 2) were surprisingly very potent inhibitors of electric eel AChE as compared to that for both JH esterase and α-NA esterase (Figure 4). For example, the acetylenic fluoro ketone with the smallest molar refractivity in Figure 4 was 3300 times more active against AChE than JH esterase, and the fluoro ketone with the next to the highest MR value was 15 000 times more active than α-NA esterase. In this sense, the acetylenic fluoro ketones are an anomaly.

Steric Restrictions around the S Atom of OTFP and the Importance of the C-3 Methyl of JH in Inhibition. A series of α- and α′-substituted OTFP analogues were synthesized and assayed as inhibitors of JH esterase. The general formula for these compounds and their inhibitory potency are illustrated in Figure 3. The addition of a methyl group in the α-position increased the potency of this compound over that of OTFP 3.5-fold, but the same substitution in the α′-position increased the activity 18-fold. The α′-Me-OTFP (3-(2-nonanylthio)-1,1,1-trifluoropropan-2-one, Figure 1) is the most potent in vitro inhibitor of JH esterase available. These results suggest that there may be a hydrophobic pocket near the active site of JH esterase that is important in inhibitor/enzyme interactions and that mimicry of the C-3 methyl of JH is an important feature in inhibition. This factor

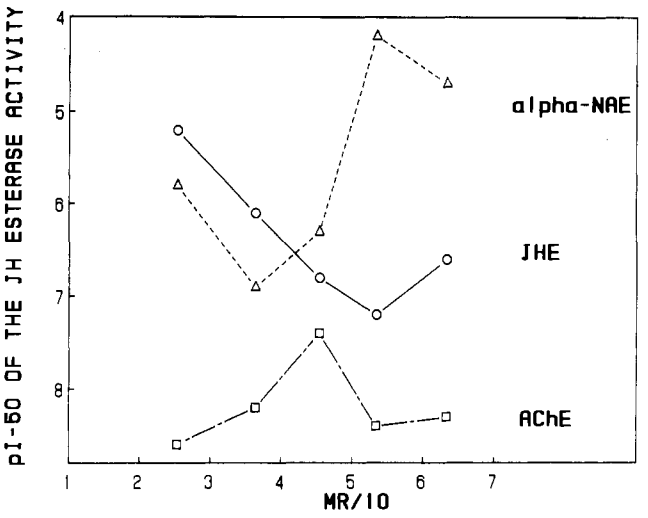


Figure 4. Relationship between the molar refractivity (MR/10) of α-acetylenic trifluoromethyl ketones (TFD-yn series, Figure 2) and pI_{50} of the hemolymph juvenile hormone esterase activity (JHE) and α-naphthyl acetate esterase activity (α-NAE) of the cabbage looper, *T. ni*, and electric eel acetylcholinesterase activity (AChE).

was taken into consideration in the design of the α,β-unsaturated trifluoromethyl ketones in the studies described previously. Additional substitutions with increasing bulk in both the α- and α′-positions resulted in a reduction in inhibitory potency (an increase in the I_{50}), but this effect was much more dramatic for substitutions in the α-position (Figure 3). Either increased bulk in the α-position is disrupting intramolecular hydrogen bonding with the S atom or the carbonyl of OTFP is operating in a more sterically restricted environment than that for interactions involving the sulfur. Sensitivity to increased bulk near the active site of JH esterase is not too surprising considering the fact that *O,O*-diisopropyl phosphorofluoridate, a potent serine esterase inhibitor, is relatively inactive against JH esterase. This is also consistent with the research of Sparks and Rose (1983) in which isopropylparaoxon did not inhibit *T. ni* JH esterase while both paraoxon and *n*-butylparaoxon were active.

Specific Inhibition with Trifluoromethyl Ketones and Unique Substrate Requirements of JH Esterase. Figure 4 demonstrates the unique structure/activity relationship that occurs for JH esterase as compared to other esterases, further supporting the earlier point of the importance of JH mimicry in JH esterase inhibition. This is also demonstrated for substitutions in the α- and α′-positions (Figure 3, data on α-NA esterase and AChE not shown). The methyl substitution in both positions increased the JH esterase inhibition but had no effect or decreased inhibition for the other esterases. The high affinity of JH esterase for JH, the selectivity for the enantiomerically correct natural substrate, the importance of JH mimicry in JH esterase inhibition, and the timing of the appearance of JH esterase argue in favor of a very specific functional role of this esterase in insect development. One of the objectives of research on JH esterase inhibition was to develop more persistent and selective in vivo inhibitors, in order to evaluate the relative importance of JH esterase in insect development. Although a number of potent and persistent organophosphorus inhibitors have been developed in our laboratories (unpublished data), these inhibitors lack the selectivity needed and inhibit both α-NA esterase and AChE. Trifluoromethyl ketone inhibitors like OTFP, on the other

hand, have for the most part demonstrated significant but not absolute specificity but are usually unstable in vivo. More recently, however, we have achieved absolute specificity and prolonged in vivo stability for at least 2 days through modifications of some of the original trifluoromethyl ketone structures (unpublished data). The availability through structure/activity studies, of selective and potent in vivo inhibitors of JH esterase, now makes possible a clearer evaluation of the relative importance of JH metabolism to JH biosynthesis in the regulation of JH titer.

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