Pesticide Biochemistry and Physiology 151 (2018) 40–46

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Insecticidal and repellent properties of novel trifluoromethylphenyl amides II

Maia Tsikolia[a], Ulrich R. Bernier[b], Natasha M. Agramonte[a,1], Alden S. Estep[c], James J. Becnel[b], Nurhayat Tabanca[d,1], Kenneth J. Linthicum[b], Aaron D. Gross[a,1], Patrick M. Guerin[e], Thomas Kröber[e], Jeffrey R. Bloomquist[a,*]

[a] Emerging Pathogens Institute, Department of Entomology and Nematology, University of Florida, Gainesville, FL 32610-0009, USA
[b] U.S. Department of Agriculture-Agricultural Research Service, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL 32608, USA
[c] Navy Entomology Center of Excellence, CMAVE Detachment, Gainesville, FL 32608, USA
[d] U.S. Department of Agriculture-Agricultural Research Service, Subtropical Horticulture Research Station, 13601 Old Cutler Rd., Miami, FL 33158, USA
[e] Institute of Biology, Faculty of Science, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

ARTICLE INFO

Keywords:
Aedes aegypti
Anopheles gambiae
Drosophila melanogaster
Mosquito

ABSTRACT

This project focused on the design, synthesis, and testing of trifluoromethylphenyl amides (TFMPAs) as potential mosquitocides and repellents. Fourteen compounds were evaluated for toxicity against larvae and adults of *Aedes aegypti*. Several compounds were toxic against *Aedes aegypti* (larval, adult and feeding bioassays) and *Drosophila melanogaster* (glass-surface contact assay), but were much less toxic than fipronil, with toxicity ratios ranging from 100-fold in the larval assay to 100,000-fold for topical application to adult insects. In repellency bioassays to determine minimum effective dosage (MED), compound *N*-(2,6-dichloro-4-(trifluoromethyl)phenyl)-2,2,3,3,3-pentafluoropropanamide (**7b**) repelled *Ae. aegypti* females at lower concentration, 0.017 (± 0.006) μmol/cm^2, than *N*, *N*-diethyl-meta-toluamide (DEET) 0.026 (± 0.005) μmol/cm^2. 2-Chloro-*N*-(3-(trifluoromethyl)phenyl)acetamide (**6a**) performed better than DEET against two species of mosquitoes: it repelled *Ae. aegypti* females at 0.013 (± 0.006) μmol/cm^2 and *Anopheles gambiae* females (in a warm body repellent assay), at a standard exposure of 2 nmol/cm^2. These studies revealed novel active structures that could further lead to compounds with better repellent activity.

1. Introduction

To find more effective tools for mosquito control, we focus on the development of new repellents and insecticides to prevent mosquito bites and so reduce disease risk to humans. It is known that fluorine-containing chemicals can be useful for controlling agricultural pests (Maienfisch and Hall, 2004). The inclusion of single fluorine atoms and trifluoromethyl groups into small molecules can significantly increase their biological activity by promoting electrostatic interactions with biological targets, increasing their metabolic stability, as well as improving cellular membrane permeability and corresponding bioavailability (Muller et al., 2007; Purser et al., 2008; Nagib and MacMillan, 2011; Hagmann, 2008; Yamazaki et al., 2009; Theodoridis, 2006; Yale, 1959). Many amides show mosquito repellent activity, such as *N,N*-diethyl-*meta*-toluamide (DEET, today's gold standard), carboxamides (Katritzky et al., 2010), acylpiperidines (Katritzky et al., 2008), etc., as well as insecticidal activity (Dong et al., 2012; Park et al., 2002; Hwang and Mulla, 1980; Blade, 1990).

This study continues our previous research (Tsikolia et al., 2013) on trifluoromethylphenyl amides (TFMPAs), where we designed and synthesized twenty TFMPAs from an extensive search of the literature for compounds with pesticidal or mosquito repellent activity. The initial twenty amides were subsequently used as the basis for designing a second generation of fourteen additional derivatives (De La Rosa et al.,

Abbreviations: TFMPA, trifluoromethylphenyl amides; DEET, N,N-diethyl-m-toluamide; MED, minimum effective dosage; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; TLC, thin layer chromatography; mp, melting point

* Corresponding author at: Emerging Pathogens Institute, 2055 Mowry Road, Department of Entomology and Nematology, University of Florida, Gainesville, FL 32610-0009, USA.

E-mail address: jbquist@epi.ufl.edu (J.R. Bloomquist).

[1] *Current addresses:* Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30333 USA (NMA); Department of Entomology, Virginia Tech, Blacksburg VA 24061 USA (ADG).

https://doi.org/10.1016/j.pestbp.2018.08.006
Received 7 March 2018; Received in revised form 7 August 2018; Accepted 10 August 2018
Available online 12 August 2018
0048-3575/

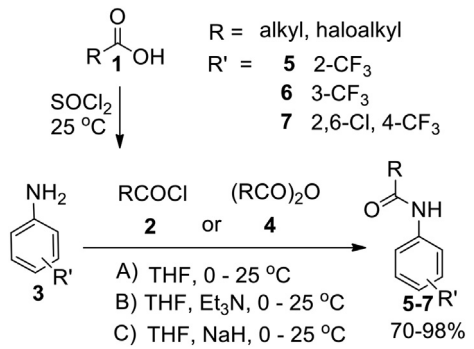


Fig. 1. Synthesis of TFMPAs **5–7**.

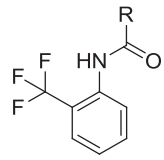
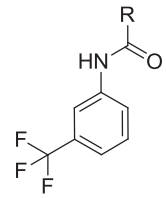
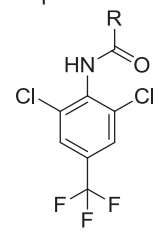
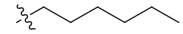
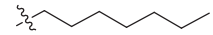
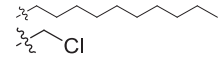
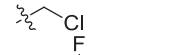
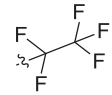
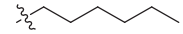
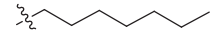
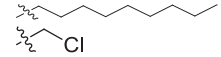
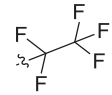
2006; Fukui et al., 1958; Baruffini et al., 1967; Du and Ouyang, 2010; Kuragano et al., 1996). (Fig. 1, Table 1). These compounds were synthesized and evaluated for insecticidal activity against *Aedes aegypti* larvae and adults, and for repellency against adult female *Ae. aegypti*. Selected compounds were also evaluated for toxicity against *Drosophila melanogaster* and repellency against female *Anopheles gambiae*. The goal of this research was to find new repellents and mosquitocides by synthesizing novel structures, and also to collect biological activity data for future quantitative structure activity relationship (QSAR) modeling.

2. Materials and methods

2.1. Synthesis of trifluoromethylphenyl amides 5–7

Fourteen compounds were synthesized (Fig. 1, Table 1). Acid chlorides **2** were prepared *in situ* by overnight reaction of the corresponding carboxylic acids **1** with 20–25% excess of neat thionyl chloride (Tsikolia et al., 2013) at 25 °C. Pentafluoropropionic anhydride (**4**) was purchased from commercial sources. Reaction of 1.05 equivalents of acyl chloride **2** or acid anhydride **4** with one equivalent of corresponding trifluoromethylphenyl amines **3** in tetrahydrofuran (THF) (10 mL) at 0–25 °C led to the production of trifluoromethylphenyl amides **5–7** in yields of 70–98% (Fig. 1). Triethylamine (Et_3N) for **7a** (Fig. 1, route A) and sodium hydride (NaH) for **7b** (Fig. 1, route B) were used as bases in the reaction.

Table 1
Average mortality of 1st instar *Ae. aegypti* larvae at 24 h post-exposure to a range of test TFMPAs 5–7 at 10.0 μM.

ID	Name	Structure	R	% Mortality (SEM)[b]
5a	2-chloro-*N*-(2-(trifluoromethyl)phenyl)acetamide			6.7 (11.5)
5b[a]	2,2,3,3,3-pentafluoro-*N*-(2-(trifluoromethyl)phenyl)propanamide			0.0
5c	*N*-(2-(trifluoromethyl)phenyl)heptanamide			0.0
5d	*N*-(2-(trifluoromethyl)phenyl)octanamide			0.0
5e	*N*-(2-(trifluoromethyl)phenyl)decanamide			0.0
5f[a]	*N*-(2-(trifluoromethyl)phenyl)undecanamide			73.3 (26.7)
6a	2-chloro-*N*-(3-(trifluoromethyl)phenyl)acetamide			33.3 (17.6)
6b[a]	2,2,3,3,3-pentafluoro-*N*-(3-(trifluoromethyl)phenyl)propanamide			33.3 (17.6)
6c	*N*-(3-(trifluoromethyl)phenyl)heptanamide			6.7 (6.7)
6d[a]	*N*-(3-(trifluoromethyl)phenyl)octanamide			0.0
6e	*N*-(3-(trifluoromethyl)phenyl)decanamide			0.0
6f[a]	*N*-(3-(trifluoromethyl)phenyl)undecanamide			0.0
7a	2-chloro-*N*-(2,6-dichloro-4-(trifluoromethyl)phenyl)acetamide			93.3 (6.7)
7b	*N*-(2,6-dichloro-4-(trifluoromethyl)phenyl)-2,2,3,3,3-pentafluoropropanamide			100 (0)
Fipronil				100 (0)
DMSO				0 (0)

[a] Denotes novel compounds. For known compounds, see references **5a** (De La Rosa et al., 2006); **5c**, **5d**, **5e**, **6c**, **6d** and **6e** (Fukui et al., 1958); **6a** (Baruffini et al., 1967); **7a** (Du and Ouyang, 2010); **7b** (Kuragano et al., 1996).
[b] Standard error of the mean.

2.1.1. General methods and materials

Melting points were determined on a hot-stage apparatus and are uncorrected. Nuclear Magnetic Resonance (NMR) analyses were performed at the NMR Facility of the University of Florida in Gainesville, FL, USA. NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 with TMS (tetramethylsilane) as the internal standard for ^1H (500 MHz) and $CDCl_3$ or DMSO-d_6 as the internal standard for ^{13}C (125 MHz). Accurate masses were measured at the Mass Spectrometry Facility of the Department of Chemistry, University of Florida, using a 6220 TOF-MS (Agilent Technologies) equipped with an electrospray and atmospheric pressure chemical ionization source. Direct analysis in real time (DART) was done with IonSense DART source (IonSense, Inc). Samples were dissolved in dichloromethane and solutions introduced via direct injection. All reactions were carried out under argon atmosphere in anhydrous THF obtained from Acros Organics, NJ, USA. The progress of a reaction was monitored by thin layer chromatography (TLC).

*2.1.2. Procedures for the preparation of trifluoromethylphenyl amides **5–7***

*2.1.2.1. Preparation of **5a–f, 6a–f**.* To a solution of trifluoromethylphenyl amine **3** (10 mmol) in THF (12 mL), acid chloride **2** (10.5 mmol) was added at 0 °C and stirred continuously for 0.2–4 h at 25 °C to produce compounds **5a–f, 6a–f** (Fig.1, route A). The reaction mixture was diluted and extracted with ethyl acetate (40 mL), washed with saturated aqueous $NaHCO_3$ (3 × 60 mL) and the organic layer dried over anhydrous Na_2SO_4. Evaporation of the solvent and recrystallization from ethanol (for **5a**), hexane (for **5d**,**e**) or hexane/ethyl acetate (for **5c,f**), or purification by silica gel column chromatography using hexane/ethyl acetate as the eluent (for **6e,f**) gave pure compounds **5a–f, 6a–f** in 70–98% yields.

*2.1.2.2. Preparation of **7a**.* To a solution of 2,6-dichloro-4-(trifluoromethyl)phenyl amine **3** (10 mmol) in THF (12 mL), acid chloride **2** (10.5 mmol) (for **7a**) was added at 0 °C in the presence of Et_3N (10.1 mmol) and stirred continuously for 24 h at 25 °C (Fig.1, route B). The reaction mixture was diluted and extracted with ethyl acetate (40 mL), washed with sat. aq. $NaHCO_3$ (3 × 60 mL) and the organic layer dried over anhydrous Na_2SO_4. Evaporation of the solvent and recrystallization from ethanol gave pure compound **7a** in 84% yield.

*2.1.2.3. Preparation of **7b**.* To a solution of 2,6-dichloro-4-(trifluoromethyl)phenyl amine (10 mmol) in THF (12 mL), NaH (60%, 10.4 mmol) was added and stirred continuously for 40 min at 0 °C (Fig.1, route C). Pentafluoropropionic anhydride (**4**) (10.5 mmol) was then added and stirred continuously for 2 h at 25 °C. The reaction was quenched with water (10 mL), extracted with ethyl acetate (40 mL), washed with sat. aq. $NaHCO_3$ (3 x 60 mL) and dried over anhydrous Na_2SO_4. Evaporation of the solvent and recrystallization from ethanol resulted in compound **7b** in 95% yield. See Supporting Information for the yields, melting points, NMR and mass spectral data.

2.2. Larval and adult bioassays with Ae. aegypti mosquitoes

The *Ae. aegypti* used for these biological assays are from a susceptible strain originally established in Orlando, FL, USA (1952), and maintained at the Mosquito and Fly Research Unit at the United States Department of Agriculture-Agricultural Research Service, Center for Medical, Agricultural, and Veterinary Entomology (USDA-ARS CMAVE) in Gainesville, FL, USA. Standard rearing and colony propagation conditions have been described previously (Pridgeon et al., 2009a), with hatching of larvae performed under vacuum for 2 h to better synchronize eclosion. Preliminary screening bioassays of compounds against first instar *Ae. aegypti* larvae were performed as described by *Pridgeon* et al. (2009b). Five larvae were placed into individual wells of a 24-well plate containing 950 μL of deionized water and 40 μL of a 2:1 aqueous suspension of alfalfa:pot belly pig chow. A 1 M solution of each test chemical was solubilized in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA), and 10 μL of this 1 M stock was added to the wells for a final concentration of 10 mM. Mortality was recorded at 24 h, 48 h, and 72 h post-exposure. Controls included 10 μL of the following: water (untreated), DMSO (carrier) and fipronil (10 mM concentration, purity 97.5%) (Sigma, St. Louis, MO, USA). For LC_{50} estimates, assays were set up as described above with serial dilutions of test chemical. Fipronil was used as a positive control, and DMSO and untreated larvae were used as negative controls. After three replicates, the data were analyzed using PoloPlus probit analysis software v2.0 (LeOra Software, Petaluma, CA, USA).

For the adult bioassay, four to seven-day old non-blood fed adult female mosquitoes were subjected to cold anesthetization at 4 °C. After approximately 60 min, the mosquitoes were sorted on ice into 3.5 oz. clear plastic cups (Solo TK-35) with ten mosquitoes per cup using soft touch forceps to minimize damage. The test chemicals were solubilized in DMSO to produce 4 M or 2 M stock solution. The stock solutions were diluted with acetone to produce a 200 mM DMSO/acetone treatment solution used to make three serial dilutions (1:1) in acetone. Each mosquito in a group of 10 was treated with 0.5 μl of each dilution applied to the thorax using a 700 series syringe and a PB 600 repeating dispenser (Hamilton, Reno, NV, USA). The dose applied to each mosquito using this method was 100, 50, 25, and 12.5 nanomoles. After the topical application, mosquitoes were transferred back into a plastic assay cup, capped with a square of tulle mesh, held at 26 ± 1 °C and 80 ± 1% RH, and provided with a 10% sucrose solution on a cotton ball daily. Mortality data was recorded at 24, 48 and 72 h post topical application for determination of LD_{50}. Fipronil was used as a positive control, while acetone and untreated mosquitoes were used as negative controls. Three replicates were completed and SigmaPlot (Version11) was used to calculate the LD_{50}.

2.3. Feeding bioassays with Ae. Aegypti mosquitoes

Mosquitoes reared as above were collected from the colony cage, anesthetized at 4 °C, and sorted on ice into groups of 10 as described above. Mosquitoes were allowed to recover under ambient conditions and starved for 24 h with no access to water or sucrose. A 10% solution of sucrose and deionized water was prepared and 10 mL was placed into 15 mL tubes (ThermoFisher, Waltham, MA). Using a 100 mg/mL stock solution of compounds in DMSO, dilutions of 100, 50, 10, 5, and 1 μM were prepared for each compound. A positive control containing fipronil was made in the same concentrations. The negative control was a sucrose solution with equivalent amounts of DMSO. A 1 mL volume of each solution was applied to a 1 cm piece of dental wick placed on the tulle mesh that covered the top of the holding cup. Each dilution was applied to three cups of ten mosquitoes. Mosquitoes were left at ambient temperature (25 ± 1 °C). Knockdown was scored at 60 min after application and mortality was scored at 24 h. The experiment was repeated 3 times on successive days.

2.4. Contact toxicity bioassays with Drosophila melanogaster flies

Contact toxicity for a selected number of TFMPAs was determined against an insecticide-susceptible (Oregon-R) *D. melanogaster* strain, maintained in culture at the University of Florida since 2009. *D. melanogaster* were reared in plastic vials on artificial media (Carolina Biological Supply, Burlington, NC, USA). Surface-contact toxicity assays were performed by dissolving the test compounds in certified acetone (Fisher Scientific). Each test compound in acetone (250 μL) was delivered to 85 cm^2 test tube (Fisher Scientific), which was manually rotated for several minutes to evenly coat the tube. The solvent from test tubes was allowed to dry completely for 30 min before the addition of 20 adult female *D. melanogaster* that were 5–7 days post-emergent. Test tubes were stoppered with a cotton ball containing 10% sucrose solution (1 mL) allowing the flies to feed ad libitum. A test tube coated with

acetone-only served as the negative control. Mortality was determined 24 h after the files were placed into the tube. The LD_{50} was calculated using the PROC PROBIT method in SAS 9.3 (Cary, NC).

2.5. Repellency bioassays with Ae. Aegypti mosquitoes

The mosquito species used for testing was *Ae. aegypti* (Orlando strain, 1952, see *2.2*). Newly emerged mosquitoes were maintained on 10% sugar water and kept in laboratory cages at an ambient temperature of 28 ± 1 °C and RH of 35–60%. Nulliparous 6- to 8-day-old female mosquitoes were pre-selected from stock cages using a hand-draw box and trapped in a collection trap (Posey and Schreck, 1981). After 500 (± 10%) females were collected in the trap, they were transferred to a test cage (approximately 59,000 cm^3 with dimensions 45 cm × 37.5 cm × 35 cm) and allowed to acclimate for 17.5 (± 2.5) min before initiating testing (Barnard et al., 2007).

The MED is a measurement used to estimate the concentration level of repellent, which fails to prevent mosquito bites through a piece of treated cloth (US Department of Agriculture, 1977). For amide treatment, 1 mL of acetone solution was transferred into a 2-dram vial containing a cloth patch. The following series of dosages were used: 0.625, 0.313, 0.156, 0.078, 0.039, 0.020, and 0.010 μmol/cm^2. DEET was used as a positive control and acetone as a negative control. Prior to the start of testing, the cloth was removed from the vial and affixed with staples onto two sections of card stock (5 cm × 2.5 cm). Approximately 5 cm of masking tape was affixed to the edges of the card stock. After the cloth and card stock were treated, they were placed on a drying rack and allowed to dry for at least 3 min prior to testing. The MED calculation was initiated using the middle range (0.078 μmol/cm^2) treated cloth and followed by use of higher or lower dosage treatments as necessary until all subjects had evaluated the cloths and pinpointed the dosage at the 1% (5 bites) failure point. There were 3 volunteers (all male) that tested each cloth. During each test, all volunteers wore a patch treated with a specific compound and tested it for a 1 min interval. Patches were then rotated among the volunteers. No patch was evaluated for > 10 min after the 3 min drying period to avoid any bias that may result from evaporative loss of treatment from the cloth throughout the test. All procedures were approved by the University of Florida Human Use Institutional Review Board and informed consent was provided by all participants (Project # 636-2005).

Each volunteer participating in the bioassay test wore a specially designed sleeve that exposed only a small area of the forearm to the mosquitoes (Fig. 2). The hand of each human volunteer was protected by a powder-free latex glove (Diamond Grip, Microflex Corporation, Reno, NV). The gloved hand and arm were then placed inside a knee-high stocking (Leggs Everyday Knee Highs, Winston-Salem, NC). A plastic sleeve constructed of polyvinyl was then placed over the arm and stocking. The sleeve had a lengthwise Velcro seam to allow sealing over the arm. There was a window cut into the sleeve (4 × 8 cm opening) approximately half way between the wrist and elbow. This window allowed odors from the volunteer's skin surface to escape from the sleeve through the opening, over which the treated cloth was placed.

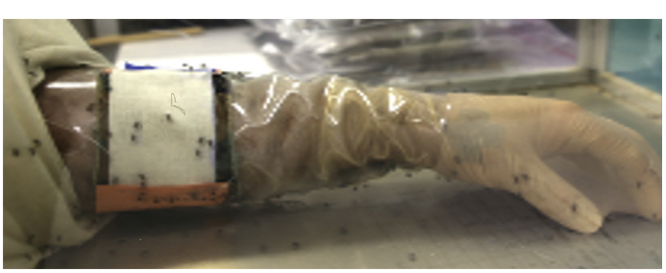


Fig. 2. Cloth patch bioassay to determine an MED of repellency for mosquitoes (Photo credit: Greg Allen).

The arm, sleeve and cloth were inserted into the mosquito cage for 1 min to determine if the compound and dosage on cloth were repellent to the mosquitoes. The number of fed mosquitoes was determined by shaking the arm briskly after 1 min and counting the number of mosquitoes that remained biting through the cloth. During the testing process, no > 10 compounds were assayed in succession with a caged population of test mosquitoes before allowing a 15 min recovery period. This approach was necessary because following prolonged and repeated repellent exposure, mosquitoes fatigue and exhibit decreased response to attractant (skin) odors.

2.6. Repellency bioassays with An. gambiae mosquitoes

A colony of *An. gambiae* Giles (ss strain 16CSS derived in 1974 from wild caught adults originating from Lagos, Nigeria, West Africa) were maintained as described in Kröber et al. (2010). Responses of female mosquitoes were evaluated using a cylindrical warm body (WB, 60 mm diameter disk, 20 mm thick; Kröber et al., 2010). A heat source, a low voltage electrical current (power 33 W) was split in parallel over 3 resistors fixed in a triangular arrangement to the inner wall of the black anodized aluminum corpus of the WB (Fig. 3a). Fifty (+/− 8; for precise numbers see Table 8) females aged 5–12 days old attracted to a hand covered with a plastic glove in rearing cages were released into WB-assay test cages (Fig. 3a) at least 30 min before assays were conducted during the final hours of the scotophase. Test compounds were dissolved in ethanol (p.a. ≥ 99.9%, Merck, Darmstadt, Germany) and applied at 2 and 20 nmol/cm^2. DEET (97%, Riedel de Haen, Pestanal®, Seelze, Germany) was used as positive control, and tested at 2, 4, 6 and 20 nmol/cm^2 on the WB (Kröber et al., 2010). Test products were applied using a glass micropipette in 100 μL solvent to a 60 mm diameter sandblasted Petri dish base held on the WB (Fig. 3b). The solvent was allowed to evaporate for 40 s before the WB was introduced into the test cage. Each product was tested at least in 5 different test cages. In each test cage, assays of up to six products and an ethanol control were made in a randomized design on each experimental day. On a given day, repellence values (in %) for each product were calculated from the number of landings on the WB in pooled controls minus landings on the WB treated with the test product, divided by the number of landings in the pooled controls. Repellence values were analyzed using the linear mixed model with experimental day, individual test cage, number of females per cage and level of the activating CO_2 pulse in ppm as cofactors in packages LME4 (Bates et al., 2015) and NLME (Pinheiro et al., 2010) of R (R Development Core team, 2010). The repellence indices calculated were adjusted to a level of 0% for controls and a maximum repellence of 100%. Statistical differences to the control and DEET were calculated using the Post Hoc Tukey test at a probability level of $P > 0.05$.

3. Results and discussion

Fourteen TFMPAs were synthesized by treatment of trifluoromethylphenyl amines in THF with acid chlorides **2** (**5a**, **5c–f**, **6a**, **6c–f**, **7a)** or acid anhydride **4 (5b**, **6b**, **7b)**, in the presence of Et_3N (**7a**) or NaH, 60% (**7b**) in 70–98% yields (Fig. 1, Table 1).

First instar *Ae. aegypti* of the susceptible Orlando strain were incubated with dilutions of compounds **5–7** in the standard larval bioassay procedure with three repetitions of initial activity testing indicating a range of activities at 10 μM. Most of the group **5** compounds (**5a**, **5b**, **5c**, **5d, 5e)** had low activity. Interestingly, **5f** which has a carbon chain one longer than **5e**, had a mortality of 73% at the 10 μM concentration. Activity of compounds **6a–f** was more variable. Mortality from **6a** and **6b** amounted to 33%. Compounds **6c**, **6d**, **6e**, and **6f** all showed only minimal mortality that was not much greater than the negative controls. Compounds **7a**, and **7b** were very active at the doses tested with mortality at or above 93%. Fipronil was also 100% effective at this concentration. Both the untreated and DMSO treated

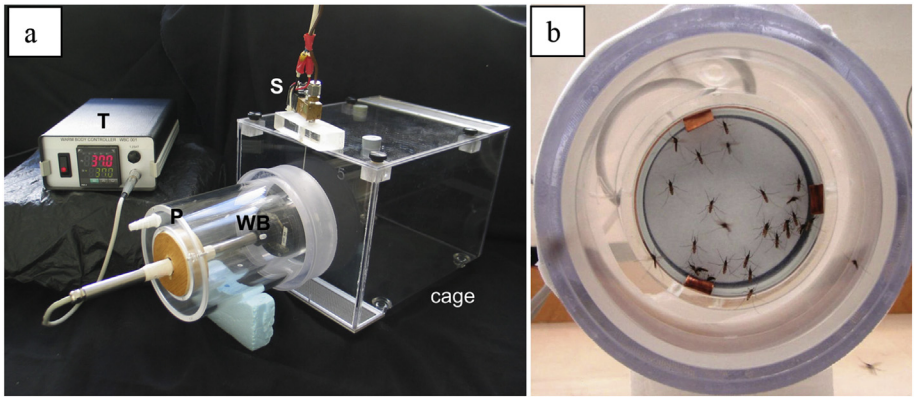


Fig. 3. (a) The warm body (WB) repellent assay setup with the test cage (right), the WB inserted into the introduction port (P) 155 mm long, temperature controller (T) and solenoid valve (S) for CO_2 pulse delivery. (b) Frontal view of the WB from the opposite side of the test cage with *An. gambiae* females landing on the Petri dish base covering the WB to which test products are applied (Photo credit: Sébastien Kessler).

Table 2
Estimates of LC_{50} for selected 2 TFMPAs against larval *Ae. aegypti* 24 h post application.

ID	LC_{50}[a], μM (95% CI)	n[b]	Slope (SEM), χ^2	Df[c]
7a	1.18 (1.04–1.62)	165	9.24 (2.29), 13.47	31
7b	1.14 (1.02–1.44)	185	1.84 (0.31), 169.79	35
Fipronil	0.0136 (0.0128–0.0143)	360	7.69 (1.16), 4.75	31

[a] LC_{50} is an estimate, and therefore a range is given,
[b] Number of insects tested in total,
[c] Degrees of freedom.

negative controls had no mortality.

Based on these initial results, low activity compounds from group **5** and group **6** were excluded from additional testing. Compounds **7a** and **7b** were evaluated with an additional series of dilutions to develop LC_{50} values. Fipronil was used for comparison (Table 2). Compounds **7a** and **7b** were similar in activity and had overlapping confidence intervals (**7a**: 1.04–1.62 μM; **7b**: 1.02–1.44 μM) indicating they were not significantly different from one another. The fipronil control resulted in an LC_{50} of 0.0136 μM which is about ~87 times more effective than **7a** or **7b**. Some structure-activity trends were observed: most compounds with the halogen-free aliphatic acyl substituents (**5c–e**, **6c–f**), showed less or no larvicidal activity compared to most compounds with the halogenated acyl substituents (**5a**, **6a**, **b**, **7a**, **b**). The *meta*-TFMPAs with halogenated moiety at the carbonyl carbon (**5a**, **b**) were more active than their *ortho*-isomers (**6a**, **b**). The presence of 2,6-dichloro and 4-trifluoromethyl moieties at the phenyl ring (**7a**, **b**) increased mortality in *Ae. aegypti* larvae compared to otherwise identical *ortho*- and *meta*-TFMPAs (**5a**,**b**, **6a**,**b**). In our previous work (Tsikolia et al., 2013), the presence of a 2,6-dichloro-substitution on the phenyl ring of the amide had an influence on the larvicidal and repellent activity of *para*-TFMPAs against *Ae. aegypti*. Specifically, the presence of a 2,6-dichloro substitution increased the larvicidal and repellent activity of *para*- substituted TFMPAs when a trifluoromethyl- or an aromatic group (2,6-dinitro or 2-methylbenzamide) was attached to the carbonyl carbon. Larvicidal and repellent activity decreased when an alkyl group such as 3-pentyl or *n*-pentyl was attached to the carbonyl carbon[15].

Adult activity testing (Table 3) on female *Ae. aegypti* also showed many of the compounds had little activity even at the highest dose (100 nmol). Inactive compounds from group **5** included **5b**, **5c**, **5e**, and **5f**. Compound **5d** produced 81.7 ± 12.2% mortality at the 100 nmol dose. This initial activity was reduced in further dilutions to 41.7 ± 11.9% and 1.7 ± 1.7% at the 50 and 25 nmol doses. The most active compound in group **5** was **5a**, which produced 100% mortality at the 100 nmol dose. At 50 nmol, mortality was still slightly above 50% and was 31.7 ± 6.5% at 25 nmol. This level of activity triggered additional investigation of this compound. Among the group **6** compounds, a similar range of activity existed with compounds **6a**, **6d**, and **6e** having minimal activity. Compounds **6b**, **6c** and **6f** killed 68–90% at100 nmol and then activity was reduced at the next dilution. Activity in **6b** was negligible at 50 nmol whereas **6f** had an activity of 43.3 ± 23.3% at the same dose. The most active compound in this group, **6c**, caused mortality of about 70% at the high dose, 66.7 ± 3.3% at 50 nmol, and 8.3 ± 1.7% at 25 nmol. This compound was then used for LD_{50} determination. In group **7**, complete mortality was caused by **7b** at the highest of the three doses used for initial testing. Compound **7a** was much less effective as an adulticide and had only minimal activity at the 10 μmol dose. Compound **7b** along with **6c** and **5a** were tested to determine LD_{50}.

Table 3
Average mortality of adult female *Ae. aegypti* at 24 h post-exposure to a range of test TFMPAs **5–7**, with doses starting at 100 nmols.

ID	% Mortality (SEM)		
	100 nmol	50 nmol	25 nmol
5a	100 (0)	53.3 (6.1)	31.7 (6.5)
5b	26.7 (1.4)	13.3 (3.3)	10.0 (4.4)
5c	5.0 (3.4)	1.7 (1.7)	10.0 (3.7)
5d	81.7 (12.2)	41.7 (11.9)	1.7 (1.7)
5e	1.7 (1.7)	3.3 (2.1)	3.3 (2.1)
5f	5.0 (2.2)	0.0 (0)	3.3 (2.1)
6a	1.7 (1.7)	3.3 (2.1)	5.0 (3.4)
6b	68.3 (20.0)	1.7 (1.7)	3.3 (2.1)
6c	70.0 (8.6)	66.7 (3.3)	8.3 (1.7)
6d	5.0 (2.2)	3.3 (2.1)	6.7 (2.1)
6e	3.3 (2.1)	1.7 (1.7)	8.3 (3.1)
6f	90.0 (5.8)	43.3 (23.3)	26.7 (21.9)
7a	3.3 (2.1)	3.3 (2.1)	13.3 (4.2)
7b	95.0 (2.2)	85.0 (3.4)	4.2 (1.1)
Fipronil	100 (0)	100 (0)	100 (0)
DMSO	0.0	0.0	0.0
Untreated	0.0	0.0	0.0

For LD_{50} values, each compound was tested in various doses on at least 330 mosquitoes (Table 4). Compound **5a** was the least active with an LD_{50} of 30.1 nmol. Compound **6c** was slightly more active at 24.5 nmol. The slopes of **5a** and **6c** were similar and the 95% confidence intervals overlapped indicating activity levels were similar. LD_{50} for compound **7b** was 7.0 nmol. In toxicity bioassays against

Table 4
Estimates of LD_{50} for selected 3 TFMPAs against adult female *Ae. aegypti* 24 h post topical application.

ID	LD_{50}, nmol (95% CI)	n	Slope (SEM), χ^2	Df
5a	30.1 (21.5–51.6)	348	2.73 (0.287), 66.42	16
6c	24.5 (17.4–47.1)	333	2.98 (0.309), 96.13	16
7b	7.0 (5.0–8.8)	352	3.28 (0.358), 43.16	16
fipronil	8.3×10^{-5} (7.3×10^{-5}-9.5×10^{-5})	330	4.90 (0.291), 37.21	9

Table 5
Feeding bioassay knockdown of adult female *Ae. aegypti* after 24 h post-exposure to a range of selected TFMPAs, with concentrations starting at 100 μM.

ID	24 h % Knockdown (SEM)			
	100 μM	**50 μM**	**10 μM**	**5 μM**
5a	22.5 (22.4)	1.1 (1.9)	3.3 (3.3)	0.0
5d	0.0	1.7 (2.4)	3.3 (0.0)	0.0
6a	3.3 (3.3)	1.1 (1.9)	2.5 (4.3)	2.2 (3.8)
6b	2.2 (3.8)	0.0	1.1 (1.9)	0.0
7a	1.1 (1.9)	2.2 (1.9)	2.2 (3.8)	1.1 (1.9)
7b	57.8 (38.9)	55.8 (37.1)	1.1 (1.9)	1.1 (1.9)
Fipronil	95.6 (7.7)	92.1 (8.4)	91.1 (3.8)	82.2 (19.5)
DMSO	0.0	0.0	0.0	0.0

female *Ae. aegypti*, the most active compounds (**5a, 7b**) had a halogenated moiety attached to a carbonyl carbon, as was observed in bioassays against larvae, although not all compounds that had a halogen-containing group attached to the carbonyl carbon (**5b, 6a** and **7a)** showed higher activity. Amides with the longer aliphatic acyl moieties (**5d, 6c** and **6f)** also showed activity that was considerably reduced at lower concentrations.

Starved *Ae. aegypti* females were given access to toxicant laced sugar baits for select TFMPAs to assess dietary activity. Compounds **5a, 5d, 6a, 6b, 7a,** and **7b** showed little effect from a range of concentrations from 100 μM to 1 μM after 1 h. Under these same feeding conditions, the fipronil control also showed < 5% mortality at all concentrations tested after 1 h. After 24 h of exposure, compounds **5a, 5d, 6a, 6b,** and **7a** resulted < 3.5% mortality at all exposure concentrations, with the exception of the 100 μM dose of **5a**, which caused 22.5% mortality (Table 5). Compound **7b** produced 57.8% and 55.8% mortality in the 100 μM and 50 μM doses respectively. The remaining concentrations for this compound showed only background activity. The fipronil control showed a consistent 82–96% mortality at all doses tested. The negative control had no mortality after 24 h of exposure. As in bioassays against larvae and adults against *Ae. aegypti,* in feeding bioassays, the most active compound was 2,6-dichloro-4-trifluoromethylphenyl amide (**7b**), with *N*-perfluoropropionyl group.

The majority of the TFMPAs did not show appreciable toxicity to *D. melanogaster* at the initial screening concentration of 25 μg/cm^2, with the exception of **6b** and **7b**, which were then further investigated in concentration-response studies (Table 6). The two toxic TFMPA compounds contained the same R substituent (pentafluoroethyl), but differed in the base structural group (Fig. 1). Compound **6b** was 1000-fold and **7b** was 135-fold less toxic than fipronil, which had an LC_{50} of 4 (2–6) ng/cm^2 against *D. melanogaster* in the glass-surface contact assay (Tsikolia et al., 2013).

Compound **6a** had the highest bite protection against female *Ae. aegypti*, with MED of repellency 0.013 μmol/cm^2 (Table 7), followed by **7b** (0.017 μmol/cm^2), which is comparable to or better than repellent activity of DEET (0.026 μmol/cm^2). Compounds **5b** and **6b** showed lower activity, with repellency MED values of 0.365 and 0.156 μmol/cm^2, respectively. Compounds **5a, 5c–f, 6c–f, 7a** did not have repellent activity up to 0.625 μmol/cm^2 (upper limit of the test). With regard to structure activity relationships, most active compounds (**5a, 6a** and **7b**) contained halogenated acyl substituents. The most active repellent against female *Ae. aegypti* was *N*-(3-(trifluoromethyl)phenyl)

Table 6
Glass Contact toxicity (24 h) of selected 2 TFMPAs against Oregon-R (susceptible strain of *D. melanogaster*).

ID	LC_{50}, μg/cm^2 (95% CI)	Slope (SEM), χ^2
6b	4 (3–5)	3.20 (0.43), 4
7b	0.54 (0.49–0.74)	2.86 (0.38), 13

Table 7
MED for repellency values for TFMPAs 5–7 against *Ae. aegypti*.

Compound	Average MED (± SEM), μmol/cm^2
DEET	0.026 (± 0.009)
5b	0.365 (± 0.121)
6a	0.013 (± 0.004)
6b	0.156 (± 0.052)
7b	0.017 (± 0.005)

Table 8
Repellence indices for compounds against *An. gambiae* mosquitoes in a warm-body assay.

ID	Dose: nmol/cm^2	Median % repellency[a]	*P* value compared to ethanol control	P value compared to DEET at 20 nmol/cm^2	n (cages)[b]
6a	**2**	52 (47–56)	< 0.001	< 0.001	232 (5)
	20	95 (92–98)	< 0.001	0.96	228 (5)
7b	**2**	20 (16–22)	nt	nt	444 (10)
	20	50 (43–54)	< 0.001	< 0.01	210 (5)
DEET	**2**	6 (2 − 13)	0.48	< 0.001	592 (11)
	4	40 (38–46)	< 0.001	< 0.001	210 (5)
	6	82 (76–88)	< 0.001	0.08	1355 (22)
	20	99 (93–106)	< 0.001	ref	517 (10)

nt denotes not tested.
[a] The repellence index is an estimate so its range is provided (see Materials and methods).
[b] Total number of insects tested with number of cages in parentheses.

chloroacetamide (**6a**), followed by *N*-(2,6-dichloro-4-(trifluoromethyl) phenyl)-2,2,3,3,3-pentafluoropropanamide (**7b**). Bite protection decreased when longer chain *N*-alkanoyl groups (hexyl, heptyl, nonyl or decyl) were present. In our previous study (Tsikolia et al., 2013), the most active repellent was trifluoroacetanilide with an *ortho*-trifluoromethyl substituent at the phenyl ring, while trifluoroacetanilides with the *meta*–trifluoromethyl and 2,6-dichloro-4-trifluoromethyl substituents at the phenyl ring had much lower repellent activity.

DEET induced dose-dependent repellency against *An. gambiae* in the WB assay (Table 8). Compound **6a** showed the highest repellent activity against female *An. gambiae* with a median repellence index of 95% at 20 nmol/cm^2, not different from DEET (99%, $P = 0.96$) at the same concentration (Table 8). At 2 nmol/cm^2 compound **6a** reduced landings on the warm body by 50%, significantly better ($P < 0.001$) than DEET at the same dose. The same compound (**6a**) was more active than DEET against *A. aegypti* in the cloth patch bioassay. Compound **7b** showed a repellence index of 51% at the 20 nmol/cm^2 dose, significantly less than DEET at the same dose for *An. gambiae* (P < 0.001; Table 8). Structure **6a** has potential to be used as a lead to develop new repellents against broad spectrum of species. It should be noted that unlike their longer chain perfluorinated acyl analogues amides with the shorter chain perfluorinated acyl moieties are not so easily hydrolyzed to produce perfluorinated acids which are problematic from an ecotoxicological standpoint.

In conclusion, fourteen TFMPAs (five of which were structurally novel) were designed and synthesized. All of them were evaluated for insecticidal and/or repellent activity against *Ae. aegypti* larvae and adult females, and insecticidal activity against *D. melanogaster* adults. The two most active compounds against *Ae. aegypti* larvae were 100- to 1000-fold less active than fipronil. Similarly, topical activity of the most active compounds against adults was 84- to 360-fold lower compared to fipronil. In feeding bioassays against *Ae. aegypti* adults, the compounds were even less active than by topical application, and also showed weak activity against *D. melanogaster* adults in a glass contact assay. However, the TFMPAs showed better performance as repellents. Two compounds (**6a** and **7b**) showed comparable repellent activity to DEET

against *Ae. aegypti* females. Moreover, compound (**6a**) showed repellence against *An. gambiae* females better than DEET at 2 nmol/cm^2 and similar to DEET at 20 nmol/cm^2. In almost all bioassays, the most active insecticides or repellents, were those that had halogenated acyl moieties for all three groups (**6** and **7**) *ortho*- (**5**), *meta*- trifluoromethyl (**6**) or 2,6-dichloro-4-trifluoromethyl (**7**) phenyl amides. Structure **6a** has potential to be used as a lead to develop new repellents against a broad spectrum of species.

Supporting information

Yields, melting points, NMR and mass spectral data for TFMPAs is available in the supporting information.

Funding

This work was supported by the Deployed War-Fighter Protection Research Program and funded by the United States Department of Defense through the Armed Forces Pest Management Board [Agreement 60-0208-4-001 and under USDA Specific Cooperative Agreements 58-0208-0-068 and 58-0208-5-001].

Acknowledgements

We thank Nathan Newlon, Greg Allen, Katelyn C. Chalaire and Jessica Louton (USDA-Agricultural Research Service-Center for Medical, Agricultural, and Veterinary Entomology) for laboratory technical support with the mosquito bioassays; Nucleic Magnetic Resonance and Mass Spectrometry facilities of the University of Florida.

References

Barnard, D.R., Bernier, U.R., Xue, R.-D., Debboun, M., 2007. Standard Methods for Testing Mosquito Repellents. In: Debboun, M., Frances, S.P., Strickman, D. (Eds.), Insect Repellents: Principles, Methods, and Uses, second ed. CRC Press, Boca Raton, FL, pp. 103–110.

Baruffini, A., Borgna, P., Pagani, G., Gialdi, F., 1967. Selective phytotoxic anilides which inhibit chlorophyll photosynthesis. Farmaco; Ed. Sci. 22, 895–916.

Bates, D., Maechler, M., Bolker, B., Walker, S.F., 2015. Linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48. https://doi.org/10.18637/jss.v067.i01.

Blade, R.J., 1990. Some aspects of synthesis and structure-activity in insecticidal lipid amides. Spec. Publ. R. Soc. Chem. 79, 151–169.

De La Rosa, M., Kim, H.W., Gunic, E., Jenket, C., Boyle, U., Koh, Y.-H., Korboukh, I., Allan, M., Zhang, W., Chen, H., et al., 2006. Tri-substituted triazoles as potent non-nucleoside inhibitors of the HIV-1 reverse transcriptase. Bioorg. Med. Chem. Lett. 16, 4444–4449. https://doi.org/10.1016/j.bmcl.2006.06.048.

Dong, W.-L., Xu, J.-Y., Xiong, L.-X., Li, Z.-M., 2012. Synthesis, structure and insecticidal activities of some novel amides containing N-pyridylpyrazole moieties. Molecules 17, 10414–10428. https://doi.org/10.3390/molecules170910414.

Du, H., Ouyang, G., 2010. Synthesis and characterization of aroyl thioureas derivatives containing phenoxy acetamide. Huaxue Yu Shengwu Gongcheng 27, 42–44.

Fukui, K., Kitano, H., Ijiri, R., Inamoto, Y., Matsufuji, T., 1958. Studies on aromatic fluorine compounds II. Preparations of fluorine-containing anilines and their derivatives. Nippon Kagaku Zasshi 79, 889–894. https://doi.org/10.1246/nikkashi1948.79.889.

Hagmann, W.K., 2008. The many roles for fluorine in medicinal chemistry. J. Med. Chem. 51, 4359–4369. https://doi.org/10.1021/jm800219f.

Hwang, Y.S., Mulla, M.S., 1980. Insecticidal activity of alkanamides against immature mosquitoes. J. Agric. Food Chem. 28, 1118–1122. https://doi.org/10.1021/jf60232a013.

Katritzky, A.R., Wang, Z., Slavov, S., Tsikolia, M., Dobchev, D., Akhmedov, N.G., Hall, C.D., 2008. Synthesis and bioassay of novel mosquito repellents as predicted from chemical structure. PNAS USA 105, 7359–7364. https://doi.org/10.1073/pnas.0800571105.

Katritzky, A.R., Wang, Z., Slavov, S., Dobchev, D.A., Hall, C.D., Tsikolia, M., Bernier, U.R., Elejalde, N.M., Clark, G.G., Linthicum, K., 2010. Novel carboxamides as potential mosquito repellents. J. Med. Entomol. 47, 924–938. https://doi.org/10.1093/jmedent/47.5.924.

Kröber, T., Kessler, S., Frei, J., Bourquin, M., Guerin, P.M., 2010. An *in vitro* assay for testing mosquito repellents employing a warm body and carbon dioxide as a behavioral activator. J. Am. Mosq. Control Assoc. 26, 381–386.

Kuragano, T., Nakamura, S., Minami, K., Minamida, I., Okauchi, T., 1996. Preparation of β-(phenylamino) styrene and analogs as insecticides and fungicides. Jpn. Kokai Tokkyo Koho (JP 08295663 A 19961112).

Maienfisch, P., Hall, R.G., 2004. The importance of fluorine in the life science industry. Chimia 58, 93–99. https://doi.org/10.2533/000942904777678091.

Muller, K., Faeh, C., Diederich, F., 2007. Fluorine in pharmaceuticals: looking beyond intuition. Science 317, 1881–1886. https://doi.org/10.1126/science.1131943.

Nagib, D.A., MacMillan, D.W.C., 2011. Trifluoromethylation of arenes and heteroarenes by means of photoredox catalysis. Nature 480, 224–228. https://doi.org/10.1038/nature10647.

Park, I.-K., Lee, S.-G., Shin, S.-C., Park, J.-D., Ahn, Y.-J., 2002. Larvicidal activity of isobutylamides identified in Piper nigrum fruits against three mosquito species. J. Agric. Food Chem. 50, 1866–1870. https://doi.org/10.1021/jf011457a.

Pinheiro, J.C., Bates, D.M., Debroy, S.D.S., 2010. Nlme: an R package for fitting and comparing gaussian linear and non-linear mixed-effects models. In: The Comprehensive R Archive Network Website, http://www.stats.bris.ac.uk/R. (accessed 11 January 2013).

Posey, K.H., Schreck, C.E., 1981. An airflow apparatus for selecting female mosquitoes for use in repellent and attraction studies. Mosq. News. 41, 566–568.

Pridgeon, J.W., Bernier, U.R., Becnel, J.J., 2009a. Toxicity comparison of eight repellents against four species of female mosquitoes. J. Med. Entomol. 25, 168–173.

Pridgeon, J.W., Becnel, J.J., Clark, G.G., Linthicum, K.J., 2009b. A high-throughput screening method to identify potential pesticides for mosquito control. J. Med. Entomol. 46, 335–341. https://doi.org/10.1603/033.046.0219.

Purser, S., Moore, P.R., Swallow, S., Gouverneur, V., 2008. Fluorine in medicinal chemistry. Chem. Soc. Rev. 37, 320–330. https://doi.org/10.1039/B610213C.

Theodoridis, G., 2006. Fluorine-containing agrochemicals: an overview of recent developments. In: Tressaud, A. (Ed.), Advances in Fluorine Science (Fluorine and the Environment: Agrochemicals, Archaeology, Green Chemistry and Water), first ed. University Bordeaux, Bordeaux, France, pp. 121–175. https://doi.org/10.1016/S1872-0358(06)02004-5.

Tsikolia, M., Bernier, U.R., Coy, M.R., Chalaire, K.C., Becnel, J.J., Agramonte, N.M., Tabanca, N., Wedge, D.E., Clark, G.G., Linthicum, K.J., Swale, D.R., Bloomquist, J.R., 2013. Insecticidal, repellent and fungicidal properties of novel trifluoromethylphenyl amides. Pestic. Biochem. Physiol. 107, 138–147. https://doi.org/10.1016/j.pestbp.2013.06.006.

US Department of Agriculture, 1977. Repellent activity of compounds submitted by Walter Reed Army Institute of Research Part I. Protection Time and Minimum Effective Dosage Against *Aedes aegypti* Mosquitoes (US Dept Agriculture, Washington, DC), Technical Bulletin. (No 1549).

Yale, H.L., 1959. The trifluoromethyl group in medicinal chemistry. J. Med. Pharm. Chem. 1, 121–133.

Yamazaki, T., Taguchi, T., Ojima, I., 2009. Unique properties of fluorine and their relevance to medicinal chemistry and chemical biology. In: Ojima, J. (Ed.), Fluorine in Medicinal Chemistry and Chemical Biology first ed. John Wiley and Sons, Ltd, Chichester, UK, pp. 3–46. https://doi.org/10.1002/9781444312096.ch1.