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Difluoromethyl ketones: Potent inhibitors of wild type and carbamate-insensitive G119S mutant *Anopheles gambiae* acetylcholinesterase

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ABSTRACT

Malaria is a devastating disease in sub-Saharan Africa, and current vector control measures are threatened by emerging resistance mechanisms. With the goal of developing new, selective, resistance-breaking insecticides we explored α-fluorinated methyl ketones as reversible covalent inhibitors of *Anopheles gambiae* acetylcholinesterase (*Ag*AChE). Trifluoromethyl ketones **5** demonstrated remarkable volatility in microtiter plate assays, but **5c**,**e**–**h** exhibited potent (1–100 nM) inhibition of wild type (WT) *Ag*AChE and weak inhibition of resistant mutant G119S mutant *Ag*AChE. Fluoromethyl ketones **10c**–**i** exhibited submicromolar to micromolar inhibition of WT *Ag*AChE, but again only weakly inhibited G119S *Ag*AChE. Interestingly, difluoromethyl ketone inhibitors **9c** and **9g** had single digit nanomolar inhibition of WT *Ag*AChE, and **9g** had excellent potency against G119S *Ag*AChE. Approach to steady-state inhibition was quite slow, but after 23 h incubation an IC_{50} value of 25.1 ± 1.2 nM was measured. We attribute the slow, tight-binding G119S *Ag*AChE inhibition of **9g** to a balance of steric size and electrophilicity. However, toxicities of **5g**, **9g**, and **10g** to adult *A. gambiae* in tarsal contact, fumigation, and injection assays were lower than expected based on WT *Ag*AChE inhibition potency and volatility. Potential toxicity-limiting factors are discussed.

Malaria is a devastating disease, responsible for an estimated 584,000 deaths world-wide in 2013.[1] Efforts to control the vector *Anopheles gambiae* have significantly reduced malaria mortality, through the use of insecticide treated nets (ITN) and indoor residual spraying (IRS).[2] To date these methods rely on only two biological targets: the voltage-gated sodium ion channel and acetylcholinesterase (AChE).[3] Pyrethroids modulate the sodium channel and are approved by the World Health Organization (WHO) for IRS and for use on ITNs.[4] Organophosphate and carbamate AChE inhibitors (AChEI) are approved only for IRS.[4] Due to the growing emergence of pyrethroid-resistant strains of *A. gambiae*,[5] there is increased interest in developing classes of AChEI that might be safe and effective on ITNs. Our group has previously reported that γ-branched 2-substituted aryl methylcarbamates (e.g., **1**, Fig. 1) can be highly selective for inhibition of *A. gambiae* AChE (*Ag*AChE) over human AChE (*h*AChE).[6] We have also developed five-membered ring heterocycle core carbamates and carboxamides (e.g., **2** and **3**, Fig. 1) that offer good toxicity against the carbamate-resistant (Akron) strain *A. gambiae*.[7] This strain of *A. gambiae* is known to carry a G119S mutant AChE,[7a,8] and the smaller core structure of these heterocyclic carbamates and carboxamides may partly account for their good inhibition of G119S *Ag*AChE.

To further address the need for new insecticides, we sought to investigate underexplored AChEI chemotypes **4**–**8** (Fig. 1). Trifluoromethyl ketones (e.g., **4**, **5a**,**b**) have been studied as inhibitors of AChE[9] and juvenile hormone esterase.[10] Despite the remarkable (picomolar) potencies that can be achieved,[11] with few exceptions (e.g., **4**)[10b] this class of compounds has received little attention as insecticides. These highly electrophilic compounds form tetrahedral intermediates with the catalytic serine of AChE, as demonstrated by X-ray crystallography.[12] Zifrosilone (**5b**) was

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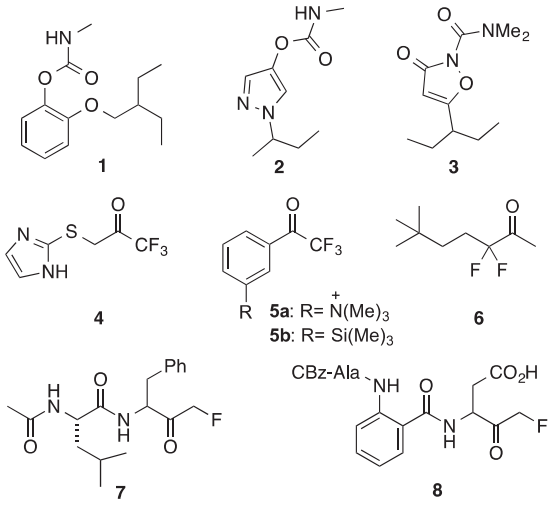
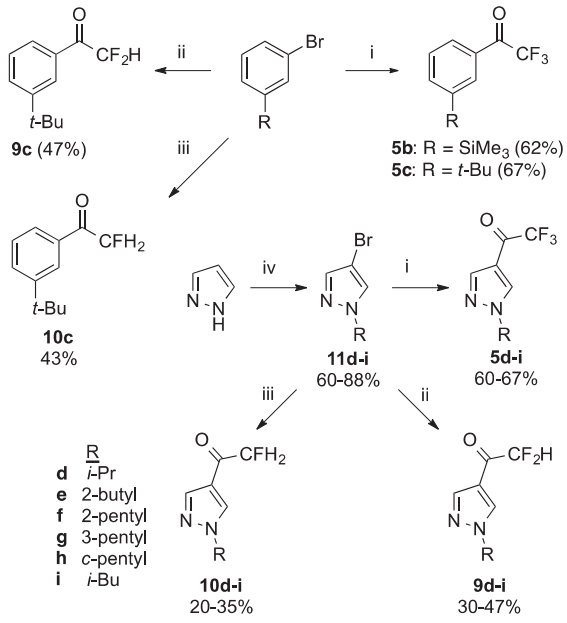


Figure 1. Mosquitocidal AChE inhibitors **1**–**3** and select fluorinated ketones (**4**–**8**) used as AChE or serine/cysteine protease inhibitors.

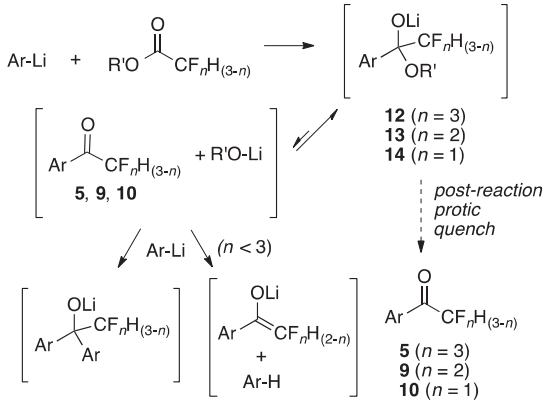
evaluated as an AChEI for the treatment of memory loss in Alzheimer's disease,[13] suggesting that the CNS penetrance needed for insecticidal action could be achieved with appropriate structural modification. Difluoromethyl ketones have received limited attention as AChE inhibitors, although α,α-difluoroalkyl ketone **6** was shown to be quite potent (K_i = 1.6 nM) at electric eel AChE.[9b] More commonly, these difluorinated ketones have been explored as inhibitors of serine proteases such as chymotrypsin,[14] α-lytic protease,[15] human leukocyte elastase,[15] and thrombin.[16] Perhaps due to the expectation that reduced electrophilicity would adversely impact inhibition potency, fluoromethyl ketones have (to our knowledge) not been reported as AChE inhibitors. Although **7** proved to be a very weak inhibitor of the serine protease chymotrypsin,[14] dipeptidyl aspartyl fluoromethyl ketones such as **8** can be potent inhibitors of cysteine proteases.[17]

We thus synthesized a series of tri-, di-, and (mono)fluoromethyl ketones bearing substituted benzene and pyrazol-4-yl substituents (Scheme 1). Trifluoromethyl ketones **5b–i** were prepared by the literature route for **5c**: metal–halogen exchange of the appropriate aryl/heteroaromatic bromide and trapping with CF_3CO_2Me (Scheme 1).[9d] The requisite *N*-alkyl-4-bromopyrazoles **11d–i** were prepared in two steps from pyrazole.[18] The preponderance of α-branched alkyl groups selected reflects the observation that these substituents increase *Ag*AChE inhibition potency of pyrazol-4-yl[7a] carbamates and 3-oxoisoxazole-2(3*H*)carboxamides (e.g., **2**, **3**).[7b] Difluoromethyl ketones **9c–i** and fluoromethyl ketones **10c–i** were prepared by trapping with CF_2HCO_2Me and CFH_2CO_2Et, respectively. Yields of the trifluoromethyl ketones **5b–i** were moderate, and in part reflects the high volatilities of these compounds. However, yields of the difluoro- and fluoromethyl ketones **9c–i** and **10c–i** were only poor to fair. We attribute these poor yields to partial collapse of tetrahedral adducts **13** and **14** to the fluorinated methyl ketones **9** and **10** prior to quench, and reaction with Ar–Li (Scheme 2). Based on the relative electrophilicity of the fluorinated ketones, the extent of collapse prior to protic quench should be **14** > **13** > **12**, which could account for the trend in chemical yield. Finally to assess the structure of these compounds in aqueous solution, ^{19}F NMR spectroscopic studies[9c] were performed at pH 7.7. Pyrazol-4-yl trifluoromethyl ketone **5g** was 67% hydrated, difluoromethyl ketone **9i** was 22% hydrated, and fluoromethyl ketone **10i** was <5% hydrated (24 h, see Supplementary data).[19]

Enzyme inhibitory activity of the compounds were assessed using a modified Ellman assay[20] in a 96-well microtiter plate



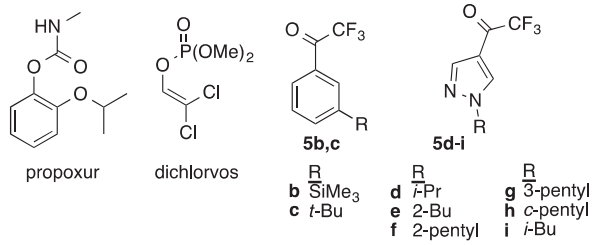
Scheme 1. Synthesis of α-fluorinated ketones. Reagents and conditions: (i) *n*-BuLi, THF, −78 °C, 2 h; $CH_3OC(O)CF_3$, −78 °C to RT, overnight; (ii) *n*-BuLi, THF, −78 °C, 2 h; $CH_3CH_2OC(O)CF_2H$, −78 °C, 5 min; (iii) *n*-BuLi, THF, −78 °C, 2 h; $CH_3CH_2OC(O)CFH_2$, −78 °C, 5 min; (iv) NBS, H_2O, 1 h; NaH, DMF, 0 °C, 1 h; R-Br, RT, overnight.



Scheme 2. Possible side reactions in the synthesis of fluorinated ketones **5**, **9**, **10**.

format previously reported.[7a] Because time-dependent inhibition of AChE by trifluoromethyl ketones is well-documented,[9a,c,e] enzyme velocities (v/v_0) were measured as a function of inhibitor concentrations [*I*] at incubation times of 10 min and 60 min. Sigmoidal plots of residual enzyme activity (v/v_0) versus [*I*] were constructed, from which the IC_{50} values were obtained. For the purpose of comparison we also examined the commercial carbamate propoxur, since it has excellent contact activity against susceptible (G3) strain *A. gambiae*, but poor toxicity against carbamate-resistant (Akron) strain *A. gambiae*.[6b,7a] Like all carbamate insecticides, propoxur carbamoylates the active site serine of AChE,[21] and its time-dependent inhibition of *h*AChE and WT *Ag*AChE is evident in Table 1. However this compound was a very weak inhibitor of G119S *Ag*AChE at 10 or 60 min incubation times (IC_{50} > 10,000 nM), consistent with the carbamate resistance phenotype this mutation confers. The organophosphate inhibitor dichlorvos was also examined, and the potency and time-dependence of its inhibition of *h*AChE and WT *Ag*AChE was similar to that of propoxur. However unlike propoxur, dichlorvos

Table 1
Inhibition IC_{50} values for propoxur, dichlorvos and trifluoromethyl ketones **5b–i** against *h*AChE and *Ag*AChE (WT and G119S)



Compound	Incubation time (min)	*h*AChE IC_{50}[a] (nM)	WT *Ag*AChE IC_{50}[a] (nM)	G119S *Ag*AChE IC_{50}[a] (nM)
Propoxur	10	2300 ± 50	182 ± 3	>10,000
	60	590 ± 19	43.6 ± 1.0	>10,000
Dichlorvos	10	2310 ± 60	324 ± 7	1650 ± 44
	60	627 ± 12	60.1 ± 0.9	300 ± 7
5b	10	285 ± 15	848 ± 44	>10,000
	60	92.9 ± 5.8	257 ± 15	10,100 ± 500
5c	10	14.6 ± 0.2	53.4 ± 0.8	>10,000
	60	5.00 ± 0.16	18.1 ± 0.4	20,800 ± 1900
5d	10	77.5 ± 2.2	142 ± 5	>10,000
	60	121 ± 3	285 ± 7	>10,000
5e	10	6.47 ± 0.15	2.47 ± 0.04	8180 ± 450
	60	8.84 ± 0.28	2.29 ± 0.17	2340 ± 90
5f	10	7.15 ± 0.11	3.74 ± 0.08	19,900 ± 2400
	60	8.37 ± 0.16	2.06 ± 0.06	2000 ± 140
5g	10	3.77 ± 0.06	2.75 ± 0.04	>10,000
	60	2.27 ± 0.04	0.68 ± 0.01	1730 ± 140
5h	10	5.20 ± 0.15	2.87 ± 0.07	>10,000
	60	6.11 ± 0.18	1.54 ± 0.08	3520 ± 140
5i	10	591 ± 11	950 ± 19	>10,000
	60	678 ± 15	1040 ± 20	>10,000

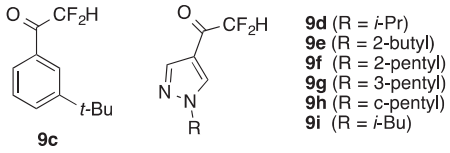
[a] Measured at 23 ± 1 °C, pH 7.7, 0.1% (v/v) DMSO; all enzymes are recombinant. Average Hill slopes at WT *Ag*AChE and *h*AChE are 1.0 ± 0.2.

exhibited significant inhibition of G119S *Ag*AChE, likely due to the smaller structure of its enol leaving group. The 5-fold ratio of G119S and WT IC_{50} values is very similar to that measured in a previous study.[22]

Trifluoromethyl ketones **5b–i** showed varying degrees of time-dependence to their inhibition of *h*AChE and WT *Ag*AChE; those bearing a phenyl group (**5b**,**c**) showed time-dependent inhibition of both *h*AChE and *Ag*AChE. However, those trifluoromethyl ketones bearing a pyrazol-4-yl substituent approach steady-state inhibition within 10 min, and **5e–h** gave single-digit nanomolar IC_{50} values. In contrast, compound **5i**, which bears a β-branched isobutyl group, was considerably less potent at *h*AChE and WT *Ag*AChE than any of the pyrazol-4-yl compounds bearing α-branched substituents (**5d–h**). Although potent inhibition of WT *Ag*AChE can be achieved with a pyrazol-4-yl trifluoromethyl ketone, this structure confers no inhibition selectivity against *h*AChE (Table 1).

In addition, none of these inhibitors offered potent inhibition of G119S *Ag*AChE, most likely due to crowding in the oxyanion hole caused by the glycine to serine mutation. Compound **5g** proved most potent at this enzyme, but its 1730 nM IC_{50} value after 60 minutes incubation is roughly 2500-fold greater than the 0.7 nM value observed for WT *Ag*AChE. Finally **5d** and **5i** curiously exhibit higher *h*AChE and WT *Ag*AChE IC_{50} values at 60 min than at 10 min. We attribute this phenomenon to rapid attainment of steady state, and evaporation of **5d** and **5i** out of the well plate during the longer incubation. As we will illustrate below, fluorinated compounds can be remarkably volatile, and **5d** has the lowest molecular weight of all the trifluoromethyl ketones tested.

Table 2
Inhibition IC_{50} values for difluoromethyl ketones **9c–i** against *h*AChE and *Ag*AChE (WT and G119S)



Compound	Incubation time (min)	*h*AChE IC_{50}[a] (nM)	WT *Ag*AChE IC_{50}[a] (nM)	G119S *Ag*AChE IC_{50}[a] (nM)
9c	10	6.05 ± 0.11	9.12 ± 0.31	1650 ± 100
	60	8.69 ± 0.18	9.79 ± 0.32	996 ± 39
9d	10	802 ± 30	354 ± 8	8290 ± 400
	60	869 ± 20	430 ± 7	8380 ± 420
9e	10	110 ± 2	26.3 ± 0.4	452 ± 12
	60	85.9 ± 1.6	25.2 ± 0.6	185 ± 7
9f	10	149 ± 2	23.4 ± 0.4	797 ± 20
	60	158 ± 3	29.1 ± 0.4	297 ± 6
9g	10	28.8 ± 0.7	1.01 ± 0.02	680 ± 43
	60	35.2 ± 0.7	1.22 ± 0.03	125 ± 6
	330	ND	ND	36.7 ± 1.7
	1380	ND	ND	25.1 ± 1.2
9h	10	208 ± 5	106 ± 2	3220 ± 150
	60	244 ± 5	134 ± 2	3390 ± 110
9i	10	9780 ± 470	3000 ± 80	>10,000
	60	11,000 ± 400	3950 ± 80	>10,000

[a] Measured at 23 ± 1 °C, pH 7.7, 0.1% (v/v) DMSO;. ND signifies not determined. Average Hill slopes at WT *Ag*AChE, *h*AChE, and G119S *Ag*AChE (**9c**–**9g** only for this enzyme) are 0.9 ± 0.1.

Difluoromethyl ketones **9c–i** were then examined for their enzyme inhibition properties (Table 2). As expected, IC_{50} values for the difluoromethyl ketones at *h*AChE and WT *Ag*AChE were generally higher than the values for the corresponding trifluoromethyl ketones (Table 1), with a few noteworthy exceptions. Difluoromethyl ketone **9c** was more potent than trifluoromethyl analog **5c** at both *h*AChE and WT *Ag*AChE, and difluoromethyl ketone **9g** was similar in potency to trifluoromethyl analog **5g** at WT *Ag*AChE. Both compounds have rather large alkyl substituents (*t*-Bu and 3-pentyl, respectively), which suggests that in some cases the smaller size of the $-CF_2H$ group compared to $-CF_3$ can compensate for its lower electron-withdrawing ability. This effect also appears to be operative in the inhibition of G119S *Ag*AChE, which has a more crowded oxyanion hole than WT *Ag*AChE. As can be seen in Table 2, G119S *Ag*AChE IC_{50} values of difluoromethyl ketones **9c–h** are uniformly lower than those of the corresponding trifluoromethyl ketones (Table 1). In addition, time-dependent inhibition is seen for the G119S enzyme: after a 60 min incubation the G119S *Ag*AChE IC_{50} value of **9g** is 125 nM, 13-fold lower than that of trifluoromethyl ketone **5g**, and 2-fold lower than that of dichlorvos. Steady-state inhibition of G119S *Ag*AChE by **9g** is nearly attained after 330 min incubation, but after 1380 min (23 h) an IC_{50} value of 25.1 ± 1.2 nM was measured (Table 2). Thus in the case of **9g**, the smaller size of the $-CF_2H$ group effectively compensates for its lower electron-withdrawing power, creating a slow, tight-binding inhibitor of G119S *Ag*AChE.

To rationalize the drastically different potencies of trifluoromethyl ketone **5g** at WT *Ag*AChE and G119S *Ag*AChE (IC_{50} values 0.68 and 1730 nM, respectively, at 60 min), and the high potency of difluoromethyl ketone **9g** for G119S *Ag*AChE, we examined the computed structures of these compounds bound to the enzymes (Fig. 2). As a starting point for docking studies we used previously developed homology models of WT and G119S *Ag*AChE[7a] based on the published X-ray structure of mouse AChE complexed to **5a** (PDB ID 2H9Y).[12c] Flexible ligand docking of the tetrahedral intermediates derived from **5g** and WT *Ag*AChE, and of **9g** and

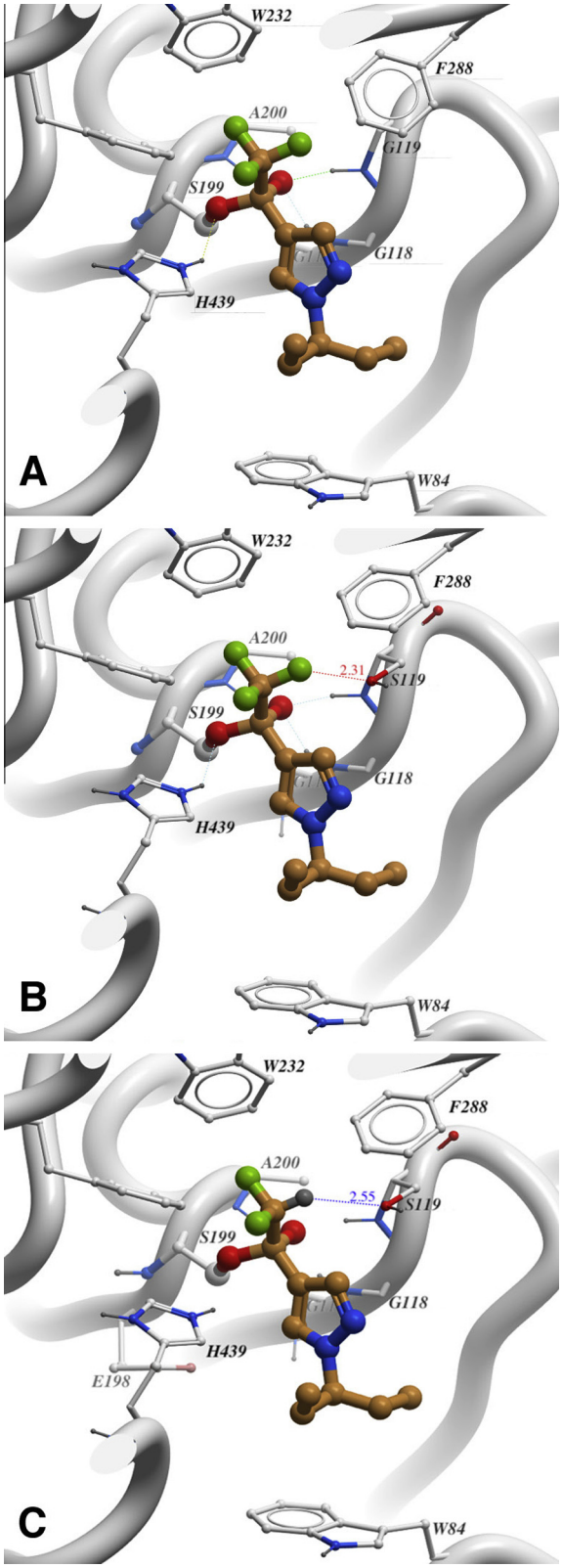


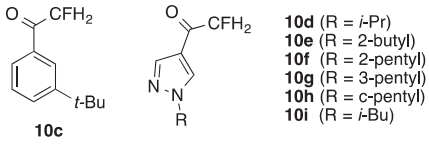
Figure 2. (A) Trifluoromethyl ketone **5g** bound to S199 of WT *Ag*AChE. (B) **5g** bound to S199 of G119S *Ag*AChE; repulsive non-bonded contact of the S119 hydroxy group with the CF_3 group is evident. (C) Difluoromethyl ketone **9g** bound to S199 of G119S *Ag*AChE; a potential hydrogen bond from the CF_2-H of **9g** to the oxygen of S119 is indicated.

G119S *Ag*AChE were performed in ICM using default settings for 'covalent' docking mode (ICM-Docking module, Molsoft) to generate Figure 2A and C.[23] To identify steric conflicts associated with the G119 to S119 mutation, the covalent adduct of **5g** with WT *Ag*AChE was superimposed with the G119S *Ag*AChE apo structure (Fig. 2B). As can be seen in Figure 2A, trifluoromethyl ketone **5g** can easily bind to the catalytic serine (S199) of WT *Ag*AChE, as expected from the X-ray structures of **5a** bound to *Tc*AChE and *m*AChE (PDB ID 1AMN[12a] and 2H9Y,[12c] respectively). However, as shown in Figure 2B, replacement of G119 with S119 causes steric repulsion between the S119 hydroxy group and one of the fluorine atoms of the CF_3 group of **5g**: the indicated O–F distance of 2.31 Å is significantly shorter than the sum of the respective van der Waals radii (2.99 Å). However in the complex of G119S *Ag*AChE with the analogous difluoromethyl ketone **9g** (Fig. 2C), this unfavorable interaction is replaced with a potential hydrogen bond from the hydrogen of the HCF_2 group to the S119 oxygen. The CF_2H group is a known H-bond donor;[24] in this way the excellent inhibitory potency of **9g** for G119S *Ag*AChE can be rationalized.

Turning to the fluoromethyl ketones **10c–i**, much weaker inhibition of *h*AChE and WT *Ag*AChE is seen compared to that of difluoromethyl ketones **9c–i** and trifluoromethyl ketones **5c–i** (Table 3). This outcome is understandable in view of the low electrophilicity of the fluoromethyl ketones. Two results stand out, however. Firstly, fluoromethyl ketone **10g** is a ~350 nM inhibitor of WT *Ag*AChE. At a 10 min incubation time, its IC_{50} value is roughly 2-fold higher than that of propoxur (Table 1). Secondly, compound **10g** also displayed micromolar inhibition of G119S *Ag*AChE. Thus given the appropriate pyrazol-4-yl substituent, fluoromethyl ketones evidence inhibition of both WT and G119S *Ag*AChE.

During our microtiter plate inhibition assay development we were surprised to find that trifluoroketones appeared to migrate from high [*I*] wells to the adjacent inhibitor-free control wells.[25] We further observed that the spatial extent of this migration was significantly enhanced when the microtiter plate was covered with its accompanying 'non-sealing' polystyrene lid. We believe the loose cover provided by the lid serves to direct evaporation to the neighboring wells rather than to allow it to escape to the atmosphere. Progressive 'distillation' of **5g** in such a loosely covered microtiter plate format is demonstrated in Figure 3: although the inhibitor was placed only in wells D6–D7, after

Table 3
Inhibition IC_{50} values for fluoromethyl ketones **10c–i** against *h*AChE and *Ag*AChE (WT and G119S)



Compound	Incubation time (min)	*h*AChE IC_{50}[a] (nM)	WT *Ag*AChE IC_{50}[a] (nM)	G119S *Ag*AChE IC_{50}[a] (nM)
10c	10	578 ± 13	726 ± 12	>10,000
	60	715 ± 15	953 ± 18	>10,000
10d	10	>10,000	>10,000	>10,000
	60	>10,000	>10,000	>10,000
10e	10	16,900 ± 1200	2750 ± 60	>10,000
	60	18,000 ± 1900	2860 ± 60	>10,000
10f	10	24,200 ± 2700	3520 ± 70	>10,000
	60	17,600 ± 1800	3290 ± 80	>10,000
10g	10	5290 ± 250	355 ± 11	2990 ± 160
	60	5190 ± 270	337 ± 11	3000 ± 160
10h	10	2500 ± 80	5860 ± 160	>10,000
	60	1310 ± 30	1540 ± 40	>10,000
10i	10	>10,000	>10,000	>10,000
	60	>10,000	>10,000	>10,000

[a] Measured at 23 ± 1 °C, pH 7.7, 0.1% (v/v) DMSO; Average Hill slopes at WT *Ag*AChE and *h*AChE are 0.9 ± 0.1 and 0.8 ± 0.1, respectively (compounds **10d**, **10i** excluded).

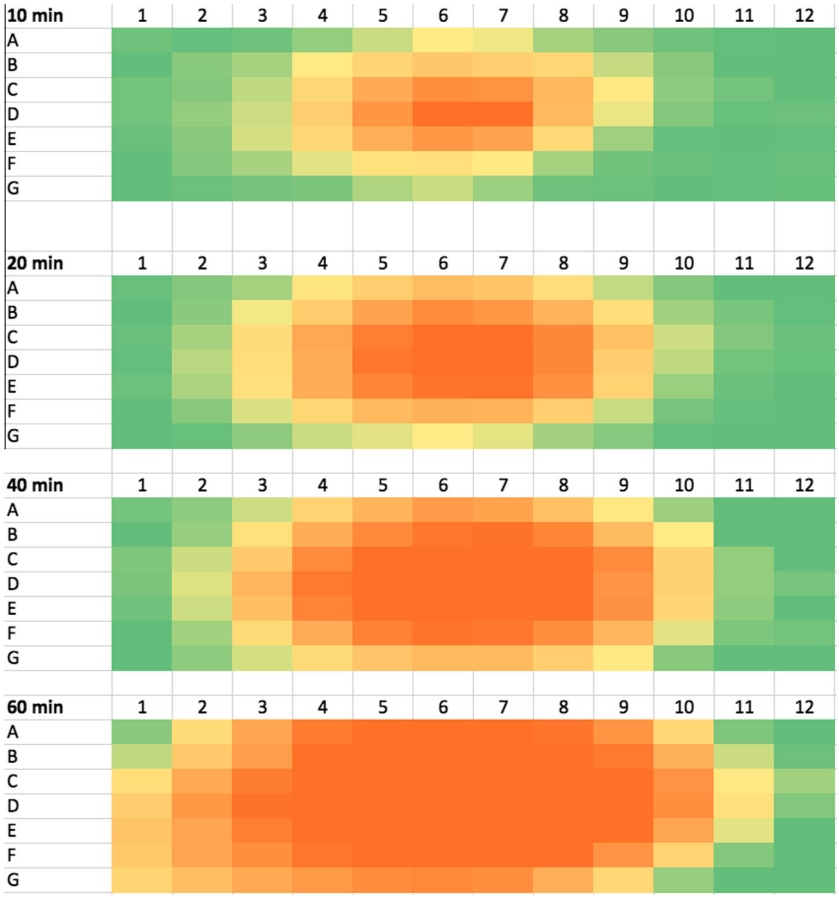


Figure 3. Microtiter plate heat maps of WT *Ag*AChE residual activity in which only wells D6–D7 of the microtiter plates were charged with 10,000 nM of inhibitor **5g** for the indicated incubation time (10–60 min, 23 ± 1 °C) before the addition of substrate. Data for Row H (enzyme-free background wells) are not shown. Color coding: red, ⩽10% residual activity; yellow, ⩽75% residual activity; green ⩾93% residual activity. Progressive vapor phase diffusion of **5g** over 60 min is evident. See Supplementary data for residual activity values.

10 min significant enzyme inhibition is visible up to 3 wells away. Further spreading is evident at 20 and 40 min, and at 60 min nearly every well evidences contamination by **5g**. In a 10 min incubation study of a homologous series of compounds, volatility was seen to decrease from **5g** (CF_3) to **9g** (CF_2H) to **10g** (CFH_2) (Fig. 4). Whereas trifluoromethyl ketone **5g** diffuses over 3 wells from D6–D7 in 10 min, difluoromethyl ketone **9g** diffuses only 1 well. In contrast, no diffusion of fluoromethyl ketone **10g** seen over 10 min. Thus the degree of fluorination plays a dominant role in the volatility of this series of analogs. Interestingly, no diffusion of dichlorvos was seen over 10 min, suggesting it is less volatile than **5g** and **9g** (see Fig. S1, Supplementary data). Finally, as expected, no diffusion of the propoxur control is seen.

As noted above, several of the fluorinated methyl ketone inhibitors (**5c**–**h**, **9e**–**h**) demonstrated potent inhibition of WT *Ag*AChE. We thus examined the tarsal contact toxicity of select compounds to adult susceptible (G3) strain *A. gambiae*, using the recommended WHO treated paper protocol[26] (Table 4). As can be seen none of the compounds tested approach the contact toxicity of propoxur, despite the fact that many (**5c**, **5e**–**5h**, **9c**, **9e**–**9g**) are much more potent inhibitors of WT *Ag*AChE than propoxur after a 60 min incubation. The most toxic compounds tested were fluoromethyl ketones **10d** and **10g** (85% mortality at 1000 μg/mL). Yet these compounds differ dramatically in their inhibition of WT *Ag*AChE, giving IC_{50} values of >10,000 nM and 337 ± 11 nM, respectively (60 min incubation). Therefore there is no apparent correlation between tarsal contact % mortality and AChE inhibition potency for these compounds. The procedure for this toxicity assay[26] mandates that treated papers be dried overnight to remove the solvent vehicle prior to mosquito exposure. Could extensive evaporation of tri- and difluoroketones (all liquids) from the treated papers prior to mosquito exposure account for the low and variable toxicities seen in Table 4? To assess this possibility, we measured the evaporative weight loss of **5g** at room temperature, and compared it to propoxur (mp 91 °C) and dichlorvos (liquid at room temperature).

As can be seen in Table 5, water evaporated completely within 1 day, and propoxur showed no weight loss over 28 days. The liquid organophosphate inhibitor dichlorvos lost 32 ± 1% of its mass over 12 days, and 51 ± 1% over 28 days. Trifluoromethyl ketone **5g** proved even more volatile than dichlorvos, losing 68 ± 1% of its mass over 12 days, and 99 ± 1% over 28 days. However over 1 day, **5g** lost only 9 ± 1% of its mass. Thus the ~25-fold lower tarsal contact toxicity of **5g** (40% mortality at 1000 μg/mL) relative to propoxur (LC_{50} = 39 μg/mL) cannot be attributed solely to compound evaporation.

Since poor penetration of the exoskeleton following tarsal contact could impede delivery of these compounds to the mosquito CNS, we explored the toxicity of the most potent trifluoromethyl, difluoromethyl and fluoromethyl ketone inhibitors of *Ag*AChE-WT (**5g**, **9g**, and **10g**, respectively) in two other assays. First we examined the toxicity of these compounds in a fumigation assay,[27] whereby *A. gambiae* were placed in a sealed 1 L vessel containing a known mass of an AChE inhibitor, but prevented from direct physical contact with the compound (Table 6). As expected, the nonvolatile compound propoxur was completely non-toxic at a high nominal concentration of 1000 ng/mL.

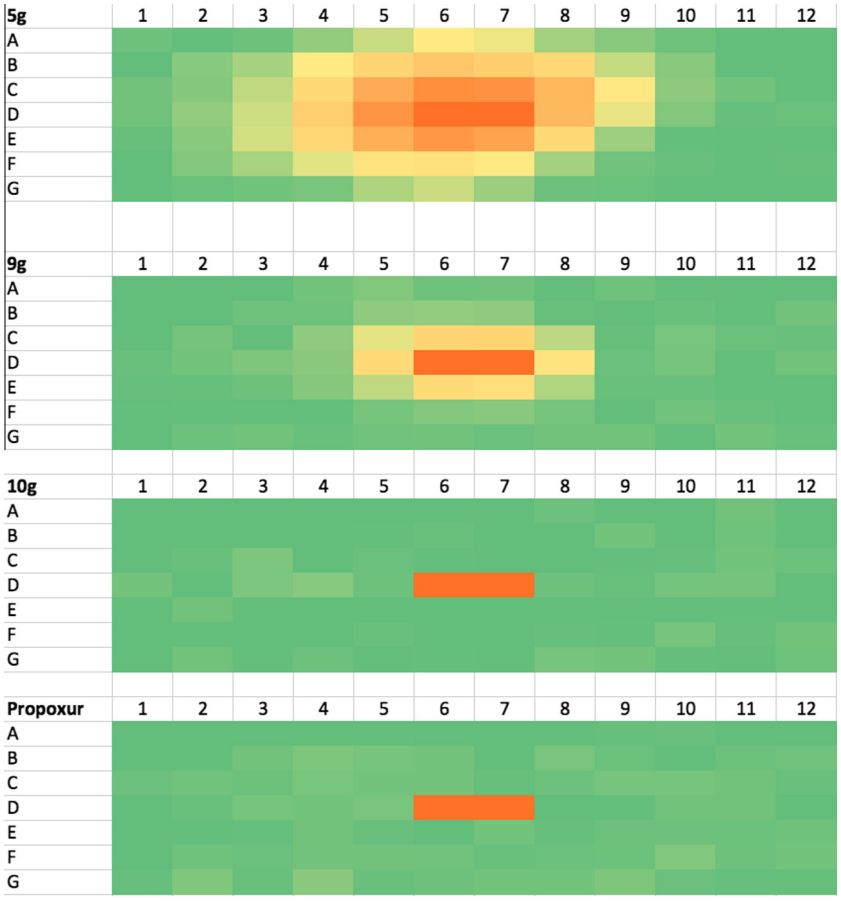


Figure 4. Microtiter plate heat maps of WT *Ag*AChE residual activity (10 min incubation) in which only wells D6–D7 of the microtiter plates were charged with 10,000 nM of inhibitor (**5g**, **9g**, **10g**, propoxur: 23 ± 1 °C). Data for Row H (enzyme-free background wells) are not shown. Color coding follows that of Figure 3. Vapor phase diffusion of **5g** and **9g** is evident. See Supplementary data for residual activity values.

Table 4
Tarsal contact[a] toxicity of select fluorinated methyl ketones to susceptible (G3) strain adult *A. gambiae*

Compound	LC_{50} (μg/mL) or % mortality at 1000 μg/mL	Compound	LC_{50} (μg/mL) or % mortality at 1000 μg/mL
Propoxur	39 (32–45)[b]	**9f**	0
5b	10	**9g**	0
5c	8	**9h**	0
5d	20	**10c**	50
5e	30	**10d**	85
5f	0	**10e**	65
5g	40	**10f**	20
5h	40	**10g**	85
9c	20	**10h**	0
9d	50	**10i**	20
9e	50		

[a] Papers were treated with ethanolic solutions of fluorinated methyl ketones and allowed to dry overnight. Mosquitoes were exposed (1 h) and mortality was recorded after 24 h.
[b] LC_{50} values derive from the concentrations of inhibitor used to treat the paper; 95% confidence limits are given in parenthesis. Data for propoxur were reported previously.[7a]

In contrast dichlorvos, which is known for its vapor phase insecticidal action,[27] displayed 100% mortality at a 100-fold lower nominal concentration (10 ng/mL). However **5g**, which is 100-fold more potent than dichlorvos at WT *Ag*AChE (Table 1), and more volatile (Table 5), proved to be >100-fold less toxic than dichlorvos (86 ± 7% mortality at 1000 ng/mL). Thus the low fumigation

Table 5
Evaporative weight loss of AChE inhibitors (and water control) at room temperature[a]

Compound	% mass lost by evaporation			
	1 day	6 days	12 days	28 days
Propoxur	0 ± 1	0 ± 1	1 ± 1	0 ± 1
Dichlorvos	2 ± 1	16 ± 1	32 ± 1	51 ± 1
5g	9 ± 1	33 ± 1	68 ± 1	99 ± 1
Water	99 ± 1	99 ± 1	100 ± 1	100 ± 1

[a] Compounds (starting masses 8–15 mg) were placed in open 1 dram vials in a fume hood at room temperature.

Table 6
Fumigation and injection toxicity of select AChE inhibitors

Compound	Fumigation mortality at indicated nominal concentration[a]	Injection LD_{50} (ng/insect) or mortality at 50 ng/insect[b]
Propoxur	0%@1000 ng/mL	0.24 (0.20–0.34)
Dichlorvos	100%@10 ng/mL	ND
5g	86 ± 7%@1000 ng/mL	83 ± 6%
9g	57 ± 25%@1000 ng/mL	65 ± 4%
10g	8 ± 6%@1000 ng/mL	21 ± 8%

[a] Measured % mortality after 24 h in a 1 L sealed vessel, see Supplementary data for experimental details. Nominal concentration is calculated from the mass of AChE inhibitor delivered and the volume of the vessel.
[b] See Supplementary data for protocol; the 95% confidence interval for the propoxur LD_{50} value is given in parenthesis. ND signifies 'not determined'.

toxicity of **5g** relative to dichlorvos must be due to some other factor. Compounds **9g** and **10g** had similar low toxicities.

As a final assessment of intrinsic toxicity, injection of these compounds into the mosquito thorax was performed. Propoxur was chosen as the positive control, and it gave a low LD_{50} value of 0.24 ng/insect. Compounds **5g**, **9g**, and **10g** were then assessed, but significant mortality from these compounds was seen only at the 200-fold higher dose of 50 ng/insect. Since **5g** and **9g** are significantly more potent inhibitors of *Ag*AChE than propoxur (Tables 1 and 2), it is again clear that factors beside exoskeleton penetrability significantly limit the toxicity of the tri- and difluoromethyl ketones. Obviously many phenomena could be at play in mitigating the toxicity of these compounds. But given the demonstrated propensity of these compounds to form hydrates, it is possible that phase II metabolism (i.e., glycosidation[28] of the hydrate) and excretion is one factor that contributes to the detoxification of these potent AChE inhibitors.

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Supplementary data

Supplementary data (experimental protocols, additional figures, synthetic procedures, analytical characterization data for the trifluoro-, difluoro-, and fluoromethyl ketones, residual activity values for Figures 3 and 4) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.09.019.

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