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DESIGN, SYNTHESIS AND INSECTICIDAL ACTIVITY OF 3-ARYLISOXAZOLINE-*N*-ALKYLPYRAZOLE-5-CARBOXAMIDE DERIVATIVES AGAINST *TETRANYCHUS URTICAE* KOCH

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Abstract – Starting from ethyl 5,5-dimethoxy-2,4-dioxopentanoate **2**, 3-arylisoxazoline-*N*-alkylpyrazole-5-carboxamide derivatives **1a**-**1d** were designed and synthesized with the key steps of 1,3-dipolar cycloaddition and EDCl coupling. Their insecticidal activity against *Tetranychus urticae* Koch was further evaluated and the results indicated that, compared with Fluralaner, all **1a-1d** showed moderate and less activity at 500 mg/L and 250 mg/L, respectively. This study complements the structure-activity relationship of *N*-alkylpyrazole-5-carboxamides to Fluralaner, giving further guidance in Fluralaner-based pesticide drug design and discovery.

INTRODUCTION

Tetranychus urticae Koch, acted as one kind of pest mite, damages hundreds of plants including vegetables, flowers and fruit trees, thus causing serious economic losses. Despite of insecticides widely-used in the market, insect resistance occurs as an emerging problem in the prevention and treatment of agricultural pests. Therefore, the discovery of insecticides with novel structure plays a much more vital role to tackle with agricultural pest resistance. Arylisoxazoline moiety has been widely-adopted as anti-inflammatory,[1] anticancer,[2] antibacterial[3] and antiviral agent.[4] Arylisoxazoline-based insecticides with greater structural diversity (Figure 1) have also been developed successively, exerting insecticidal activity by acting on gamma-aminobutyric acid (GABA) gate channel.

Fluralaner, the active substance of the marketed veterinary drug Bravecto™ (chewable tablets), is a new inhaled insecticide and acaricide. Studies have shown that the binding site of Fluralaner may be located

near the transmembrane segment TM1 and TM3 of the GABA receptor while further mechanism and binding site are still in progress.[5] As a novel GABA gated chloride channel interfering agent, Fluralaner exhibits no cross-resistance compared with current GABA receptor insecticides[5,6] as well as good safety and nontoxicity to non-target organisms such as mammals, zebrafish, and poultry.[7] The successful development of Fluralaner has been newly classified as GABA gated chloride channel interfering agents, which has attracted the attention and favor of pesticide[6,8-13] and chemistry scientists.[14-23] There are also arylisoxazoline GABA receptor antagonists with different chemical structures such as Afoxolaner,[24] Sarolaner,[25] Lotilaner,[26,27] Fluxametamide[28] and Isocycloseram[29] showing insecticidal and acaricidal activities, Afoxolaner, Sarolane, Lotilaner and Fluxametamide have also approved for market.

It can be found that arylisoxazoline GABA receptor antagonists own the special structure template termed as "aromatic ring + isoxazoline ring + aromatic ring + amides". Notably, most of them have additional chlorine atoms at positions 3 and 5 of the aromatic ring away from the amide moiety.

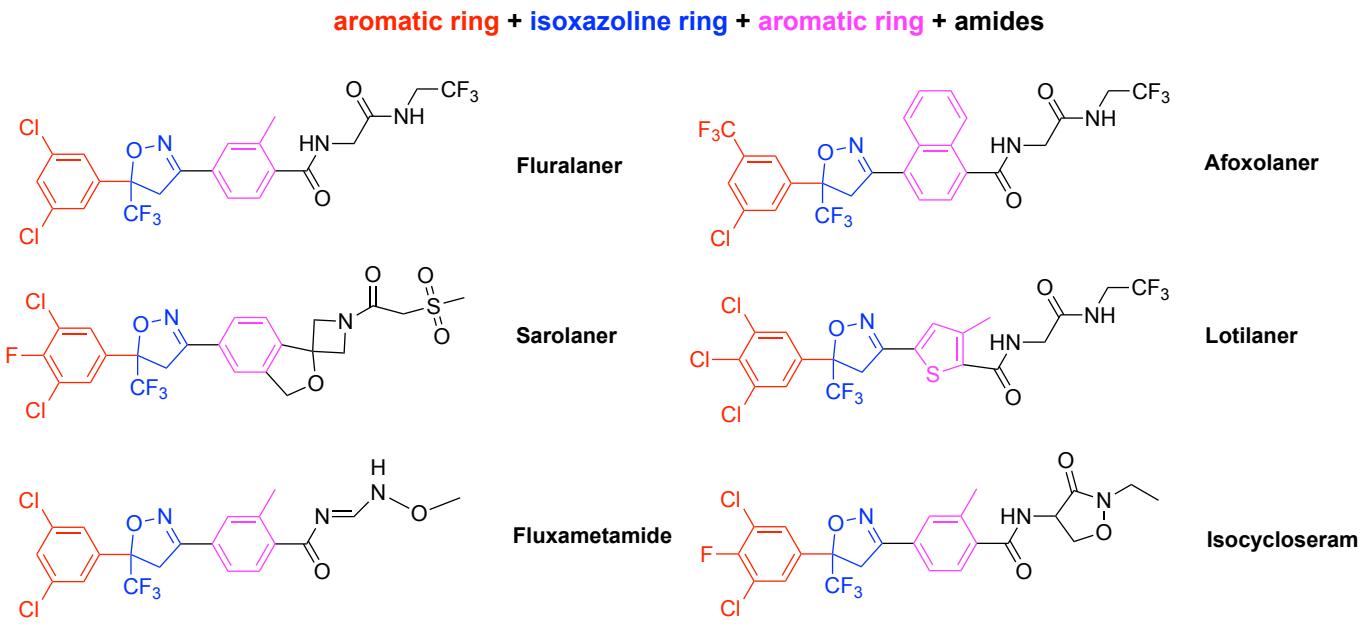


Figure 1. Chemical structure of arylisoxazoline derivatives as insecticides and acaricides

Pyrazole pesticides are another kind of heterocyclic compounds widely-used in insecticidal, acaricidal, bactericidal and herbicidal fields.[30] Among them, *N*-alkylpyrazole-5-carboxamide pesticides have been prior to others because of high safety and efficiency, no cross-resistance and small effective dosage. The representative varieties are Tebufenpyrad and Tolfenpyrad (Figure 2).

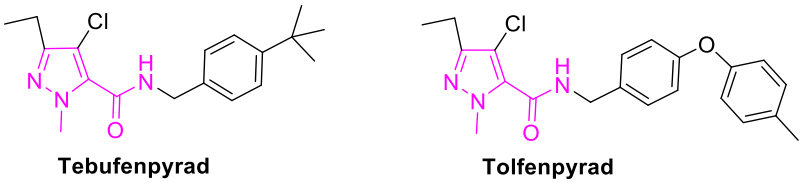


Figure 2. Chemical structure of pyrazole derivatives as insecticides and acaricides

Based on all the above, we hypothesize that the combination of molecular fragment *N*-alkylpyrazole-5-carboxamide moiety and arylisoxazoline moiety of Fluralaner could improve the insecticidal and acaricidal activity achieved by synergistic effects. So far, the structure-activity relationship of the amide-bound benzene ring in the structure of Fluralaner is not clear. Therefore, 3-arylisoxazoline-*N*-alkylpyrazole-5-carboxamide derivatives **1a-1d** (Figure 3) were designed, synthesized and evaluated for the insecticidal activity against *Tetranychus urticae* Koch. Significantly, the combinational structure maintained the fundamental framework to the best degree regarding GABA receptor antagonists "aromatic ring + isoxazoline ring + aromatic ring + amides". Taken together with the reasonable replacement of alkylbenzene with alkylpyrazole in an isosteric and bioisosteric manner, these derivatives are highly likely to exert potential insecticidal activity.

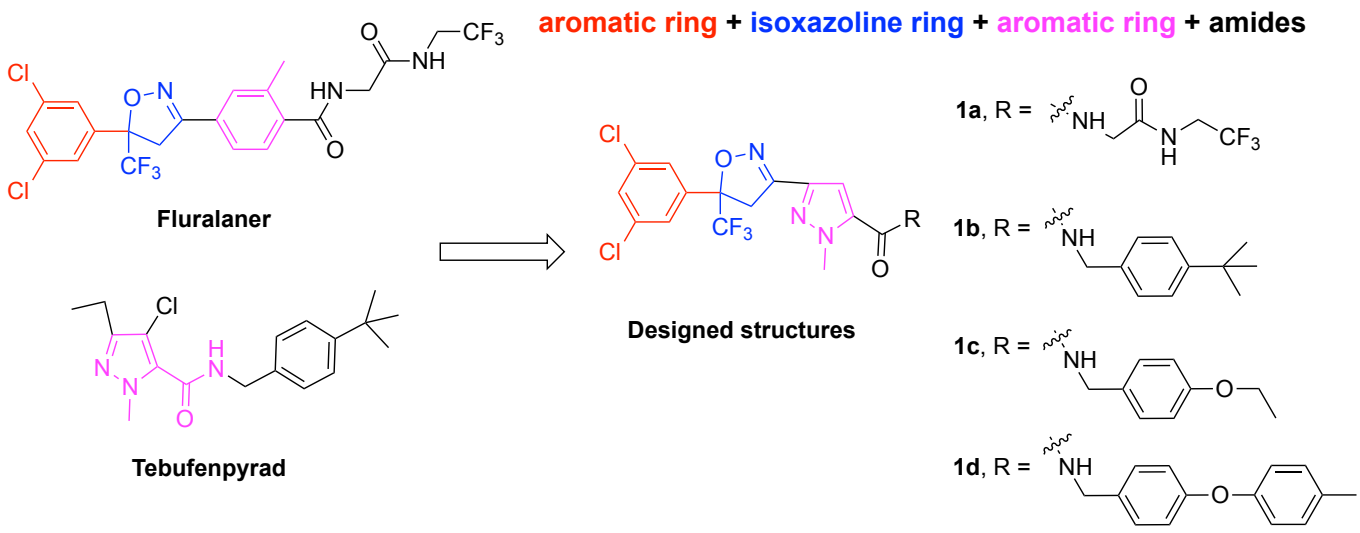
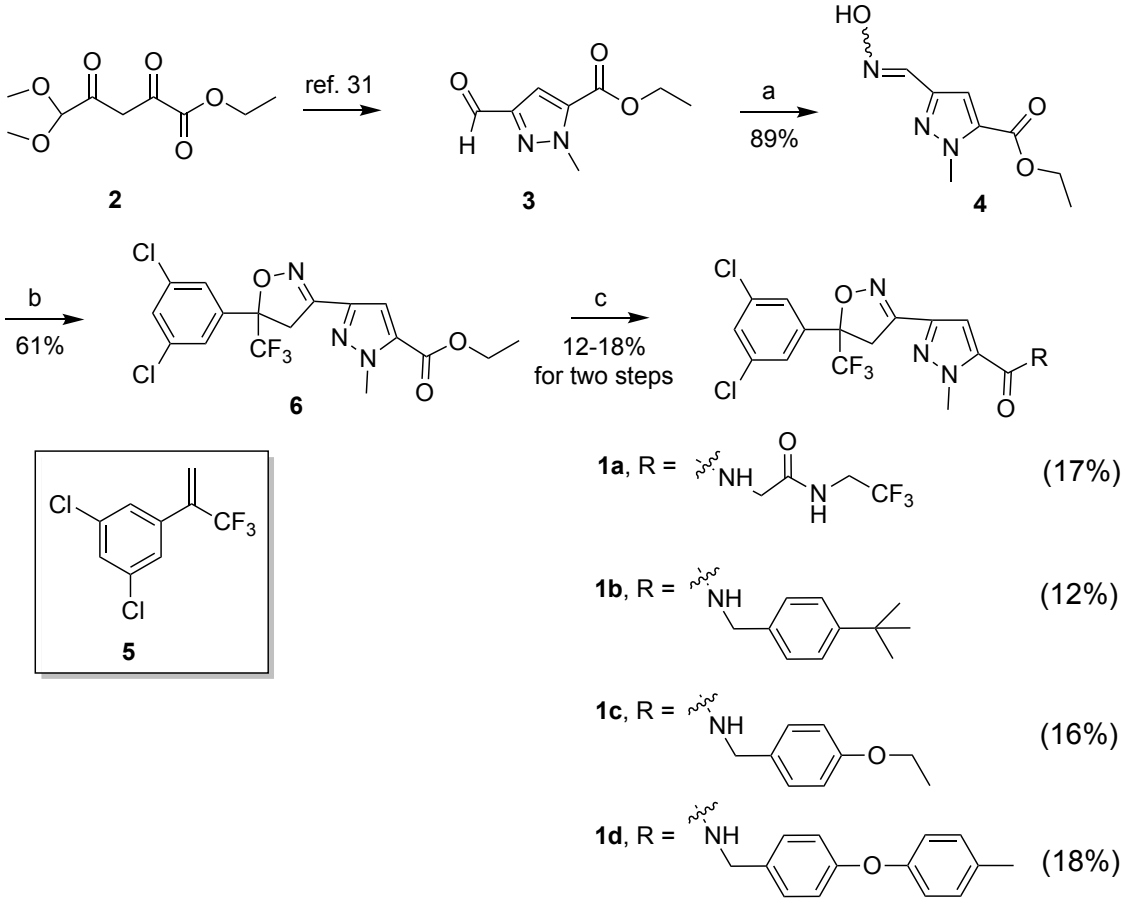


Figure 3. The rational design strategy of combining the Fluralaner molecular fragment arylisoxazoline with *N*-alkylpyrazole-5-carboxamide

RESULTS AND DISCUSSION

Synthesis. As shown in Scheme 1, ethyl 5,5-dimethoxy-2,4-dioxopentanoate **2** was converted into ethyl 3-formyl-1-methyl-1*H*-pyrazole-5-carboxylate **3** in three steps according to the known procedure.[31] Briefly, compound **2** was treated with hydrazine hydrate and AcOH to give the pyrazole, which was deprotected with 50% aqueous AcOH to afford the aldehyde. Further treatment of the aldehyde with

K_2CO_3, dimethyl sulfate and acetone afforded the 1-methyl-1*H*-pyrazole **3**. Compound **3** was treated with hydroxylamine hydrochloride and ethanol in the presence of sodium acetate to give the oxime **4**, which was synthesized by using a different previously reported method.[31] The oxime was treated with NCS to obtain the intermediate chlorohydroxyimide, which was further treated with the styrene **5** to yield the key intermediate **6** in a good yield through the 1,3-dipolar cycloaddition. The removal of the ester group of **6** with sodium hydroxide produced the crude acid. EDCl coupling of the crude acid with different amine, HOBt and CH_2Cl_2 in the presence of Et_3N could obtain the final arylisoxazolines **1a**-**1d**, respectively.



Reagents and Conditions: a) $NH_2OH{\cdot}HCl$, NaOAc, EtOH, rt, 14 h; b) i. NCS, DMF, rt, 4 h; ii. **5**, Et_3N, rt, 4 h; c) i. NaOH, THF, H_2O, 66 °C, 3 h; ii. 2-amino-*N*-(2,2,2-trifluoroethyl)acetamide, EDCI, HOBt, Et_3N, CH_2Cl_2, rt, 15 h for **1a** and 4-*tert*-butylbenzylamine for **1b** and 4-ethoxybenzylamine for **1c** and 4-(4-methylphenoxy)benzenemethanamine hydrochloride for **1d**.

Scheme 1. Synthesis of 3-arylisoxazoline-*N*-alkylpyrazole-5-carboxamide derivatives **1a**-**1d**

Among all the synthesized derivatives, the absolute configuration of arylisoxazoline (**1b**) was confirmed by X-ray crystal assay (Figure 4) and the X-ray crystal structure reflected the inherent characteristic as a racemic mixture of *R*- and *S*-enantiomers. To the best of our knowledge, commercially available Bravecto™ Chewable Tablets contain Fluralaner with one chiral center leading to the racemic mixture which are used as the active ingredient. Therefore, the insecticidal activities of all the derivatives were evaluated as the racemic mixture without separating their absolute *R* or *S* configurations.

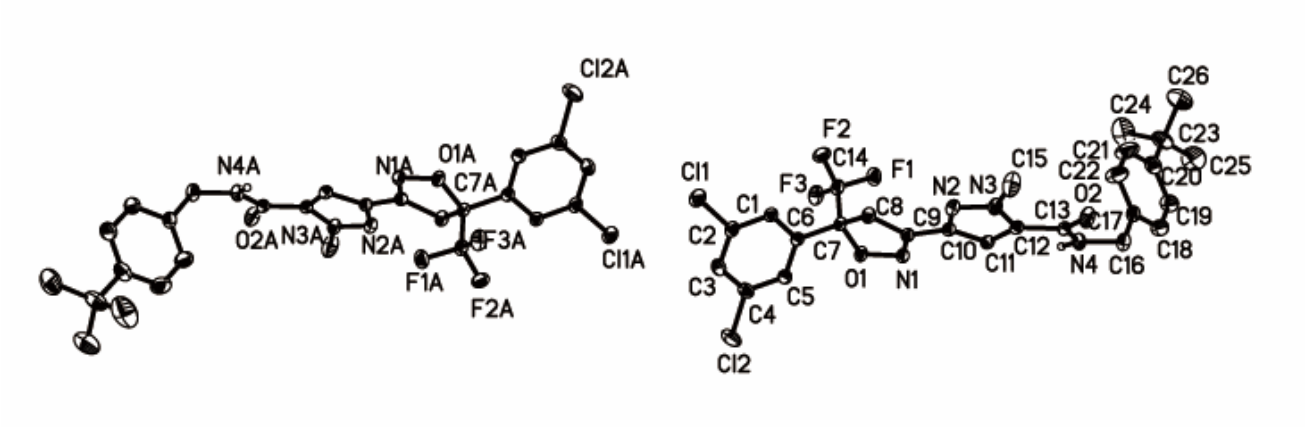
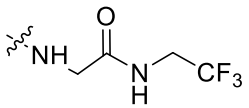
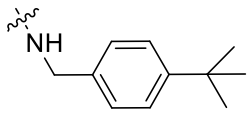
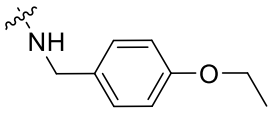
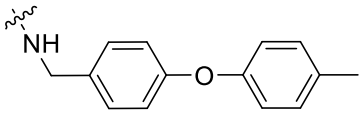


Figure 4. X-Ray crystal structure of the arylisoxazoline (**1b**)

Insecticidal activity

Compounds **1a-1d** were evaluated for insecticidal activity against *Tetranychus urticae* Koch, using a leaf dip method (Table 1).[32] The preliminary bioassays indicated that **1a-1d** showed insecticidal activity of 45%-62% mortality against *Tetranychus urticae* Koch at 500 mg/L. The followed rescreening bioassay results showed compounds **1a-1d** owned insufficient activity against *Tetranychus urticae* Koch at 250 mg/L, and compound **1c** (19%) showed the best activity among **1a-1d**. In addition, 4-ethoxyphenyl-substituted arylisoxazoline derivative showed better insecticidal activity than the other synthesized derivatives.

Table 1. The insecticidal activity of the final arylisoxazolines **1a-1d** against *Tetranychus urticae* Koch in a leaf dip assay

Compound	R	Insecticidal activity, %	
		500 mg/L	250 mg/L
1a		45±3	9±2
1b		49±4	12±3
1c		51±4	19±3
1d		62±3	11±2
Fluralaner	-	100±0	100±0

To the best of our knowledge, hydrogen bond (H-bond) interactions are the major driving factor for the binding between Fluralaner enantiomer and GABA receptor pockets. Studies have shown that the insecticidal activity of *S*-configuration is more active than *R*-configuration[33,34] with the explanation supported by homologous modeling and molecular docking.[35] Detailly, *S*-Fluralaner could bind to the GABA receptor to form two H-bonds through the amide and arylisoxazoline groups, while *R*-Fluralaner could bind to the receptor to form only one H-bond through the arylisoxazoline group, thus resulting in insecticidal difference.[35] However, this could not explain the insecticidal activity difference between our various derivatives and Fluralaner on the basis of racemic mixture utilization. In this way, it might be inferred that the introduction of pyrazole moiety mainly contributes to the reduction of insecticidal activity, especially in view of the only structural difference caused by pyrazole moiety between **1a** and Fluralaner. Also, considering the phenyl group has greater aromaticity than pyrazole group, the replacement of phenyl group with pyrazole group could possibly lead to a reduced π-π stacking interaction, resulting in a decreased binding affinity with corresponding GABA receptor, which undoubtfully requires further experimental support.

CONCLUSIONS

In summary, considering the reasonable combination effects of the arylisoxazoline moiety of Fluralaner and molecular fragment *N*-alkylpyrazole-5-carboxamide, the arylisoxazoline derivatives **1a**-**1d** were synthesized starting from compound **2**, accompanying by the 1,3-dipolar cycloaddition and EDCl coupling as the key steps, followed by the insecticidal activity evaluation against *Tetranychus urticae* Koch. The bioassay results showed that all of them exhibited moderate and insufficient insecticidal activity against *Tetranychus urticae* Koch at 500 mg/L and 250 mg/L, respectively, when compared with Fluralaner. Besides, the introduction of pyrazole moiety might serve as the major factor for the decreased insecticidal activity especially given the slight structural difference between Fluralaner and compound **1a**. Precise explanations could be provided by further replacement of pyrazole moiety with better aromaticity groups and the binding assay between related derivatives and GABA receptor.

In conclusion, this study gives supplementary evidence of structure-activity relationship and offer potential instructions of further rational design of Fluralaner-based pesticide drug design and discovery.

EXPERIMENTAL

General Procedures

Proton (^{1}H), carbon (^{13}C) and fluorine (^{19}F) NMR spectra were obtained on an Abilene 400 (400/100/376 MHz) spectrometer. Chemical shifts were reported in ppm units with Me_4Si or $CHCl_3$ as the internal standard. Infrared spectra were recorded on FT-IR (IRSpirit-T) made by Shimadzu. All reactions were

routinely carried out under an inert atmosphere of dry nitrogen. Reactions were checked by thin layer chromatography (Kieselgel 60 F254, Merck). Spots were detected by viewing under a UV light, and by colorizing with charring after dipping in a phosphomolybdic acid solution. In aqueous work-up, all organic solutions were dried over anhydrous magnesium sulfate and filtered prior to rotary evaporation at water pump pressure. The crude compounds were purified by column chromatography on a silica gel (Kieselgel 60, 70-230 mesh, Merck). Unless otherwise noted, materials were obtained from commercial suppliers and were used without purification. All solvents were purified and dried by standard techniques just before use. Methylene chloride and toluene were purified by refluxing with CaH_2.

Procedure for the preparation of 4

To a stirred solution of ethyl 3-formyl-1*H*-pyrazole-5-carboxylate **3** (3.64 g, 20.0 mmol) in EtOH (100 mL) was added hydroxylamine hydrochloride (2.57 g, 37.0 mmol), followed by the addition of sodium acetate (2.13 g, 26.0 mmol) at room temperature under N_2. After being stirred at the same temperature for 14 h, the reaction mixture was poured into water to separate out a white solid, which was filtered off with suction to give **4** (3.5 g, 89%) as a white solid.

Procedure for the preparation of 6

To a stirred solution of **4** (3.2 g, 16.23 mmol) in DMF (40 mL) was added NCS (2.75 g, 20.61 mmol) at room temperature under N_2 and the mixture was stirred at the same temperature for 4 h. To the reaction mixture, 1, 3-dichloro-5-(3, 3, 3-trifluoroprop-1-en-2-yl)benzene **5** (3.82 mL, 21.75 mmol) and Et_3N (3.02 mL, 21.75 mmol) were added and the mixture was continuously stirred at the same temperature for 4 h. The reaction mixture was partitioned between EtOAc (100 mL) and water (100 mL). The aqueous layer was washed with EtOAc (3 × 100 mL) and the combined organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated in *vacuo*. The crude product was purified by silica gel column chromatography (petroleum ether: EtOAc = 4 : 1) to give **6** (4.3 g, 61%) as a white solid: IR (cm^{-1}) 3443.7, 3093.9, 2957.2, 1729.3, 1708.6, 1572.3, 1305.2, 1270.0, 1192.4, 1175.8, 1117.9, 1077.5, 1025.2, 1009.4, 861.4, 803.1, 765.9, 698.8, 669.4; ^{1}H NMR (CD_3OD, 400MHz) δ 7.61 (d, J = 1.6 Hz, 2H), 7.57 (t, J = 2.0 Hz, 1H), 7.23 (s, 1H), 4.37 (q, J = 7.2 Hz, 2H), 4.23 (d, J = 18.4 Hz, 1H), 4.18 (d, J = 7.2 Hz, 3H), 3.93 (d, J = 18.4 Hz, 1H), 1.40-1.36 (m, 3H); ^{13}C NMR (CD_3OD, 100 MHz) δ 160.5, 153.7, 140.8, 140.6, 136.8, 136.8, 136.0, 130.8, 126.8, 126.8, 125.4 (q, J = 277.9 Hz), 110.6, 88.1 (q, J = 30.8 Hz), 62.7, 44.8, 40.5, 14.6; ^{19}F NMR (CD_3OD, 376 MHz) δ -81.4; HRMS (ESI) found 436.0451 [calc for $C_{17}H_{15}Cl_2F_3N_3O_3^+$ $(M+H)^+$ 436.0437].

Procedure for the preparation of 1a

To a stirred solution of **6** (3.93 g, 9.01 mmol) in a solution of THF (20 mL) and H_2O (20 mL) was added NaOH (0.72 g, 18.02 mmol) at room temperature under N_2 and the mixture was heated at 66 °C with stirring for 3 h. After the reaction was completed, THF was evaporated under reduced pressure and the pH of the mixed solution was adjusted to 4 with 1M HCl, a white solid was precipitated, and then filtrated to give crude acid, which was used immediately for next step without any purification. To a stirred solution of crude acid (1.0 g, 2.45 mmol) in CH_2Cl_2 (20 mL) were added EDCI (0.70 g, 3.68 mmol) and HOBt (0.50 g, 3.68 mmol) at room temperature under N_2 and the mixture was stirred at the same temperature for 30 min. To the reaction mixture, 2-amino-*N*-(2,2,2-trifluoroethyl)acetamide (0.46 g, 2.94 mmol) and Et_3N (0.68 mL, 4.90 mmol) were added and the mixture was continuously stirred at the same temperature for 15 h. The reaction mixture was partitioned between CH_2Cl_2 (50 mL) and 1M HCl (50 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layers were washed successively with saturated aqueous $NaHCO_3$ and saturated brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in *vacuo*. The residue was purified by silica gel column chromatography (petroleum ether: EtOAc = 20 : 1) to give **1a** (250 mg, 17% for two steps) as a white solid: IR (cm^{-1}) 3318.6, 2927.0, 1662.8, 1570.3, 1424.1, 1302.7, 1252.5, 1168.7, 1027.4, 1009.3, 803.0, 694.9; ^1H NMR (CD_3OD, 400MHz) δ 7.61 (d, J = 1.6 Hz, 2H), 7.58-7.57 (m, 1H), 7.23 (s, 1H), 4.23 (d, J = 18.4 Hz, 1H), 4.15 (s, 3H), 4.04 (s, 2H), 3.97-3.94 (m, 2H), 3.92-3.90 (m, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 172.1, 161.8, 153.8, 140.8, 140.5, 138.4, 136.8, 130.8, 126.8, 125.9 (q, J = 277.9 Hz), 125.4 (q, J = 283.8 Hz), 107.3, 88.1 (q, J = 30.4 Hz), 44.8, 43.3, 41.4 (q, J = 34.7 Hz), 40.2; ^{19}F NMR (CD_3OD, 376 MHz) δ -74.0 (t, J = 7.5 Hz), -81.4; HRMS (ESI) found 546.0549 [calc for $C_{19}H_{16}Cl_2F_6N_5O_3^+$ $(M+H)^+$ 546.0529].

Procedure for the preparation of 1b

Compound **1b** was prepared according to the procedure used in the preparation of **1a**: White solid; Yield: 12% for two steps; IR (cm^{-1}) 3317.7, 3138.5, 3083.6, 3057.1, 2963.8, 2868.9, 1650.7, 1567.0, 1516.9, 1303.2, 1175.7, 1106.6, 1015.9, 892.6, 862.8, 802.6, 765.5, 696.0; ^1H NMR (CD_3OD, 400 MHz) δ 7.60 (d, J = 2.0 Hz, 2H), 7.58-7.57 (m, 1H), 7.39-7.36 (m, 2H), 7.26 (d, J = 8.4 Hz, 2H), 7.19 (s, 1H), 4.49 (s, 2H), 4.22 (d, J = 18.4 Hz, 1H), 4.15 (s, 3H), 3.92 (d, J = 18.4 Hz, 1H), 1.30 (s, 9H);^{13}C NMR (CD_3OD, 100 MHz) δ 161.2, 159.9, 153.8, 140.8, 140.5, 138.9, 136.8, 131.7, 130.8, 130.1, 126.8, 125.4 (q, J = 283.7 Hz), 115.6, 106.9, 88.0 (q, J = 30.4 Hz), 64.6, 44.8, 43.7, 40.2, 15.3; ^{19}F NMR (CD_3OD, 376 MHz) δ -81.3; HRMS (ESI) found 553.1400 [calc for $C_{26}H_{26}Cl_2F_3N_4O_2^+$ $(M+H)^+$ 553.1379]. Compound **1b**: $C_{26}H_{25}Cl_2F_3N_4O_2$, M_r = 553.40, monoclinic, space group $P2_1/c$, a = 20.2063(7) Å, b =14.3059(4) Å, c = 9.7417(4) Å, a = 90°, b = 103.124(4)°, g = 90°, V = 2742.47(17) $Å^3$, T = 293(2) K, Z = 4, ρ_{calc} = 1.340 g

cm^{-3}, F (000) = 1144.0, crystal dimension 0.16×0.1×0.06 mm^3, m(Cu Kα) = 2.571 mm^{-1}, Cu Kα radiation (λ =1.54184 Å). Of 4839 reflections collected in the 2θ range from 7.64 to 134.156° using an ω scan on a Rigaku Rapid R-axis diffractometer, 4839 were unique reflections (R_{sigma} = 0.0377). The structure was solved and refined against F^2 using SHELXS97 and SHELXL97, wR_2 = 0.2335, R_1 = 0.0798, GOF = 1.050, and max/min residual electron density 0.54/-0.35 e $Å^{-3}$. CCDC 2110234 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre.

Procedure for the preparation of 1c

Compound **1c** was prepared according to the procedure used in the preparation of **1a**: White solid; Yield: 16% for two steps; IR (cm^{-1}) 3335.6, 3144.1, 3098.0, 3075.5, 2980.0, 2957.3, 2927.7, 2873.8, 1646.4, 1571.9, 1549.2, 1515.5, 1303.8, 1094.3, 1048.6, 1025.4, 1011.0, 901.9, 864.5, 805.5, 762.1, 719.7, 672.6; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.59 (d, J = 1.6 Hz, 2H), 7.56-7.55 (m, 1H), 7.25-7.23 (m, 2H), 7.16 (s, 1H), 6.87-6.85 (m, 2H), 4.44 (s, 2H), 4.21 (d, J = 18.4 Hz, 1H), 4.14 (s, 3H), 4.00 (q, J = 6.8 Hz, 2H), 3.91 (d, J = 18.4 Hz, 1H), 1.36 (t, J = 7.0 Hz, 3H); ^{13}C NMR (CD_3OD, 100 MHz) δ 161.2, 159.9, 153.8, 140.8, 140.4, 138.9, 136.8, 131.7, 130.8, 130.1, 126.8, 125.4 (q, J = 283.7 Hz), 115.6, 106.9, 88.0 (q, J = 30.3 Hz), 64.6, 44.8, 43.7, 40.2, 15.3; ^{19}F NMR (CD_3OD, 376 MHz) δ -81.3; HRMS (ESI) found 541.1019 [calc for $C_{24}H_{22}Cl_2F_3N_4O_3^+$ $(M+H)^+$ 541.1016].

Procedure for the preparation of 1d

To a stirred solution of crude acid (500 mg, 1.23 mmol) in CH_2Cl_2 (10 mL) were added EDCI (0.35 g, 1.85 mmol mmol) and HOBt (0.25 g, 1.85 mmol) at room temperature under N_2 and the mixture was stirred at the same temperature for 30 min. To the reaction mixture, 4-(4-methylphenoxy)benzenemethanamine hydrochloride (0.37 g, 1.47 mmol) and Et_3N (0.68 mL, 4.92 mmol) were added and the mixture was continuously stirred at the same temperature for 15 h. The reaction mixture was partitioned between CH_2Cl_2 (30 mL) and 1M HCl (30 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 30 mL). The combined organic layers were washed successively with saturated aqueous $NaHCO_3$ and saturated brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in *vacuo*. The residue was purified by silica gel column chromatography (petroleum ether: EtOAc = 15 : 1) to give **1d** (150 mg, 18% for two steps) as a white solid: IR (cm^{-1}) 3423.9, 2926.3, 2857.1, 1654.7, 1500.7, 1242.0, 1191.0, 1169.6, 1102.9, 1026.5, 1009.8, 801.7, 761.1, 719.6, 693.4, 670.9; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.59 (d, J = 1.6 Hz, 2H), 7.56 (t, J = 2.0 Hz, 1H), 7.32-7.29 (m, 2H), 7.18 (s, 1H), 7.15-7.13 (m, 2H), 6.92-6.89 (m, 2H), 6.86-6.84 (m, 2H), 4.48 (s, 2H), 4.22 (d, J = 18.4 Hz, 1H), 4.15 (s, 3H), 3.91 (d, J = 18.4 Hz, 1H), 2.30 (s, 3H); ^{13}C NMR (CD_3OD, 100 MHz) δ 161.2,

158.7, 156.3, 153.8, 140.8, 140.5, 138.8, 136.8, 134.4, 134.3, 131.4. 130.8, 130.3, 126.8, 125.4 (q, J = 283.6 Hz), 120.1, 119.5, 107.0, 88.0 (q, J = 30.4 Hz), 44.8, 43.6, 40.2, 20.8; ^{19}F NMR (CD_3OD, 376 MHz) δ -81.3; HRMS (ESI) found 603.1154 [calc for $C_{29}H_{24}Cl_2F_3N_4O_3^+$ $(M+H)^+$ 603.1172].

The insecticidal activity against *Tetranychus urticae* Koch

The insecticidal activity against *Tetranychus urticae* Koch of all of the final compounds **1a**-**1d** was evaluated using a leaf dip method.[32] All of the synthesized arylisoxazoline derivatives were dissolved in acetone and diluted (with 0.1% Triton X-100 aqueous solution) into a series of working concentrations (250-500 mg/L) as required. The cowpea leaves with adult female mites on were immersed into each drug solution, taken out 5 sec later, and the excess drug solution was absorbed from the mites using absorbent paper. After the samples had been dried in the shade, the back side of each was placed on the surface of the aforementioned soaked sponge with filter paper. In total, three replicates were conducted for each concentration, with 30 mites present in each replicate. Treatment with 0.1% Triton X-100 aqueous solution was used as a control. After 24 h of inspection, the bodies of the mites were touched gently with a fine brush, and those that did not move their feet were deemed dead. The mortality data for the adult females were corrected using Abbott's formula.[36] For comparative purposes, Fluralaner was used as a positive control and was tested under the same conditions. Evaluation was based on a percentage scale of 0-100 (0 = no activity, 100 = total kill).

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