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New Trifluoromethyl Ketones as Potent Inhibitors of Esterases: ^{19}F NMR Spectroscopy of Transition State Analog Complexes and Structure–Activity Relationships

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A variety of trifluoromethyl ketones (TFMKs) have been studied as inhibitors of the antennal esterases of the Egyptian armyworm *Spodoptera littoralis.* The chemicals behaved as tight slow-binding inhibitors, the β-thio derivatives being the most potent ones, particularly 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) with an IC_{50} of 0.08 μM. Other TFMKs, containing a propenyl group at the non-polar end of the molecule as in the natural pheromone structure (compounds **4, 8** and **9**), were also notably active. Correlation studies of inhibition with lipophilicity (log P) indicated that the optimum log P values for activity of the β-thio compounds are in the range of 3.03–5.11, while a higher lipophilicity (range 5.37-5.89) was required among the devoid of sulfur ketones. ^{19}F NMR studies, carried out with OTFP, showed that the inhibitor binds the enzyme in a reversible manner, forming an adduct (probably a hemiacetal) of tetrahedral geometry with the active site of the enzyme. To our knowledge, this is the first time that such a study on a natural esterase extract has been undertaken.

Hydrolytic enzymes, particularly esterases and proteases, play a pivotal role in many biological systems and their inhibition could be of high therapeutic interest (1–3). Trifluoromethyl ketones (TFMKs) have been found to be potent inhibitors of a variety of serine esterases and proteases, such as acetylcholinesterase and chymotrypsin (3, 4), juvenile hormone esterase (JHE) (5, 6), human liver microsomal carboxylesterases and porcine liver esterases (7). Inhibition studies with this type of compounds have led, f.i., to the development of renin inhibitors as potential antihypertensive agents (8).

Following our initial studies on inhibition of the antennal esterases (AE) responsible for the pheromone catabolism in the Egyptian armyworm *Spodoptera littoralis* (9), and in order to establish structure-activity relationships, we report herein the biological activity of new TFMKs with regard to the lipophilic character of the chemicals. The compounds include saturated (comp. **1-3**), monoolefinic (comp. **4-6**), dienic (comp. **7-9**) and acetylenic (comp. **10-11**) TFMKs as well as a series of β-thio derivatives **12-16.** 3-Alkylthio- and 3-arylthiotrifluoropropanones have been found to be competitive inhibitors of neuropathy target esterase and JHE (10, 11). To support the hypothesis that TFMKs bind the active site of the enzyme by formation of an adduct of tetrahedral configuration (12), we have carried out ^{19}F NMR binding studies of the AE with 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP, **13**) in the presence or not of paraoxon, a phosphorylating irreversible inhibitor. Although utilization of ^{19}F NMR has been reported to investigate the mechanism of inhibition of JHE (12), chymotrypsin (13), carbonic anhydrase (14) or carboxypeptidase A (15), no such a study on the inhibition of AE by TFMKs

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has heretofore been described. Our study could provide useful clues for the development of new and more specific inhibitors of esterases (16, 17)

MATERIALS AND METHODS

TFMKs were previously synthesized in our laboratory (18). Tritiated pheromone (specific acitivity 137 mCi/mmole) was prepared as reported by us (Guerrero and Feixas, in press), from which a stock solution (5.64×10^{-2}M in hexane/toluene 95/5) was ready-to-use for the bioassays.

Insects were reared in our laboratory on a slightly modified diet from that previously reported (19). For inhibition bioassays, males of 1-2 days old were anesthetized with CO_2 and their antennae removed. The antennae were immediately frozen in liquid nitrogen and kept at −80° until use. Crude antennal esterase preparations were obtained by homogenizing batches of frozen antennae in 20 mM Tris-HCl buffer (pH=7.4) at 680 rpm on a variable speed Heidolph ZZR-2000 mixer for 5 min in an ice bath. The contents of the tube was transferred to an Eppendorf tube, sonicated at 40 w for 10 sec and centrifuged at 12000 g for 2 min at 4°C to remove the cuticular debris. On the other hand, in borosilicate tubes, previously treated with 1-decanol (20), were placed 2 μl of a 1×10^{-4}M soln. of tritiated pheromone in ethanol, 2 μl of 0.5-250 μM of the inhibitor in ethanol and 71 μl of Tris-HCl buffer. After vortexing for 10 sec, an aliquot fraction (25 μl) of the esterase extract equivalent to 2 antennae was added to the mixture, vortexed for 30 sec. and incubated at 25°C for 5 min on a thermostatized bath. In preincubation experiments the inhibitor and the extract in buffer soln. were incubated for 10 min before addition of the labeled substrate. In control experiments no inhibitor was added and instead 73 μl of buffer soln. were used. Incubation was stopped by addition of 200 μl of hexane:ethyl acetate 7:3. After vortexing for 1 min, aliquots of the organic solution were spotted on silica gel TLC plates on which samples of unlabeled pheromone acetate and alcohol had been previously deposited. Elution with hexane:ethyl acetate 4:1 and visualization with iodine afforded two spots (r_f=0.2 and 0.5 cm), corresponding to pheromone alcohol and acetate respectively, which were cut into polyethylene scintillation vials. The vials were diluted with 10 ml of Optiphase ''Hisafe'' 2, allowed to stand for 24 h and counted on a Rakbeta 1217 LKB scintillation counter. Every test was run in triplicate. Blank assays were also included in every set of experiments by incubation of 2 μl of substrate in 100 μl of buffer soln. The extent of hydrolisis was calculated by the ratio of radioactivity of the resulting tritiated alcohol with regard to the total radioactivity found. To the initial hydrolysis values of the inhibition and control experiments were subtracted those of the blank and the resulting data used for the inhibition calculation. Inhibitory potency of the chemicals was determined by percentage of the relative decrease of hydrolysis in the presence of inhibitor in relation to the hydrolysis values obtained in control experiments. IC_{50} of each compound was calculated by least squares regression analyses in duplicate experiments considering three different concentrations of inhibitor.

^{19}F NMR spectra were recorded on a Varian Unity 300 spectrometer, operating at 282 MHz, and the values are expressed in δ scale relative to trichlorofluoromethane used as internal standard. The measurements conditions were the following: pulse width 10 μs, acquisition time 1.0 s, spectral width 75329 Hz, pulse decay 1 s, 16 K data points, number of scans 320-1600. Spectra were run in 5 mm o.d. tubes under continuous or single-frequency decoupling. Samples were prepared as follows. To an extract of 160 antennae in 250 μl of 20 mM Tris-HCl buffer soln. (pH 7.4) was added 25 μl of a 14 mM soln. of OTFP in ethanol and the resulting solution was brought to 500 μl with D_2O. Thus, final concentration of the inhibitor was 0.7 mM. The mixture was incubated at 25°C for 15 min, cooled with ice and immediately placed into the NMR tube. In competition assays, 2 μl of a 16 mM soln. of paraoxon in ethanol were also added to give a final concentration of 64 μM of paraoxon. In experiments devoid of enzyme, 50 μl of a 14 mM soln. of OTFP in ethanol was used, followed by the addition of 225 μl of $(CD_3)_2CO$ and dilution to 500 μl with D_2O or CD_3OD.

RESULTS AND DISCUSSION

The compounds used as inhibitors were selected 1) to probe structural parameters at the active site of the AE, and 2) to provide new clues for the synthesis of more specific substrates and inhibitors. Most of the compounds analyzed were TFMKs but 3-octylthio-1,1,1-trifluoropropan-2-ol (OTFP-OH) was also included in our study since it had exhibited a unique inhibition effect on JHE (21). As shown in Table 1, some of the compounds are very potent inhibitors of the AE of the insect, particularly compounds **8, 9, 13, 14** and **4.** In general, β-thio derivatives resulted better inhibitors than the corresponding parent, devoid of sulfur, compounds, particularly in pre-incubation experiments. The chemicals exhibited higher potency when tested with a pre-incubation period, pointing out that the TFMKs tested are tight slow-binding inhibitors, as previously shown for other TFMKs (9, 22). Within the non-sulfur compounds and without pre-incubation, the most potent inhibitors resulted those whose structures closely mimic the

TABLE 1
Inhibition of Antennal Esterases of *Spodoptera littoralis* by Trifluoromethyl Ketones 1–16

$RCOCF_3$ R	Comp	Log P[a]	IC_{50} (μM)[b]	
			No preinc	With preinc
C_9H_{19}	**1**	3.59	38.2	N.D.
$C_{15}H_{31}$	**2**	6.70	10.9	4.17
$-(CH_2)_{10}-$	**3**	2.62	9.3	N.D.
Z11:$C_{14}H_{27}$	**4**	5.78	2.8	0.23
Z11:$C_{16}H_{31}$	**5**	6.82	4.9	1.59
Z9:$C_{18}H_{35}$	**6**	7.85	13.7	N.D.
Z9Z12:$C_{18}H_{33}$	**7**	7.45	10.7	N.D.
Z9E11:$C_{14}H_{25}$	**8**	5.37	1.4	0.14
Z10E12:$C_{15}H_{27}$	**9**	5.89	1.4	0.60
YN11:$C_{16}H_{29}$	**10**	6.41	6.3	N.D.
Z14YN12:$C_{17}H_{29}$	**11**	6.52	5.6	N.D.
$C_6H_{13}SCH_2$	**12**	1.99	14.9	0.14
$C_8H_{17}SCH_2$	**13**	3.03	1.5	0.08
$C_{10}H_{21}SCH_2$	**14**	4.07	2.2	0.18
$C_{12}H_{25}SCH_2$	**15**	5.11	4.9	1.18
$C_{15}H_{31}SCH_2$	**16**	6.67	17.6	9.55

[a] Log P values were calculated by the FRAGMENT method of Hansch and Leo (25) and corrected by Rekker and Mannhold (26).

[b] IC_{50} values were calculated by least squares regression analysis ($r^2 > 0.94$) from three separate inhibitor concentrations in duplicate experiments.

natural pheromone (Z,9-E,11-tetradecadienyl acetate, Z9E11:$C_{14}H_{25}OAc$), i.e. dienes **8** and **9.** Following this trend, monoene **4** with one double bond at position 11, like in the pheromone structure, also displayed a notable inhibitory action. Introduction of another TFMK group (compound **3**) improved the activity in comparison to that of a monofunctionalized compound of similar chain length (compound **1**). Elongation of the chain at the non-polar end of the molecule or displacement of the double bond to the center of the structure, like in compounds **5** and **6,** implied decrease of activity. Replacement of a double by a triple bond did not apparently induce any effect (compare compounds **5** and **10**). On the other hand, it is interesting to note that compound **11,** a structurally related analogue of the processionary moth sex pheromone (Z,13-hexadecen-11-ynyl acetate, Z13YN11:$C_{16}H_{27}OAc$) but 2 carbons longer than diene **8,** displayed a fair inhibition effect (IC_{50} 5.6 μM). TFMKs **4, 8, 9** and **11** contain the same $CH_3CH_2CH{=}CH$ function at the far end of the molecule, as in the original pheromone structure, suggesting that this group is important for esterase inhibitory activity. Compound **11** also significantly inhibited the pheromone response in the field when mixed with the natural pheromone in several ratios (23).

β-Thiotrifluoromethyl ketones were the most potent AE inhibitors found, particularly **13** with and IC_{50} of 0.08 μM with preincubation. The inhibitory potency was chain length-dependent, and thus plotting log $1/IC_{50}$ vs number of carbon atoms in the chain resulted in a parabolic curve with an inflexion point at n=8 (not shown). OTHP-OH exhibited without preincubation a poor IC_{50} 69.9 μM, implying that the keto group is the actual reactive species for a serine residue of the enzyme.

In correlation studies activity-log P (octanol-water partition coefficient) we found that, among the β-thio derivatives, the best inhibitors exhibited log P values in the range 3.03-5.11. Plot of log P vs log $1/IC_{50}$ showed a parabolic curve with an inflection point for OTFP (Figure

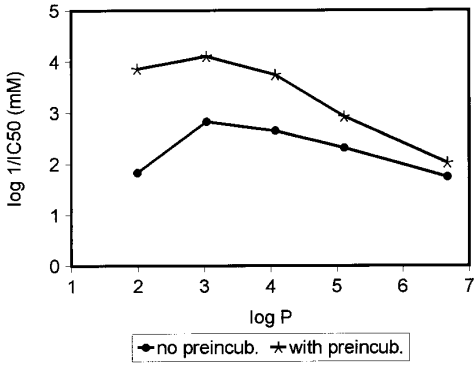

FIG. 1. Plot of inhibitory activity (log $1/IC_{50}$) vs lipophilicity (log P) of β-thiotrifluoromethyl ketones **12-16** with and without preincubation.

1). The range of optimum activity is similar to that reported by Thomas et al. (10). Higher lipophilicity was required for the parent TFMKs to elicit a prominent inhibition effect, the optimum log P range being 5.37-5.89. In this case and from the results shown in the table, it is apparent that, in addition to the lipophilic character of the chemicals, other parameters, like some structural and electronic features of the compounds, are also important factors in the interaction enzyme-inhibitor.

^{19}F NMR studies have also been conducted in order to study more precisely the type of interaction enzyme-inhibitor (Figure 2). Among the TFMKs we selected OTFP and found that this ketone is hydrated in a 1:1 mixture of Tris-HCl buffer soln. and D_2O giving one signal at δ-83.63 ppm (A in Fig. 2). In the presence of the enzyme, the spectrum showed a much broader signal (linewidth 156 Hz) centered at δ-83.32 ppm, clearly indicating binding of the inhibitor with the enzyme. Moreover, the chemical shift corresponded to a species of tetrahedral geometry, pointing out to the presence of a hydrate or hemiacetal (B in Fig. 2). To rule out the possibility of an acetal type species, a 1:1 mixture of OTFP and CD_3OD in $(CD_3)_2CO$ showed two peaks at δ-84.14 and -80.25 ppm, the former being transformed into the latter after standing at room temperature for 24 h (not shown). It was concluded that the hydrate initially formed (δ-84.14) was directly converted into the acetal (δ-80.25) with no hemiacetal being detected. When OTFP was mixed with the esterase extract in the presence of an excess of paraoxon, an irreversible phosphorylating enzyme inhibitor, the linewidth of the signal decreased to 51 Hz and shifted upfield to δ-83.7 ppm, a very close value to that assigned previously to the free hydrate in D_2O. Therefore, paraoxon irreversibly displaced the enzyme-OTFP complex and since phosphorylation occurs specifically by binding a serine residue of the active site (24), it is likely that the interaction inhibitor-active site occurs through formation of an hemiacetal (C in Fig. 2). In a control experiment, run with OTFP and paraoxon in buffer soln. but without enzyme, the spectrum clearly ressembled that obtained without paraoxon, i.e. with the hydrate as the main signal (δ-83.63 ppm) (D in Fig. 2). No apparent interaction OTFP-paraoxon was therefore observed. In general, these results are in agreement with those reported by Linderman et al. (12) on the mode of action of OTFP as inhibitor of acetylcholinesterase.

Our studies confirm that some of the TFMKs tested are potent AE inhibitors, reversibly binding the active site residue of the enzyme by the carbonyl group and forming an adduct (probably an hemiacetal) of tetrahedral geometry. Our results also show that not only the high electrophilic character of the TFMK is necessary for inhibitory activity, but also certain structural features of the inhibitors. The trifluoromethyl group plays undoubtedly the major role,

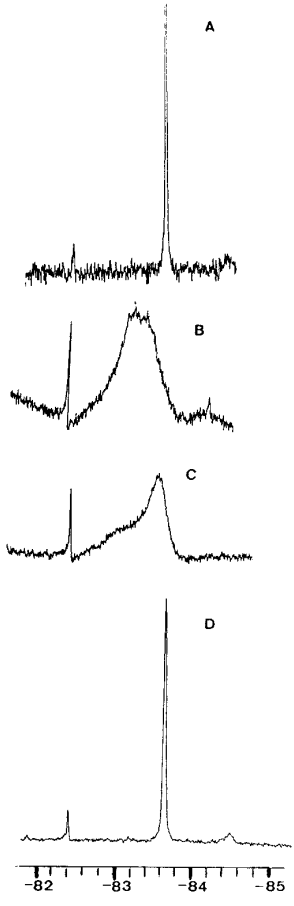

FIG. 2. ^{19}F NMR spectra of the inhibition of ARE of *Spodoptera littoralis* by OTFP. (A) The ketone is hydrated in a 1:1 mixture of D_2O with buffer soln. (pH 7.4). (B) Signal corresponding to OTFP-enzyme bound complex is shifted downfield and markedly broadens. (C) In the presence of paraoxon, the OTFP-enzyme complex is displaced by the phosphorylating inhibitor, causing upfield shift of the signal. The linewidth also decreases (part of the OTFP-enzyme complex remains bound). (D) Spectrum of OTFP in the presence of paraoxon. Absorption at δ −82.4 ppm corresponds to an impurity.

but the non-polar end of the molecule, containing the propenyl group as in the natural pheromone structure, is also important for a successful interaction with the enzyme.

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