PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 17, 76–88 (1982)

Trifluoromethylketones as Possible Transition State Analog Inhibitors of Juvenile Hormone Esterase

BRUCE D. HAMMOCK, KEITH D. WING,[1] JAMES MCLAUGHLIN,[2] VICTOR M. LOVELL,[3] AND THOMAS C. SPARKS[4]

Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616

Received August 12, 1981; accepted November 3, 1981

A series of compounds containing the trifluoromethylketone group have been synthesized utilizing either a modified Grignard procedure or by reacting selected aliphatic bromides or tosylates with the Collman reagent [$Na_2Fe(CO)_4$]. When tested *in vitro* as inhibitors of crude juvenile hormone esterase from the hemolymph of the cabbage looper, *Trichoplusia ni* (Noctuidae), the most active compounds were trifluoromethylketones possessing either a juvenoid-like structure or a straight aliphatic chain. The logarithm of the inhibitory potency of the aliphatic compounds was proportional to their chain length, up to 1,1,1-trifluorotetradecan-2-one ($I_{50} = 1 \times 10^{-7}$ *M*). This powerful inhibition was found to be highly selective for JHE, reversible, competitive by Lineweaver–Burk analysis, and was characterized by high affinity of the inhibitor for the esterase ($K_i = 3.2 \times 10^{-9}$ *M*, K_m JH III $= 2 \times 10^{-7}$ *M*). Other trifluoromethylketones were shown to be inhibitors of *T. ni* α-naphthylacetate esterase and bovine trypsin. By analogy with the mechanism of trypsin action, trifluoromethylketones are probably potent inhibitors due to their resemblance to a tetrahedral transition state on the reaction coordinate to the acylated enzyme.

INTRODUCTION

The juvenile hormones (JHs)[5] regulate a myriad of developmental and reproductive events in insects, and metamorphosis in holometabolous insects is certainly among the most striking of these events. The reduction in JH titer initiating metamorphosis in the Lepidoptera examined is caused by catabolism of the JHs as well as by reduction in their rates of biosynthesis (1, 2). Hydrolysis of the methyl ester of JH to yield JH acid is apparently the major route of metabolism (1–3). In the cabbage looper (Noctuidae, *Trichoplusia ni*) hydrolysis is due largely to a single enzyme, JH esterase (JHE, EC 3.1.1.), which is present mainly in the hemolymph and fat body (4–6).

The properties of JHEs appear to be unique. In most insects they are weakly inhibited by commonly used inhibitors of carboxylesterases such as diisopropyl phosphofluoridate (DFP), eserine, or triorthocresol phosphate (TOCP). However, they are surprisingly sensitive to a number of phosphoramidates such as methamidophos or *O*-ethyl-*S*-phenyl phosphoramidothiolate (EPPAT) (7–11). Sparks and Hammock (11) have demonstrated that the development of *T. ni* can be disrupted by the application of some of these compounds, indicating that highly potent inhibitors of JH esterase could be developed as selective insect control agents.

As part of a further search for effective,

[1] Current address: Department of Entomology, University of California, Berkeley, California, 94720.

[2] Current address: Department of Chemistry, University of California, Riverside, California, 92521.

[3] Current address: University of Kansas Medical School, Kansas City, Kansas, 66103.

[4] Current address: Department of Entomology, Louisiana State University, Baton Rouge, Louisiana 70803.

[5] Abbreviations used: JH(s), juvenile hormone(s); JHE(s), juvenile hormone esterase(s); DFP, diisopropyl phosphofluoridate; TOCP, triorthocresol phosphate; EPPAT, *O*-ethyl-*S*-phenyl phosphoramidothiolate; NMR, nuclear magnetic resonance spectroscopy; ir, infrared spectroscopy; TFT, 1,1,1-trifluorotetradecan-2-one; PTU, phenylthiourea; α-NA, α-naphthyl acetate; α-NAE, α-NA esterase; TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

0048-3575/82/010076-13$02.00/0

selective inhibitors of JHEs, trifluoromethylketones were found to be potent. This report describes the synthesis of these compounds and their inhibitory activity on selected enzymes. A hypothesis to explain this inhibitory action is proposed which may facilitate the development of related compounds as physiological and biochemical probes as well as selective insect growth regulators.

MATERIALS AND METHODS

Synthesis

Two basic procedures were used to synthesize the fluoromethylketones discussed in this paper. In each case the resulting compounds were judged to be pure based upon analysis by gas–liquid and thin-layer chromatography and by nuclear magnetic resonance (NMR), infrared (ir), and ultraviolet (uv) spectroscopy. In both procedures dry glassware and solvents as well as an inert atmosphere are essential. 4′-Methoxytrifluoroacetophenone was prepared by a modified Grignard procedure (12, 13). To a suspension of magnesium turnings (0.504 mol), in anhydrous ether freshly distilled from lithium aluminum hydride (200 ml) was added *p*-bromoanisole (0.499 mol, Aldrich) at a rate sufficient to maintain reflux after the reaction had been initiated. After addition was complete the reaction was refluxed for 1 hr. Following cooling to room temperature (22°C) a solution of trifluoroacetic acid (0.149 mol, Aldrich) in anhydrous ether (40 ml) was added over 0.5 hr. After the second addition was complete the reaction was refluxed for 3 hr. The reaction mixture was then poured over a mixture of ice and excess hydrochloric acid. The layers were separated and the aqueous phase extracted with ether (4 × 25 ml). The combined organic phases were then washed with 5% sodium bicarbonate solutions (100-ml portions until aqueous remained basic) and a saturated sodium chloride solution (1 × 100 ml). The resultant ethereal solution was then dried over anhydrous sodium sulfate. Filtration followed by removal of the ether gave a yellow oil (62.2 g) which was distilled at reduced pressure. After a forerun of material distilling at >2.5 mm and <72°C, a yellow oil (15.77 g, 51.8%) distilled at 74–82°C/2.1–2.5 mm. The ketone (**24**, Table 1) showed ir absorption at 1701 cm^{-1} (lit. 1709 cm^{-1}). For further confirmation the ketone (29 mmol) was dissolved in 95% ethanol (100 ml) and reduced under 30 psi of hydrogen for 5 hr in the presence of 900 mg 10% Pd/C. The uv and ir spectra indicated that reduction was complete and a single spot was observed on thin-layer chromatography. Addition of new catalyst and further exposure to hydrogen for 8 hr resulted in no further reduction. The alcohol (**25**) was recovered in 67% yield following distillation at 86–90°C/1.2 mm. 1,1,1-Trifluorotetradecan-2-one (TFT, 7) and several other aliphatic ketones were prepared in a similar manner from 1-bromododecane or the appropriate bromide. The Grignard procedure

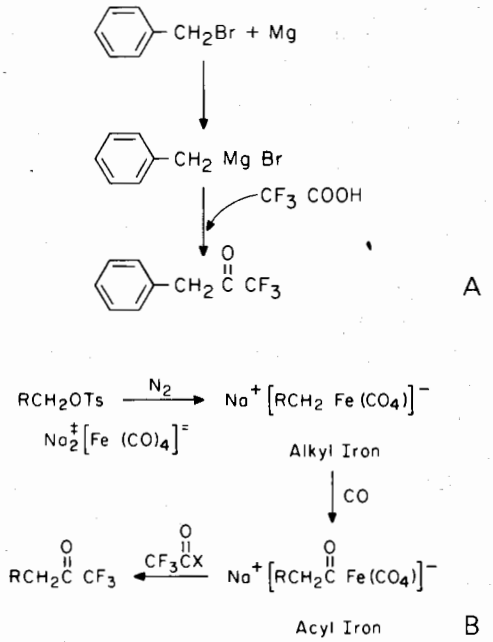


FIG. 1. *Synthesis of trifluoromethylketones via a modified Grignard procedure (A) and via iron tetracarbonyl and either trifluoroacetic anhydride or trifluoroacetyl chloride (B).*

applied to aliphatic bromides consistently gave 30% isolated yield (Fig. 1A).

Alternatively, aliphatic trifluoromethylketones were prepared by slight modifications of the method of Collman *et al.* (14, 15). The Collman reagent [iron tetracarbonyl, $Na_2Fe(CO)_4$, Alfa-Ventron, 10 mmol] was transferred to a dry, round-bottom flask in a glove bag under N_2. A stirring bar was added and the flask was capped with a tight-fitting septum which was folded over and taped down with electrical tape. A N_2 inlet and bubbler tube were attached and 65 ml dry tetrahydrofuran (freshly distilled from lithium aluminum hydride) were added via syringe. The light tan slurry was vigorously stirred and 10 mmol of the respective aliphatic bromide or tosylate was added via syringe causing a mild exothermic reaction accompanied by a gradual change from a light tan slurry to a red–brown solution. Disappearance of the starting bromide or tosylate as monitored by thin-layer chromatography showed alkylation to be complete in approximately 2 hr. The reaction was then stirred under a CO atmosphere for 5 hr and 30 mmol trifluoroacetic anhydride (Aldrich, ≥99% gold label) was added via syringe to the stirred solution with noticeable exothermicity. Trifluoroacetyl chloride could also be used with a slight increase in yield. After 24 hr under a CO blanket the reaction was diluted with ether (25 ml), poured into 80 ml acidified brine and extracted with ether (50 ml, 2×). The ether extract was washed with water (20 ml, 2×), saturated brine (25 ml, 2×), and then dried over anhydrous magnesium sulfate. Removal of the solvent and chromatography on Florisil or silica gel using a hexane-to-ether step gradient resulted in the trifluoromethylketone being eluted in a lightly colored fraction containing iron by-products. The ketone-containing fractions were then either rechromatographed or vacuum-distilled to obtain analytically pure samples of colorless oils. The aliphatic trifluoromethylketones could be readily identified by a characteristic two proton triplet at δ 2.62 upon NMR in carbon tetrachloride and a strong carbonyl band at 1760 cm^{-1} when examined by ir (Fig. 1B).

Compounds **27** and **28** were from Aldrich and **29** and **30** from Peninsular Chem Research. Other compounds were prepared by analogous procedures from either commercially available starting materials or by routes previously described in the literature. However, when 2-isopropoxybenzylbromide (a propoxur analog) was exposed to the Collman reagent under conditions described above, the reaction mixture detonated.

Enzyme Assays

JHE analyses were performed using hemolymph drawn by piercing a proleg with a sharp dissecting needle from last (fifth)-instar *T. ni* larvae on early morning Day 2, when hemolymph JHE titers are very high (ca. 35 nmol JH III cleaved/min-ml hemolymph) (5, 16). Independent tests have shown that only very low levels of lactate dehydrogenase, an enzymatic marker for fat body cell cytosol, are found in hemolymph collected in this manner and that blood cells contribute negligible JHE activity (4, 5). The hemolymph was diluted with sodium phosphate buffer (pH 7.4, I = 0.2 *M*, with 0.01% (w/v) phenylthiourea, subsequently referred to as PTU buffer, 4°C) so that linear rates of JH hydrolysis were obtained in subsequent assays. Candidate inhibitors were added in 1 μl of ethanol to 100 μl of the diluted hemolymph and preincubated for 10 min at 30°C. Substrate (C10-^{3}H JH III, 10 Ci/mmol, New England Nuclear and unlabelled E, E JH III, Calbiochem) were added such that a final concentration of 5×10^{-6} *M* JH III containing ~30,000 dpm/assay was obtained unless otherwise indicated. The assays were run at 30°C, halted by the addition of basic methanol and isooctane, and the aqueous phase was analyzed according to the partition method of Hammock and Sparks (17).

α-Naphthyl acetate (α-NA) hydrolysis

was monitored in hemolymph taken from last-instar Day 3 larvae bled 7 hr after "lights on" and diluted 10× in PTU buffer (16). After a 10-min preincubation of 100 μl of the enzyme at 30°C with candidate inhibitors added in 1 μl ethanol, 1 ml of 2.5×10^{-4} *M* α-NA was added in PTU buffer to the enzyme-inhibitor sample. Hydrolysis of α-NA by esterase activity (α-NAE) was then monitored as described previously (8, 16, 18).

Trypsin (EC 3.4.21.4) activity was monitored using a modification of a procedure described in (19). A 500-μl aliquot containing 0.08 μg trypsin (Sigma, bovine pancreatic) in Tris–HCl buffer (pH 8.1, 0.04 *M*, 0.01 *M* $CaCl_2$) was preincubated with the candidate inhibitor added in 10 μl of ethanol for 3 min at room temperature in 10 × 75-mm culture tubes (this procedure gave results identical to those obtained using a 10-min preincubation at 30°C in a shaking incubator). A 500-μl aliquot containing 2×10^{-4} *M* *p*-toluenesulfonyl-L-arginine methyl ester (TAME) in Tris buffer was then added to give a total of 1 ml of 0.08 μg trypsin and 1×10^{-4} *M* TAME. This solution was equilibrated for 2 min at 30°C in the spectrophotometer beam, and the absorbance at 247 nm was read continuously for at least 3 min. The reaction in the absence of inhibitor was linear for at least 5 min. Controls containing 1% ethanol showed no inhibition of trypsin activity in the assay system used above. I_{50} values for all inhibitors and enzymes were determined from semilog plots (5, 9, 11).

In each case at least three replicates each of two points above and below the I_{50} were used to determine the I_{50}. Each inhibitor was compared in the same run with inhibitors of similar potency. The inhibition constant, K_i, for TFT inhibition of JHE was determined from Lineweaver–Burk double-reciprocal plots (20) and a plot of the slopes of the resulting lines (K_m/V_{max}) vs inhibitor concentration (21).

Protein concentration was monitored using a commercial Coomassie brilliant blue G-250 solution (Bio-Rad Laboratories) (22, 23), with bovine serum abumin (Sigma, fraction V) as a standard. A 300-μl aliquot of concentrated dye solution diluted 5× in distilled water was mixed with 30 μl of the protein sample and read on a Gilson EIA Manual spectrophotometer in Gilson Cuvette-paks.

Reactivation of Inhibited JHE

Hemolymph was diluted 2× with PTU buffer and incubated with either 1×10^{-3} *M* EPPAT or 1×10^{-4} *M* TFT. When diluted and assayed as described above, 0 and 10% of the original JHE activity was observed. The inhibited samples were placed on identical but separate gel filtration columns prepared from the same batch of Sephadex G-25 (9 ml bed volume, 24 cm height, 0.69 cm i.d.) and run under identical conditions (6°C, 8.5 min/0.25-ml fraction with PTU buffer). The green hemolymph pigment proved to be a useful marker for the excluded protein fractions which eluted in a total of 3 ml. The combined protein fractions collected from the EPPAT- and TFT-treated samples were further diluted 2× and 10×, respectively, and were immediately assayed using a 1-hr and a 15-min incubation time, respectively.

In Vivo Effects of Inhibitors

Three experiments, described earlier by Sparks and Hammock (11) were used to discern the *in vivo* effects of one of the JHE inhibitors. In each case EPPAT was used as a positive control. First, larvae were treated at a single time topically with inhibitors, then groups of three larvae were bled at six times following treatment as described previously (4, 5, 11, 16). The JHE activity in the hemolymph of treated larvae was then compared with that in the hemolymph of control or ethanol-treated control larvae. The second experiment involved Day 2 or Day 3 larvae which were treated with 100 nmol of inhibitor in 1 μl of ethanol (or ethanol alone) followed by 200 nmol of JH I (C 10-^{3}H, 13.5 Ci/mmol, New England

Nuclear). Six hours later the proportion of the total dose which appeared in the hemolymph and which cochromatographed with JH and its major metabolites was determined by thin-layer chromatography and liquid scintillation counting. Finally, the inhibitor was topically applied in the morning and afternoon to larvae in Day 1 and Day 2 of the last larval instar. The larvae were observed for time of pupation and for behavioral or morphological abnormalities until adult eclosion. Selected compounds were also applied topically in acetone to the abdomens of adult female susceptible (NAIDM) house flies with mortality and symptoms recorded over a 48-hr period. An estimate of mammalian toxicity was made by intraperitoneal administration of selected compounds in corn oil to male Swiss–Webster mice.

RESULTS

Synthesis

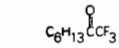
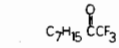
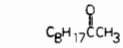
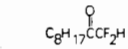
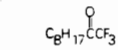
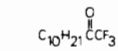
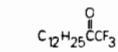
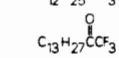
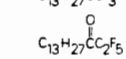
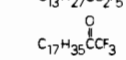
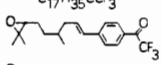
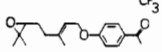
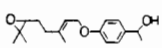
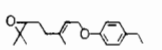
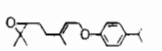
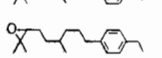
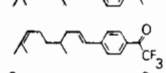
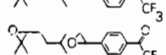
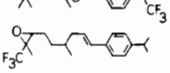
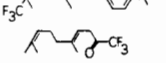
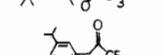
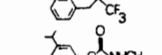
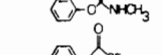
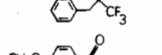
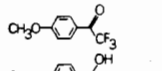
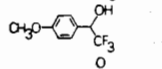
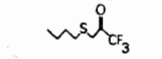
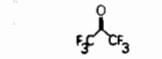
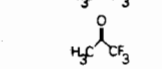
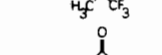
The modified Grignard procedure resulted in a 50–60% yield of the desired trifluoromethylketones when aromatic bromides were used as the starting materials. Consistent isolated yields of ca. 30–35% were obtained with aliphatic bromides. The Collman procedure gave erratic results regardless of whether bromides or tosylates were used as starting materials and whether tirfluoroactyl chloride or anhydride were used as electrophiles. In many instances no trifluoromethylketone was formed, and the starting material could be recovered quantitatively and used successfully in a subsequent reaction. When successful, the Collman method proceded cleanly with 65–78% isolated yields. No differences were noted in yields between reactions which were maintained under carbon monoxide and those in which nitrogen was substituted for carbon monoxide once the acyl iron had been formed. In cases where both the Collman and Grignard procedures were used to prepare the same compound, no differences could be found in the chemical or biochemical properties of the two products.

Inhibitor Potency on JHE

Although not kinetically valid, the I_{50} values presented in Table 1 clearly indicate the relative potency of the inhibitors tested. Under these conditions DFP and TOCP have I_{50} values of greater than 1×10^{-4} *M* on JH esterase while EPPAT and paraoxon have I_{50} values of 1 and 2×10^{-6} *M*, respectively. The importance of the trifluoromethylketone moiety to inhibitor activity can clearly be seen by comparing several compounds. In the aliphatic series 2-decanone (**3**) causes negligible inhibition when compared to 1,1,1-trifluorodecan-2-one (**5**). In the juvenoid series the acetophenone (**12**), hydroxyethyl (**13**), isopropyl (**15**), and ethyl (**14, 16**) substituents clearly impart minimal inhibitory activity when compared to similar compounds possessing the trifluoromethylketone moiety (**11, 17, 18**). The inhibitory potency of the anisole derivative (**24**) vanishes upon reduction of the trifluoromethylketone to the corresponding alcohol (**25**). The importance of a juvenoid-like structure to potency can be observed by comparing the highly active trifluoroacetophenone derivatives, (**11, 17**), with the much less active diepoxide (**18**) and anisole derivatives (**24**). The 3-phenyl-1,1,1-trifluoroacetone derivatives (**21, 23**) showed surprisingly low inhibitory activity; however, the *m*-isopropyl derivative (**21**) was a better inhibitor in the millimolar range than *m*-isopropylphenyl-*N*-methyl carbamate (**22**) as could be expected from previous studies (4, 9, 10). If, as indicated by previous studies with JHE (24), an α, β-unsaturated carbonyl is important for interaction with the enzyme, the trifluoroacetophenone derivatives (**11, 17, 18 24**) would be expected to be better inhibitors than the phenyltrifluoroacetone derivatives (**21, 23**) and even the geranyl phenyltrifluoroacetone derivative (**20**). Perhaps the poor activity of 1,1,1-trifluoro-4-trifluoromethylpentene-2-one (**30**)

TABLE 1

Inhibition of Juvenile Hormone Esterase and Bovine Trypsin by Trifluoromethylketones and Related Compounds

Compound		$I_{50} \pm SD$ (M)[a]	
Number	Structure	JH esterase	Trypsin
1		$2.2 \pm 0.2 \times 10^{-4}$	1.6×10^{-5}
2		$6.8 \pm 1.1 \times 10^{-5}$	$>10^{-4}$
3		7×10^{-3}	
4		$1.0 \pm 0.3 \times 10^{-5}$	$>10^{-4}$
5		$6.7 \pm 1.3 \times 10^{-6}$	$>10^{-4}$
6		$2.2 \pm 0.5 \times 10^{-7}$	$>10^{-4}$
7		1.0×10^{-7}	7×10^{-5}
8		$3.9 \pm 0.6 \times 10^{-6}$	$>10^{-7}$
9		$>10^{-3}$	7×10^{-6}
10		1.4×10^{-5}	$>10^{-4}$
11		2×10^{-7}	2×10^{-5}
12		$>10^{-4}$	
13		$>10^{-4}$	
14		$>10^{-4}$	
15		$>10^{-4}$	
16		$>10^{-4}$	
17		3×10^{-7}	1.8×10^{-6}
18		$5.0 \pm 0.4 \times 10^{-4}$	$>10^{-4}$
19		$>10^{-4}$	3×10^{-5}
20		$>10^{-3}$	$>10^{-4}$
21		$>10^{-3}$	$>10^{-4}$
22		$>10^{-3}$	
23		$>10^{-3}$	$>10^{-4}$
24		7×10^{-5}	$>10^{-4}$
25		$>10^{-4}$	
26		$1.5 \pm 0.2 \times 10^{-6}$	3×10^{-5}
27		$>10^{-2}$	$>10^{-3}$
28		$>10^{-3}$	
29		$>10^{-3}$	
30		$>10^{-3}$	

[a] I_{50} values obtained from at least three separate determinations of two assays per treatment with points both above and below the I_{50}. Substrate concentrations: 5×10^{-6} M JH III, 1×10^{-4} M TAME, respectively.

can be attributed to the highly polarized nature of the ketone or the molecule's small size. The sulfur derivative (**26**) may also support this argument because a sulfur can in some cases mimic an olefin. Further studies are certainly needed to test such a hypothesis, but compound **26** is clearly a more potent inhibitor than the aliphatic trifluoromethylketones of similar length (**1, 2**).

Several conclusions may be drawn from the aliphatic ketones tested. For instance, 1,1,1,2,2-pentafluorosextadecan-3-one (**9**) is a much poorer JHE inhibitor than either much shorter (**1, 2, 5–8**) or longer (**10**) trifluoromethylketones. 1,1-Difluorodecan-2-one (**4**) is slightly weaker inhibitor than 1,1,1-trifluorodecan-2-one (**5**), although both are still potent inhibitors. A dependence between inhibitor potency and carbon chain length is clear from Table 1. A plot of log inhibitor potency vs carbon chain length shows a linear increase in potency from 1,1,1-trifluorooctan-2-one through 1,1,1-trifluorotetradecan-2-one (r^2-0.97 for compounds C = 6–12, Fig. 2). The ability of

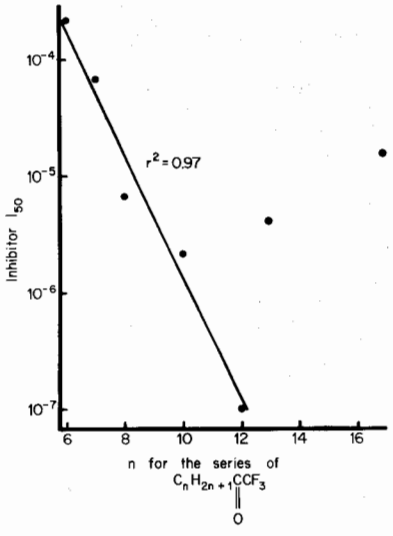


FIG. 2. *Relationship between inhibitory potency expressed as molar I_{50} to JHE and carbon chain length of a series of normal hydrocarbon trifluoromethylketones. Substrate concentration, 5 × 10⁻⁶ M JH III. The r^2 value was calculated based on carbon chain lengths of n = 6–12.*

two compounds of still greater chain length (**8, 10**) to inhibit JHE is decreased dramatically. Neither hexafluoroacetone (**27**), 1,1,1-trifluoroacetone (**28**), nor the 1,1,1-trifluoro-3-bromopropanone (**29**) inhibited JHE even at very high concentrations.

None of the ketones tested caused more than slight inhibition of the α-NAE activity in *T. ni* hemolymph. The best inhibitors were **23** and **24** with I_{50} values of 3 and 4 × 10^{-5} *M*, respectively. The other compounds caused 50% inhibition at 1 × 10^{-4} *M* or above. In contrast to hemolymph JHE activity which in *T. ni* appears to be largely due to a single protein, the α-NA hydrolysis observed is a composite of the individual activities of numerous hemolymph enzymes which vary in relative and absolute activities during development (4, 6, 7).

A screen of the trifluoromethylketone compounds for their activity in inhibiting commercial bovine pancreatic trypsin (Table 1) showed that several compounds blocked the ability of this enzyme to cleave an aromatic arginine methyl ester. In particular, compound **17**, a nonepoxidized aromatic nonadiene exhibited an I_{50} for trypsin at about two orders of magnitude less than the substrate concentration (1 × 10^{-4} *M*). In addition, compound **9**, which contains a pentafluoroethyl rather than the trifluoromethylketone moiety, was an effective inhibitor with an I_{50} of about 7 × 10^{-6} *M*. This molecule was inactive as a JHE inhibitor. Several other compounds were also effective inhibitors with I_{50} values about an order of magnitude lower than the TAME concentration (**1, 7, 11**). The presence of the *S*-heteroatom in compound **26** did not seem to increase the inhibitory potency of compound **1** on trypsin as it did with JHE. The remainder of the compounds showed little activity close to their limits of solubility. However, for many of the poorly inhibitory compounds, enzymatic rates were very low for a few seconds after the addition of TAME during which time the substrate was probably displacing the trifluoromethylketone.

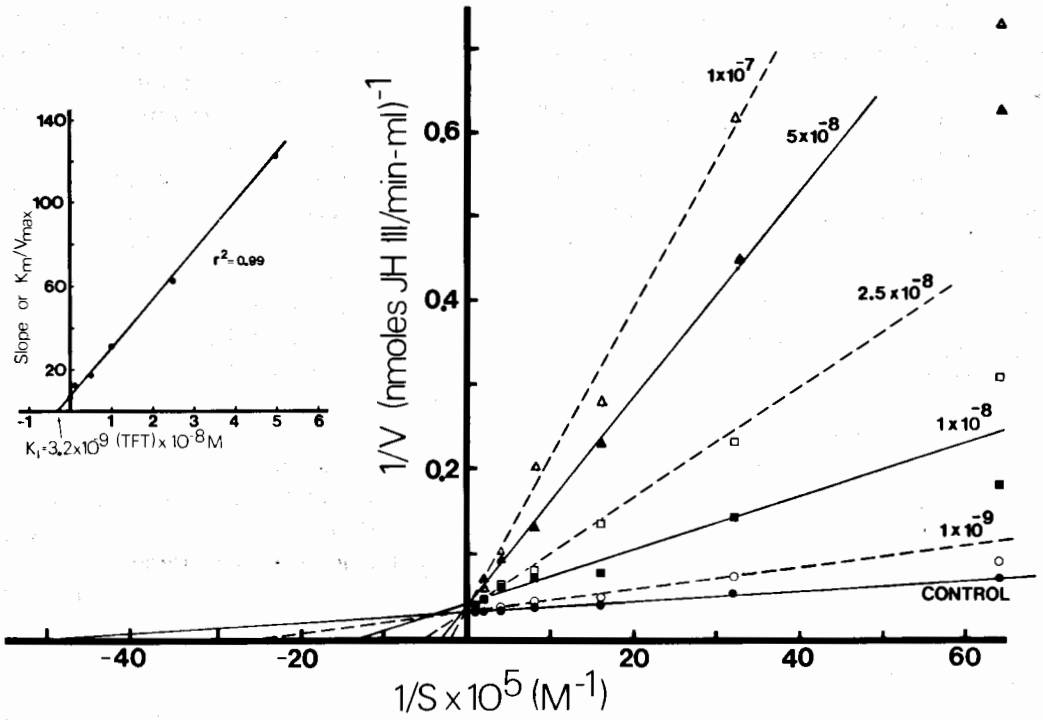


FIG. 3. *Lineweaver–Burk analysis of the inhibition of JH III hydrolysis by JHE using varying molar concentrations of TFT. Inset: Plot of the slopes of the double-reciprocal graphs vs inhibitor concentration to determine a K_i of 3.2 × 10^{-9} M for TFT.*

Kinetic Analysis

The double-reciprocal analysis of TFT-inhibited hemolymph clearly indicates competitive inhibition (Fig. 3). The uninhibited K_m of *T. ni* hemolymph for JH III was calculated to be 2.0 × 10^{-7} *M*, which is lower than the 1.4 × 10^{-6} *M* value reported for the tobacco hornworm, *Manduca sexta* (25). The slopes of all double-reciprocal plots involving inhibitors were calculated based on JH III concentrations of 1.0 × 10^{-5} to 3.1 × 10^{-7} *M*, and the lines had r^2 values of 0.98 or above. However, the rate of JH acid formation using 1.56 × 10^{-7} *M* JH III was in each case higher than would be anticipated based upon the rates observed using higher JH concentrations. There are many possible explanations for this observation including the hypothesis that as the substrate concentration approached the enzyme concentration, formation of the acyl enzyme could be the observed rate-limiting step rather than total substrate turnover involving the slower deacylation step. When the slopes of the double-reciprocal lines (K_m/V_{max}) are plotted vs inhibitor concentration from 0 to 5 × 10^{-8} *M* TFT (Fig. 3), a linear relationship (r^2 = 0.998) is obtained indicating a K_i of 3.2 × 10^{-9} *M* for TFT.

The K_i data for TFT should not be over interpreted. First, crude hemolymph rather than a purified enzyme was used for the determination. Although the JH carrier protein would have no influence on JH hydrolysis under the conditions used, other proteins could have an effect. One argument against such a nonspecific protein effect is that TFT I_{50} values are identical when hemolymph from different stages is diluted to yield identical rates of JH hydrolysis but vastly different protein concentrations. A second caution is that only data

from *T. ni* are reported in this study. TFT inhibits JHE from a variety of lepidopterous larvae, but its I_{50} on the tobacco hornworm, *Manduca sexta,* is an order of magnitude higher than that for *T. ni* under assay conditions where EPPAT is 10-fold more potent than in *T. ni*. A third caution is that the data reported here were obtained for JH III (V_{max} = 41 nmol JH III cleaved/min/ml hemolymph, $K_m = 2 \times 10^{-7}$ *M*). In *T. ni* an identical I_{50} is obtained for TFT when JH I (V_{max} = 80 nmol JH III/min/ml hemolymph, $K_m = 3.2 \times 10^{-7}$ *M*) is used as a substrate. However, in *M. sexta* TFT is 10-fold more potent at inhibiting JH III hydrolysis than JH I hydrolysis. Finally, TFT gives a very shallow inhibition slope. Although 50% inhibition is obtained at 1×10^{-7} *M*, 25 times this concentration is needed for 90% inhibition.

Reactivation of Inhibited JHE

Quantitative recovery of *T. ni* JHE activity is observed following gel filtration; however, no JHE activity was detected following gel filtration of EPPAT-treated hemolymph. In contrast, gel filtration and subsequent dilution led to a rescue of 62% of the original JHE activity in hemolymph pretreated with TFT. The lack of total recovery is consistent with reversible inhibition and can be attributed to incomplete removal of free TFT as well as both specific and nonspecific binding of TFT to proteins.

In Vivo Effects

When 100 nmol of TFT was applied topically to Day 1 or Day 2 *T. ni* larvae, hemolymph JHE activity was only 13–15% below that in control larvae for 6 hr following treatment. Although this reduction is statistically significant ($\alpha = 0.05$), its biological significance is questionable since previous studies indicate that one can inhibit over half of the *in vivo* JHE without detectable metamorphic effects (16). In contrast, 100 nmol of EPPAT applied to the same group of larvae inhibited 85–95% of the JHE activity (11). When insects were treated 3 hr after lights on during Day 2 of the last instar with TFT and radiolabeled JH I, only a slight stabilization of the JHE was noted after 6 hr. The radioactivity in the hemolymph among JH, JH acid, JH diol, JH diol acid, and polar metabolites indicated a concentration of 5.5, 4.6, 0.68, 0.13, and 5.7 nmol/ml hemolymph, respectively, following TFT treatment. Control values for ethanol treated larvae were 4.4, 8.9, 0.23, 0.98, and 3.2 nmol/ml hemolymph, respectively. Day 2 larvae have a hemolymph volume of approximately 75 μl so only about 0.41 and 0.33 nmol of the 200 nmol of JH I applied existed in the hemolymph of the TFT-treated and control insects at 6 hr.

Using the irreversible inhibitor EPPAT, Sparks and Hammock (11) demonstrated that pupation could be delayed in *T. ni* presumably by maintaining an abnormally high prothoracicotropic hormone level. When this experiment was repeated, topical application of 200 nmol EPPAT twice a day to 47, Day 1 and 2 last-instar *T. ni* larvae again blocked the appearance of any gate I larvae (which pupate after 4 days in the last larval instar) and dramatically increased the occurrence of gate 3, 4, and 5 larvae. Similar application of 200 nmol TFT did not lead to a detectable delay in the time of pupation of 45 treated vs 47 control larvae. In contrast to *N,N,N*-trimethylammonium-*m*-trifluoroacetophenone which was highly toxic to mice, apparently to inhibiting acetylcholine esterase (26), the compounds tested in this laboratory showed minimal toxicity. At a dose of 50 μg/insect both the *m*-isopropyl-*N*-methylcarbamate (**22**) and the structurally related trifluoromethylketone (**21**) led to symptoms typical of cholinesterase inhibition while TFT treated house flies appeared normal. However, at 24 and 48 hr compound **21** caused no toxicity while the carbamate-treated insects failed to recover. When an IP dose of 500 mg/kg of compound **21** or TFT was given to male Swiss–Webster mice, they appeared agitated and sweated profusely for several hours before recovering. Partial loss

of facial hair was noted after several days, but no mortality or other symptoms were observed over a period of several months.

DISCUSSION

Over 30 years ago Pauling pointed out that if an enzyme can increase the rate of a reaction and not the equilibrium constant then the affinity of the enzyme for the transition state(s) of the reaction must be much higher than for either the substrate or the product (27, 28). The idea received little attention until this decade when the concept of "transition state stabilization" became an integral component of many theories of enzyme action, and it is particularly well documented in the case of the serine proteases (29–35).

Some semantic problems are associated with the term "transition state mimic" because the synthesis of a stable mimic of a true transition state in which a bond is in the process of being formed or cleaved is unlikely. However, theoretical approaches have led to estimations that transition states may bind to an enzyme $>10^{10-14}$ better than the substrate. Thus, a mimic which has even a vague resemblance to a transition state may be a very potent inhibitor (29, 34). Some workers have distinguished between a true transition state mimic and a quasisubstrate based upon an ultimate change in bond order during the course of the reaction (26). For the purpose of this discussion "transition state analog" will be used in a broad sense to discuss substrate analogs which may approach or partially mimic an enzyme-stabilized activated complex normally occurring along the reaction coordinate leading to the formation of an acyl enzyme (36, 37).

The fluoromethylketones tested in this study meet many of the criteria for transition state analogs. The low K_i of TFT certainly attests to its affinity for the enzyme. Although TFT does not approach the theoretical limit of possessing a K_i 10^{10-14} lower than the K_m, the K_i of 3.2×10^{-9} M for JHE is almost two orders of magnitude below the K_m for JH III. This difference compares favorably with many of the most potent transition state analogs previously reported (33, 34). In contrast to EPPAT which causes irreversible inhibition of JHE apparently by phosphorylation, JHE inhibition by TFT is clearly reversible and competitive. Both of these characteristics are commonly associated with transition state analogs. However, the major criterion for a transition state analog according to Wolfenden (33) is that the compound should possess a "mechanistically understandable relationship to substrates and products." Based on numerous studies on serine proteases and esterases, a mechanism leading from the Michaelis complex to the acyl enzyme involving a tetrahedral transition state is widely accepted (Fig. 4A). Numerous lines of evidence have estab-

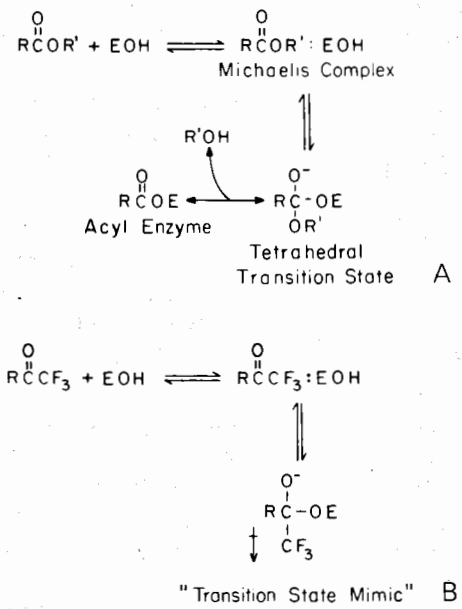


FIG. 4. *Proposed mechanism of catalysis for known serine-hydroxyl proteases and esterases (A). The enzyme lowers the activation energy of the hydrolysis reaction by stabilizing the tetrahedral transition state between the trigonal carbonyl of the ester (RCOOR') and the acyl enzyme (RCOOE). An analogous intermediate occurs during deacylation. Trifluoromethylketones may inhibit the esterase by mimicking the tetrahedral transition state with a structure approaching that of an enzyme-hemiketal (B).*

lished that chloro- and bromomethylketones form hemiketals with the serine present at the enzyme catalytic site, and that a subsequent covalent bond is formed with the halogen as a leaving group. Fluorines possess the strong electron-withdrawing properties of other halogens so that in aqueous solution fluoroketones are largely in the hydrated state (38). However, other properties of fluorine are quite distinct. Fluorines have a small atomic radius such that the steric properties of a trifluoromethyl moiety are quite similar to those of a methyl group although more hydrophobic (39). The carbon–fluorine bond strength is over 107 kcal/mol so that permanent alkylation of the enzyme is much less likely with fluoromethyl than with other halomethylketones.

Several of the trifluoromethylketones tested were potent inhibitors of bovine pancreatic trypsin, a well-characterized enzyme which is known to catalyze ester and amide bond cleavage via nucleophillic attack by a serine hydroxyl group to form a tetrahedral transition state and then an acyllated enzyme intermediate (35). Thus a reasonable hypothesis is that some of the trifluoromethylketones are substrate analogs of JH based on analogy with trypsin and other serine esterases and proteases, forming a Michaelis-like complex with JHE. The highly polarized ketone then readily reacts with the enzyme to form a hemiketal-type structure approaching that shown in Fig. 4B. Apparently the hemiketal structure has some resemblance to the normal tetrahedral transition states which occur both during the formation and the hydrolysis of the acyl enzyme.

The inhibition of serine esterases and proteases as well as other enzyme classes by polarized aldehydes and ketones is quite common. Brodbeck *et al.* (26) has demonstrated that some trifluoromethylketones are potent inhibitors of acetylcholinesterase and various trifluoromethylketones have been shown to inhibit rat liver carboxylesterase (unpublished) and bovine trypsin, as well as *T. ni* hemolymph α-NAE and JHE. However, these studies also demonstrate that a high degree of selectivity can be incorporated into the molecule. The optimum C14 carbon length of TFT as a JHE inhibitor corresponds nicely to the carbon chain backbone of JH O, I, and II. It is suggestive that the potency of such inhibitors can be further increased by the synthesis of more exact mimics of the natural hormone as well as by modifications affecting the polarity of the carbonyl moiety. Even with their current potency, the fluorinated ketones offer a tremendous potential as probes for the investigation of a wide variety of enzymes. The fluorines are characterized by strong NMR signals occurring in regions of minimal interference from biological molecules. Chemical shifts due both to nearby functionalities and due to the hydrophobicity of the molecule's environment are huge in comparison to proton signals. Thus, appropriate fluorinated ketones should facilitate both kinetic and equilibrium examinations of catalytic sites. Trifluoromethylketones have already proven useful in *in vitro* studies as selective JHE inhibitors (5, 11, 43). Since the binding to enzymes is reversible and the trifluoromethylketone moiety is chemically stable, such compounds should be ideal for affinity columns for a variety of esterases and proteases. Preliminary, unpublished studies indicate that remarkable levels of JHE purification may indeed be possible on such columns. The lack of pronounced *in vivo* activity by TFT is disappointing, but not unexpected. Reversible inhibitors commonly have much weaker *in vivo* effects than irreversible inhibitors of lower affinity for the enzyme. More potent inhibitors in this series may in fact demonstrate effects *in vivo* as inhibitors of JHE or other insect enzymes. However, the trifluoromethylketone moiety may prove useful in other aspects of insect growth regulator research.

If the trifluoromethylketone moiety can be incorporated into a biologically active juvenoid structure, the compound could

have dual action due to its inhibition of JHE. However, other factors could lead to an even greater enhancement of activity. For optimal activity juvenoids must gain entry into the correct *in vivo* pool at the appropriate time in insect development while also avoiding degradation and elimination by the host. It has been demonstrated by several workers that the JH carrier proteins of the Lepidoptera have a very low affinity for juvenoids, and thus these compounds may be poorly distributed through the insect. The high affinity of some trifluoromethylketones for JHE may facilitate their storage and distribution by the hemolymph in the critical last larval instar. As the JHE titer undergoes its normal precipitous drop near the time of prothoracicotropic hormone release, the biologically active compounds could be released at the very time when the larva is highly sensitive to morphogenetic agents.

ACKNOWLEDGMENTS

C. A. Mullin (Department of Entomology, Michigan State University) provided compound **26**. This work was supported in part, by NIEHS Grant 7 R01 ES023710-01 and B. D. Hammock was supported by Research Career Development Award 5 KO4 ES00107-03.

REFERENCES

1. C. A. D. deKort, and N. A. Granger, Regulation of the juvenile hormone titer, *Annu. Rev. Entomol.* **26**, 1 (1981).
2. B. D. Hammock, and G. B. Quistad, Metabolism and mode of action of juvenile hormone, juvenoids and other insect growth regulators, *in* "Progress in Pesticide Biochemistry" (D. H. Hutson and T. R. Roberts, Eds.), Vol. 1, p. 1, Wiley, New York, 1981.
3. M. Slade, and C. H. Zibitt, Metabolism of Cecropia juvenile hormone in insects and mammals, *in* "Insect Juvenile Hormones: Chemistry and Action" (J. J. Menn and M. Beroza, Eds.), p. 155, Academic Press, New York, 1972.
4. T. C. Sparks, and B. D. Hammock, A comparison of induced and naturally occurring juvenile hormone esterases from last instar larvae of *Trichoplusia ni, Insect Biochem.* **9**, 411 (1979).
5. K. D. Wing, T. C. Sparks, V. M. Lovell, S. O. Levinson, and B. D. Hammock, The distribution of juvenile hormone esterase and its interrelationship with other proteins influencing juvenile hormone metabolism in the cabbage looper, *Trichoplusia ni, Insect Biochem.* **11**, 473 (1981).
6. M. Rudnika, and B. D. Hammock, Approaches to the purification of the juvenile hormone esterase from the cabbage looper, *Trichoplusia ni, Insect Biochem.* **11**, 437 (1981).
7. L. L. Sanburg, K. J. Kramer, F. J. Keźdy, and J. H. Law, Juvenile hormone-specific esterases in the haemolymph of the tobacco hornworm, *Manduca sexta, J. Insect Physiol.* **21**, 873 (1975a).
8. L. L. Sanburg, K. J. Kramer, F. J. Keźdy, and J. H. Law, Role of juvenile hormone esterases and carrier proteins in insect development, *Nature (London)* **253**, 266 (1975b).
9. B. D. Hammock, T. C. Sparks, and S. M. Mumby, Selective inhibition of JH esterases from cockroach hemolymph, *Pestic. Biochem. Physiol.* **7**, 517 (1977).
10. S. M. Mumby, B. D. Hammock, T. C. Sparks, and K. Ota, Synthesis and bioassay of carbamate inhibitors of the juvenile hormone hydrolyzing esterases from the house fly, *Musca domestica, J. Agric. Food Chem.* **27**, 763 (1979).
11. T. C. Sparks, and B. D. Hammock, Comparative inhibition of the juvenile hormone esterases from *Trichoplusia ni, Tenebrio molitor,* and *Musca domestica, Pestic. Biochem. Physiol.* **14**, 290 (1981).
12. K. T. Dishart and R. Levine, A new synthesis of ketones containing one perfluoroalkyl group, *J. Amer. Chem. Soc.* **78**, 2268 (1956).
13. P. F. Camps, R. Canela, J. Coll, A. Messeguer, and A. Poca, Insect chemistry VI: Trifluoromethyl analogs of juvenile hormones, *Tetrahedron* **34**, 2179 (1978).
14. J. P. Collman, S. R. Winter, and D. R. Clark, Selective synthesis of aliphatic ketones using sodium tetracarbonyl ferrate (-II), *J. Amer. Chem. Soc.* **94**, 1788 (1972).
15. J. P. Collman and N. W. Hoffman, Synthesis of hemifluorinated ketones using disodium tetracarbonylferrate (-II), *J. Amer. Chem. Soc.* **95**, 2689 (1973).
16. T. C. Sparks, W. S. Willis, H. H. Shorey, and B. D. Hammock, Haemolymph juvenile hormone esterase activity in synchronous last instar larvae of the cabbage looper, *Trichoplusia ni, J. Insect Physiol.* **25**, 125 (1979).
17. B. D. Hammock and T. C. Sparks, A rapid assay for insect juvenile hormone esterase activity, *Anal. Biochem.* **82**, 573 (1977).
18. K. Van Asperen, A study of housefly esterases by means of a sensitive colorimetric method, *J. Insect Physiol.* **8**, 401 (1962).
19. K. A. Walsh, Trypsinogens and trypsins of vari-

ous species, *in* "Methods in Enzymology" (G. E. Perlmann and L. Laszlo, eds.), Vol. 19, p. 41, Academic Press, New York, 1970.
20. H. Lineweaver and D. Burk, The determination of enzyme dissociation constants, *J. Amer. Chem. Soc.* **56**, 658 (1934).
21. W. W. Cleland, Steady state kinetics, *in* "The Enzymes" (P. D. Boyer, Ed.), 3rd Ed., Vol. 2, p. 1, Academic Press, New York, 1970.
22. M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantites of protein utilizing the principle of protein–dye binding, *Anal. Biochem.* **72**, 248 (1976).
23. T. Spector, Refinement of the Coomassie blue method of protein quantitation, *Anal. Biochem.* **86**, 142 (1978).
24. G. Weirich and J. Wren, Juvenile-hormone esterase in insect development: A comparative study, *Physiol. Zool.* **49**, 341 (1976).
25. R. K. Vince and L. I. Gilbert, Juvenile hormone esterase activity in precisely timed last instar larvae and pharate pupae of *Manduca sexta*, Insect Biochem. **7**, 115 (1977).
26. U. Brodbeck, K. Schweikert, R. Gentinetta, and M. Rottenburg, Fluorinated aldehydes and ketones acting as quasi-substrate inhibitors of acetylcholinesterase, *Biochim. Biophys. Acta* **567**, 357 (1979).
27. L. Pauling, Molecular architecture and biological reactions, *Chem. Eng. News* **24**, 1375 (1946).
28. L. Pauling, Chemical achievement and hope for the future, *Amer. Sci.* **36**, 51 (1948).
29. R. Wolfenden, Analog approaches to the structure of the transition state in enzyme reactions, *Acc. Chem. Res.* **5**, 10 (1972).
30. J. F. Kirsch, Mechanism of enzyme action, *Annu. Rev. Biochem.* **42**, 820 (1973).
31. G. E. Leinhard, I. I. Secemski, K. A. Koehler, and R. I. Lindquist, Enzymatic catalysis and the transition state theory of reaction rates: Transition state analogs, *Cold Spring Habor Symp. Quant. Biol.* **36**, 45 (1972).
32. G. E. Lienhard, Enzymatic catalysis and transition-state theory, *Science* **180**, 149 (1973).
33. R. Wolfenden, Transition state analog inhibitors and enzyme catalysis, *Annu. Rev. Biochem.* **45**, 271 (1976).
34. A. Fersht, "Enzyme Struction and Mechanisms," p. 260, Freeman, San Francisco, 1977.
35. J. Kraut, Serine proteases: Structure and mechanism of catalysis, *Annu. Rev. Biochem.* **46**, 331 (1977).
36. E. K. Thornton and E. R. Thornton, Scope and limitations of the concept of the transition state, *in* "Transition States in Biochemical Processes" (R. D. Gandeur and R. L. Schowen, Eds.), p. 3, Plenum, New York, 1978.
37. M. F. Hegazi, D. M. Quinn, and R. L. Schowen, Transition-state properties in acyl and methyl transfer, *in* "Transition State in Biochemical Processes" (R. D. Gandour and R. L. Schowen, Eds.), p. 355, Plenum, New York, 1978.
38. J. P. Guthrie, Carbonyl addition reactions: Factors affecting the hydrate-hemiacetal and hemiacetal-acetal equilibrium constants, *Canad. J. Chem.* **53**, 898 (1975).
39. T. B. Patrick, Fluoro-organic biochemistry, *J. Chem. Educ.* **56**, 228 (1979).
40. W. H. Huestis and M. A. Raftery, A study of cooperative interactions in hemoglobin using fluorine nuclear magnetic resonance, *Biochemistry* **11**, 1648 (1972).
41. M. R. Bendell and G. Lowe, Co-operative ionisation of aspartic-acid-158 and histidine-159 in papain, *Eur. J. Biochem.* **65**, 481 (1976).
42. K. K. Yabusaki and C. E. Ballou, *S*-Trifluoroacetonyl-coenzyme A: A ^{19}F analogue of acetyl-coenzyme A, *Biochemistry* **17**, 875 (1978).
43. G. Jones, K. D. Wing, D. Jones, and B. D. Hammock, The source and action of head factors regulating juvenile hormone esterase in larvae of the cabbage looper, *Trichoplusia ni*, *J. Insect Physiol.* **27**, 85 (1981).