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Design and synthesis of *N*-2,6-difluorophenyl-5-methoxyl-1,2,4-triazolo[1,5-*a*]-pyrimidine-2-sulfonamide as acetohydroxyacid synthase inhibitor

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ABSTRACT

Triazolopyrimidine-2-sulfonamide belongs to a herbicide group called acetohydroxyacid synthase inhibitors. With the aim to discover new triazolopyrimidine sulfonanilide compounds with high herbicidal activity and faster degradation rate in soil, the methyl group of Flumetsulam (**FS**) was modified into a methoxy group to produce a new herbicidal compound, *N*-2,6-difluorophenyl-5-methoxy-1,2,4-triazolo[1,5-*a*]pyrimidine-2-sulfonamide (experimental code: **Y6610**). The enzymatic kinetic results indicated that compound **Y6610** and **FS** have k_i values of 3.31×10^{-6} M and 3.60×10^{-7} M against *Arabidopsis thaliana* AHAS, respectively. The 10-fold lower enzyme-inhibiting activity of **Y6610** was explained rationally by further computational simulations and binding free energy calculations. In addition, compound **Y6610** was found to display the same level in vivo post-emergent herbicidal activity as **FS** against some broadleaf weeds and good safety to rice, maize, and wheat at the dosages of 75–300 g ai/ha. Further determination of the half-lives in soil revealed that the half-life in soil of **Y6610** is 3.9 days shorter than that of **FS**. The experimental results herein showed that compound **Y6610** could be regarded as a new potential acetohydroxyacid synthase-inhibiting herbicide candidate for further study.

1. Introduction

Flumetsulam (**FS**), *N*-2,6-difluorophenyl-5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidine-2-sulfonamide, is the first triazolopyrimidine sulfonanilide herbicide for the control of broad-leaf weeds and grasses in soya beans, field peas and maize.[1] Its action target is acetohydroxyacid synthase (AHAS; also known as acetolactate synthase; EC 2.2.1.6; formerly EC 4.1.3.18), which catalyze the biosynthesis of branched-chain amino acids including valine, leucine, and isoleucine.[2] Selectivity of **FS** in soya beans is due to rapid metabolic deactivation. Availability of **FS** in soil is principally dependent upon soil pH and organic matter.[3,4]

However, because of the high herbicidal activity of **FS** on broad-leaf plants and slow degradation rate, its trace residues in soil will have adverse effect on following crops in quality and yield.[3,4] Therefore, it is very important to design and synthesize new triazolopyrimidine sulfonanilide compounds with high herbicidal activity and faster degradation rate in soil. The existing results indicated that methoxy-containing products always have faster rate of degradation in soils because the methoxy group could be rapidly degraded to hydroxyl group.[5–8] As a result, methoxy-containing products always have shorter half-life in soils. As shown in Scheme 1, for example, florasulam was rapidly degraded by microbial action with an average half-life of 2.4 days (range 0.7–4.5 days). The first step in the degradation pathway involved conversion of the methoxy group on the triazolopyrimidine ring to a hydroxyl group to form *N*-(2,6-difluorophenyl)-8-fluoro-5-hydroxy-1,2,4-triazolo[1,5-*a*]-pyrimidine-2-sulfonamide.[5] On the contrary, the half-life of **FS** in Hoytville clay soil was found to be 49 days at 26.1 °C.[3]

Based on the above consideration, we proposed if we modified the methyl group of **FS** into a methoxy group, the resulted compound (experimental code, **Y6610**, Scheme 1), *N*-2,6-difluorophenyl-5-methoxy-1,2,4-triazolo-[1,5-*a*]pyrimidine-2-sulfonamide, is expected to keep herbicidal activity and have shorter half-life in soils than **FS**. Herein, we described in detailed the synthesis, molecular modeling, in vitro enzyme inhibition, in vivo herbicidal activities, crop selectivity, and the results of soil degradation of compound **Y6610**.

2. Results and discussion

2.1. Synthetic chemistry and structural characterization

Compound **Y6610** was prepared by a six-step synthetic route using 3-amino-5-benzylthio-1,2,4-triazole (**1**) as starting material

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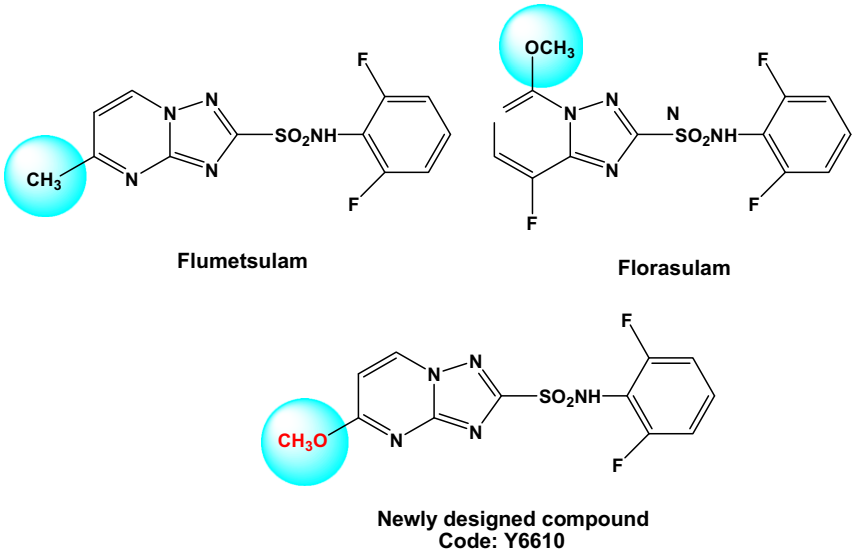


Scheme 1.

as shown in Scheme 2. The cyclization reaction of 3-amino-5-benzylthio-1,2,4-triazole (**1**) with diethyl malonate afforded 2-benzylthio-5,7-dihydroxy-1,2,4-triazolo-[1,5-*a*]pyrimidine (**2**), which was converted to 2-benzylthio-5,7-dichloro-1,2,4-triazolo[1,5-*a*]pyrimidine (**3**) by the chlorination reaction with $POCl_3$. Then, the 7-chloro of compound **3** was selectively removed by the Cu–Zn reagent to produce intermediate **4**. Subsequent substitution reaction of intermediate **4** with sodium methoxide resulted in 2-benzylthio-5-methoxy-1,2,4-triazolo[1,5-*a*]pyrimidine (**5**), which was converted easily to the key intermediate **6** with chlorine gas under ice bath. Without further purification, intermediate **6** reacted with 2,6-difluoroaniline in dichloromethane solution using pyridine as base to give the title compound **Y6610** in two-step overall yield of 38%. The structures of all intermediates and compound **Y6610** were confirmed by ^{1}H NMR, EI-MS spectral data, and elemental analyses. In addition, the crystal structure of **Y6610** was determined by X-ray diffraction analysis. As shown in Figure 1, the 1,2,4-triazolo[1,5-*a*] pyrimidine ring system is planar with a maximum deviation of 0.012 Å, the dihedral angle formed by the mean planes of triazolo-pyrimidine and benzene ring system is 43.08°.

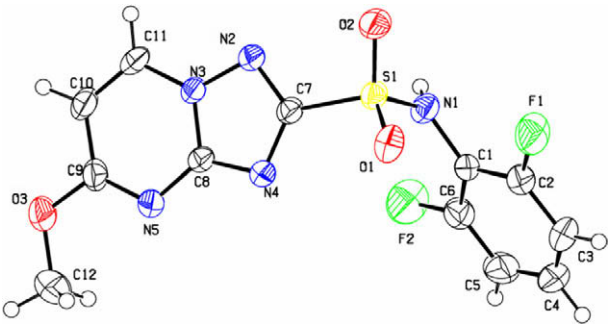
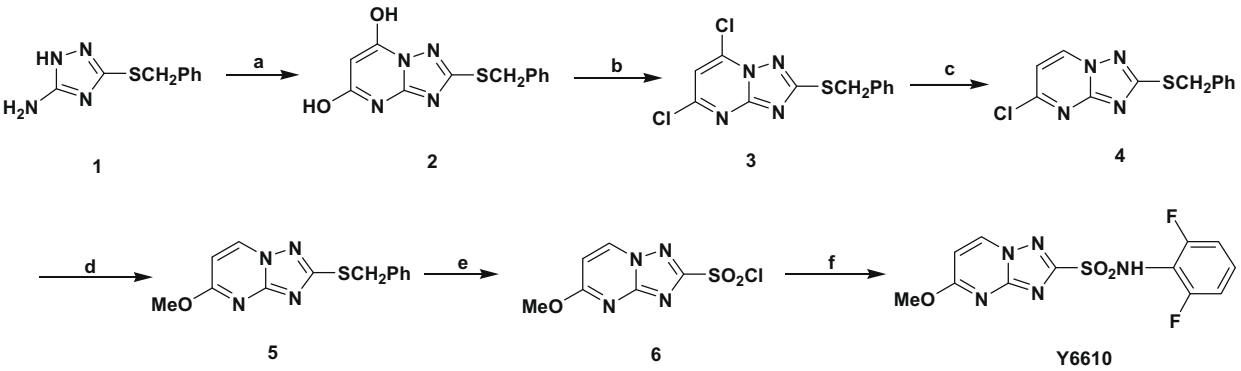


Figure 1. Crystal structure of compound **Y6610**.

2.2. Computational simulations of the interaction between Y6610 and AHAS

The k_i values of compounds **Y6610** and **FS** against *Arabidopsis thaliana* AHAS were determined according to the methods reported previously[9–11] and the results were listed in Table 1. As shown in Table 1, compound **Y6610** and **FS** have k_i values of 3.31×10^{-6} M and 3.60×10^{-7} M, respectively. These results indicated that compound **Y6610** displayed about 10-fold lower in vitro activity than **FS**. According to the equation of $\Delta G_{exp} = -RT\ln(1/K_i)$, the experimental binding free energies for **Y6610** and **FS** are calculated to be −7.49 kcal/mol and −8.80 kcal/mol, respectively. In order to understand the molecular mechanism of their different enzyme-inhibiting activity, we carried out further molecular simulations by integrating molecular docking, energy minimizations, and bind-



Scheme 2. Reagents and conditions: (a) $CH_2(COOC_2H_5)_2$, CH_3ONa, EtOH, reflux; (b) $POCl_3$, reflux; (c) Cu–Zn, THF; (d) CH_3ONa, CH_3OH; (e) Cl_2, CH_2Cl_2/H_2O; (f) 2,6-difluoroaniline, pyridine, CH_2Cl_2.

Table 1
Summary of the experimental and computational results

No.	Experimental k_i values[a] (M)	ΔG_{exp}[b] (kcal/mol)	ΔG_{cal}[c] (kcal/mol)
Y6610	3.31×10^{-6}	−7.49	−7.64
FS	3.60×10^{-7}	−8.80	−8.28

[a] Determined according to previously reported method.[10]
[b] $\Delta G_{exp} = -RT\ln k_{i,exp}$.
[c] Calculated according to the modified MM-GBSA method.[11]

ing free energy calculations. All the complex structures derived from molecular docking were performed energy minimizations using the Sander module of the AMBER 8 program until the convergence criterion of 0.001 kcal mol^{-1} $Å^{-1}$ was reached. Then, the corresponding binding free energies predicted for these two inhibitors using the modified MM-GBSA protocol used in our other studies[11–13] are −7.64 kcal/mol and −8.28 kcal/mol, respectively. The qualitative agreement between the computational and experimental results confirmed the reliability of our computational models.

As shown in Figure 2, the simulated models indicated that compound **Y6610** binds with *A. thaliana* AHAS in a similar mode to **FS**. The triazolopyrimidinyl moiety formed π–π stacking interactions with residue Trp574, which made a great contribution to the binding free energies (ΔG). However, compared with the structure of AHAS-**FS** complex, compound **Y6610** displayed a significant conformational change in the binding pocket in two ways. First, compound **Y6610** is buried deeper into the active site about ~1.2 Å than **FS** and the interactions between Trp574 and **Y6610** were improved slightly. Second, the sulfonyl group rotated about 78° along the S–C bond. As a result, the interactions between Arg377 with **Y6610** were significantly weakened. For example, there are three hydrogen bonds with residue Arg377 in the model of *A. thaliana* AHAS in complex with **FS**, but **Y6610** formed only two hydrogen bonds with residue Arg377. Therefore, the loss of one hydrogen bond resulted in about 10-fold decrease of binding affinity of compound **Y6610**.

2.3. Herbicidal activities and crop selectively

Although the in vitro test indicated that compound **Y6610** have 10-fold lower inhibition activity against *A. thaliana* AHAS than **FS**, the in vivo green house test as shown in Table 2 indicated that compound **Y6610** displayed promising and broad-spectrum post-emergence herbicidal activities as **FS**. For example, both compound **Y6610** and **FS** displayed over 80% inhibition activity even at the dosage of 37.5 g/ha against broad-leaf weeds, such as *Chenopodium album, Brassica juncea, Amaranthus retroflexus*,*Eclipta prostrate*, *Cerastium arvense*, *Portulaca oleracea*, *Abutilon theophrasti*, and *Cardamine hirsute* L. However, at the dosage of 37.5 g/ha, these two compounds did not displayed obvious herbicidal activity against monocot weeds, such as *Echinochloa crusgalli, Digitaria sanguinalis*, and *Setaria faberii*.

As shown in Table 3, rice, maize, and wheat among six tested crops exhibited highly tolerance to compound **Y6610** by post-emergence application at the dosages of 75–300 g ai/ha, whereas cotton, rape, and soybean are susceptible even at dosage as low as 75 g ai/ha. At the dosage of 300 g ai/ha, maize still exhibited highly tolerance to compound **Y6610**. It should be noted that rice, maize, cotton, rape, soybean, and wheat are highly sensitive to **FS** under the same experimental conditions. These results indicated that compound **Y6610** have much better crop safety than **FS** and might be developed as a potential herbicide used for the weed control in rice, maize, and wheat field.

2.4. HPLC determination of the half-lives of Y6610 and FS in soil

Three methods of simultaneous extraction of sulfonamide herbicides from soils, such as shake extraction, microwave-assisted extraction (MAE), and ultrasonic-assisted extraction (USE), were established. The results showed in the respects of method recovery shake extraction had the best extraction efficiency compared to MAE and USE. Therefore, shake extraction were used for extraction in all experiments. To test the linearity of the calibration curves,

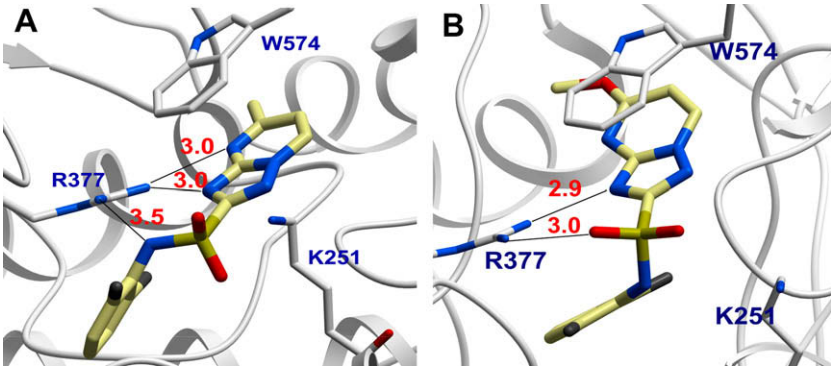


Figure 2. The binding models of **FS** (A) and **Y6610** (B) with *A. thaliana* AHAS.

Table 2
Herbicidal activity of compound **Y6610** and **FS** (post-emergency)

No.	Dosage (g ai/ha)	CA[a]	CT	BJ	AR	EP	CAR	PO	AT	CHL	EC	DS	SF
Y6610	37.5	++	+	++	++	+++	+++	+++	++	+++	–	–	–
	75	++	+	+++	++	+++	+++	+++	++	+++	–	–	–
	150	+++	++	+++	+++	+++	+++	+++	+++	+++	–	–	+
FS	37.5	–	++	++	++	+++	+++	++	++	++	–	–	–
	75	+	++	++	++	+++	+++	+++	++	+++	–	+	–
	150	+	+++	++	+++	+++	+++	+++	+++	+++	+	++	+

[a] CA for *Chenopodium album*, CT for *Cassia tora*, BJ for *Brassica juncea*, AR for *Amaranthus retroflexus*, EP for *Eclipta prostrate*, CAR for *Cerastium arvense*, PO for *Portulaca oleracea*, AT for *Abutilon theophrasti*, CHL for *Cardamine hirsute* Linn, EC for *Echinochloa crusgalli*, DS for *Digitaria sanguinalis*, and SF for *Setaria faberii*.
[b] Rating system for the growth inhibition percentage: +++, ⩾90%; ++, ⩾80%; +, 50–80%; –, <50%.

Table 3
Sensitivities of compound **Y6610** and **FS** against different crops

No.	Dosage (g ai/ha)	Rice	Cotton	Soybean	Maize	Rape	Wheat
Y6610	75	0	20	60	0	70	0
	150	0	30	80	0	80	0
	300	10	/	/	0	/	10
FS	75	60	80	80	30	70	50

Table 4
Linear equation and limits of detection of the method

Analytes	Linear range (μg/mL)	Linear equation	*r*	LOD (ng/mL)
Y6610	0.01–5	$Y = 120.8x + 0.06$	0.9989	0.254
FS	0.01–5	$Y = 120.5x - 2.41$	0.9997	0.266

the various concentrations of the standard solution ranging from 0.01 to 5 μg/mL were analyzed by HPLC. The calibration curves were constructed from peak areas counts. As shown in Table 4, a good linearity relationship is observed for two analytes with the correlation coefficients (*r*) ranging from 0.9997 to 0.9989. The limits of detection (LOD) based upon a signal-to-noise ratio of 3:1 (S/N = 3) are 0.266 ng/mL for FS, 0.254 ng/mL for **Y6610**. In addition, validation of the method was performed in terms of recovery studies. Recovery experiments were carried out by adding known volume of herbicides standard to the soil samples. Satisfied recovery results were found to be 80% and 72% for **Y6610** and **FS**, respectively. The limit of quantification (LOQ) for this method was defined as the lowest concentration of compounds in a sample that could be quantitatively determined with suitable precision and accuracy. The LOQ was determined as the sample concentration of the herbicide at peak heights of 10 times the baseline noise. The LOQ of **Y6610** was found to be 0.0852 mg/kg, and that for **FS** was 0.0752 mg/kg.

The results of the half-life for **FS** and **Y6610** are given in Figure 3. Experiments were carried out to test the degradation of the analytes in soil. Aliquots of spiked soils containing 5 mg/kg of **FS** and 5 mg/kg of **Y6610** were processed according to the proposed procedure.

According to the degradation equation:[14]

$$C = C_0 \times e^{-kT}$$

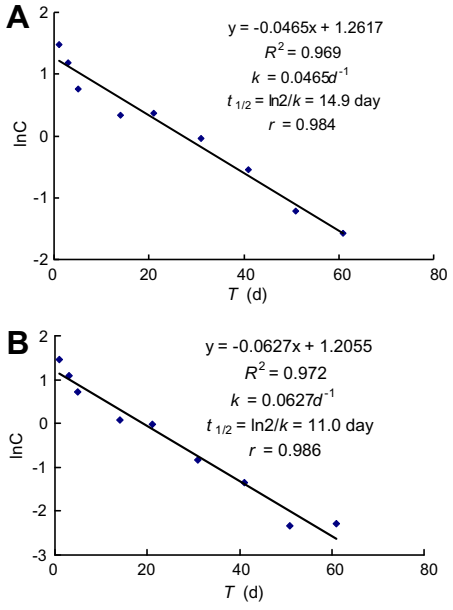


Figure 3. Determination curves of the half-life for **FS** (A) and **Y6610** (B).

The half-life can be calculated by the following equation:

$$T_{(1/2)} = \ln 2/k$$

In which, the degradation coefficient *k* equals to the slope of the linear plotting curve of ln*C* versus *T*. As can be seen, the half-life is 14.9 day for **FS** and 11.0 day for **Y6610**, respectively. The correlation coefficients (*r*) of the **FS** and **Y6610** are 0.984 and 0.986, respectively.

3. Conclusion

In conclusion, based on the known degradation mechanism of methoxy-containing triazolopyrimidine-2-sulfonamide, a new methoxy-containing analogue of **FS**, *N*-2,6-difluorophenyl-5-methoxy-1,2,4-triazolo[1,5-*a*]-pyrimidine-2-sulfonamide (**Y6610**), was designed and synthesized with the aim to discover new triazolopyrimidine2-sulfonamide with high herbicidal activity and faster degradation rate in soil. The enzymatic kinetic study indicated that **Y6610** and **FS** have k_i values of 3.31×10^{-6} M and 3.60×10^{-7} M against *A. thaliana* AHAS, respectively. Computational simulations were performed to describe the interactions between **Y6610** and AHAS, which explained rationally why **Y6610** displayed 10-fold lower enzyme-inhibiting activity than **FS**. In addition, greenhouse assay indicated that compound **Y6610** displayed the same level post-emergent herbicidal activity as **FS** against some broad-leaf weeds. Very interestingly, crop selectively test showed that **Y6610** have good safety to rice, maize, and wheat at the dosages of 75–300 g ai/ha. On the contrary, rice, maize, cotton, rape, soybean, and wheat are highly sensitive to **FS** under the same experimental conditions. Further determination of the half-lives in soil revealed that the half-life is 14.9 day for **FS** and 11.0 day for **Y6610**, respectively. The obtained results showed that compound **Y6610** could be regarded as a new potential acetohydroxyacid synthase-inhibiting herbicide candidate for further study.

4. Materials and methods

Unless otherwise noted, reagents were purchased from commercial suppliers and used without further purification, as all solvents were redistilled before use. ^{1}H NMR spectra were recorded on a Mercury-Plus 400 spectrometer in $CDCl_3$ or DMSO with TMS as the internal reference. MS spectra were determined using a Trace MS 2000 organic mass spectrometer. Elemental analyses were performed on a Vario EL III elemental analysis instrument. Melting points were measured on a Buchi B-545 melting point apparatus and are uncorrected. Intermediates **1** were prepared directly by the reaction of benzyl chloride with commercially available 3-amino-5-mercapto-1,2,4-triazolo in ethanol solution of sodium hydroxide.[15]

4.1. Preparation of 2-benzylthio-5,7-dihydroxy-1,2,4-triazolo[1,5-*a*]pyrimidine (2)

A methanol solution of 10.8 g (50 mmol) of sodium methoxide (25%) dissolved in 10 mL of absolute ethanol was treated with 4 g (25 mmol) of diethyl malonate followed by 6.8 g (25 mmol)

of 3-amino-5-benzylthio-1,2,4-triazole. The resulting solution was heated at reflux for 5 days. On cooling to room temperature the solid which had separated was collected by filtration, washed with cold ethanol and dissolved in 100 mL of water. The resulting yellow solution was acidified with concentrated HCl to precipitate a solid. The solid was collected by filtration and dried to yield 6.4 g (94%) of the desired product as a white solid, mp 202–209 °C (decomposition) [lit.,[16] 199–210 °C (decomposition)]. ^{1}H NMR spectra were in agreement with the assigned structure. ^{1}H NMR (400 MHz, DMSO): δ 4.38 (s, 2H), 7.26–7.42 (m, 6H), 12.92 (br s, 2H).

4.2. Preparation of 2-benzylthio-5,7-dichloro-1,2,4-triazolo[1,5-*a*]pyrimidine (3)

A suspension of 11.0 g (0.04 mol) of 2-benzylthio-5,7-dihydroxy-1,2,4-triazolo[1,5-*a*]pyrimidine and 57.0 mL (0.6 mol) of phosphorous oxychloride was heated at reflux for 5 h. The resulting solution was concentrated by evaporation under reduced pressure. The residue was partitioned between cold water and methylene chloride, and the organic phase was separated and dried ($MgSO_4$). The organic phase was filtered with silica and concentrated by evaporation under reduced pressure to yield the desired product as 8.7 g (70%) of yellow solid. mp 102–104 °C [lit.,[16] 97–100 °C]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.52 (s, 2H), 7.13 (s, 1H), 7.25–7.30 (m, 3H), 7.47 (d, 2H, J = 7.2 Hz).

4.3. Preparation of 2-benzylthio-5-chloro-1,2,4-triazolo[1,5-*a*]pyrimidine (4)

A zinc–copper couple was prepared by stirring 1.0 g of copper sulfate in 20 mL of water with 15.0 g of zinc dust for 2 h. The couple was collected by filtration, washed with acetone, and dried overnight under vacuum at 100 °C. To a solution of 33.0 g (106 mmol) of 2-benzylthio-5,7-dichloro-1,2,4-trizolo[1,5-*a*]pyrimidine in 12.5 mL (213 mmol) of acetic acid 50 mL of methanol and 300 mL of fresh distilled tetrahydrofuran was added 20.5 g of Zn–Cu couple. When the reaction was complete (TLC analysis) the mixture was filtered through Celite and the filtrate was concentrated by evaporation at reduced pressure. The residue was triturated with methanol to separated a solid. The solid was collected by filtration to yield the desired product as 26.6 g (91%) of orange solid, mp 123–125 °C [lit.,[16] 125–127 °C]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.50 (s, 2H), 7.05 (d, 1H, J = 7.2 Hz), 7.25–7.32 (m, 3H), 7.45 (d, 2H, J = 7.2 Hz), 8.61 (d, 1H, J = 7.2 Hz).

4.4. Preparation of 2-benzylthio-5-methoxy-1,2,4-triazolo[1,5-*a*]pyrimidine (5)

A mixture of 6.0 g (22 mmol) of 2-benzylthio-5-chloro-1,2-4-triazolo[1,5-*a*]pyrimidine in 25 mL of methanol was treated with 5.0 g (23.8 mmol) of a 25% solution of sodium methoxide in methanol. When the reaction was complete (TLC analysis) the reaction mixture was diluted with 100 mL of water and neutralized with 3 N HCL (aq). The solid which separated was collected by filtration, washed with water and dried to afford 4.5 g (76%) of the desired product as a white solid, mp 124–126 °C [lit.,[16] 126–128 °C]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.08 (s, 3H), 4.52 (s, 2H), 6.49 (d, 1H, J = 7.2 Hz), 7.26–7.34 (m, 3H), 7.46 (d, 2H, J = 7.2 Hz), 8.40 (d, 1H, J = 7.2 Hz).

4.5. Preparation of 5-methoxy-1,2,4-triazolo[1,5-*a*]pyrimidine-2-sulfonyl chloride (6)

Chlorine was bubbled into a suspension of 10.9 g (40 mmol) of 2-benzylthio-5-methoxy-1,2,4-triazolo[1,5-*a*] pyrimidine in 100 mL of methylene chloride, 50 mL of water cooled to −1 °C. During the course of the addition the temperature of the reaction mixture was maintained below 5 °C. When the reaction was complete (TLC analysis) the organic layer was separated and the aqueous layer was extracted twice with methylene chloride. The combined organic phases were dried ($MgSO_4$) and evaporated at reduced pressure to obtain crude product and it was used straight without further purification.

4.6. Preparation of *N*-(2,6-difluorophenyl)-5-methoxy-1,2,4-triazolo[1,5-*a*]pyrimidine-2-sulfonamide (Y6610)

The starting 2,6-difluoroaniline (7.8 g, 60 mmol) was dissolved in 100 mL of pyridine and the above suspension in 100 mL dichloromethane was added. The reaction mixture was stirred at room temperature for 10 h. The pyridine was removed by evaporation at reduced pressure, and the residue was treated with 200 mL of 0.5 N NaOH and 100 mL dichloromethane. After stirring to dissolve all soluble material the aqueous layer was separated and acidified with 3 N HCl. The precipitate which separated upon acidification was collected by filtration and dried to give 5.2 g (38% overall yield from **5**) of the desired product as a yellow solid. Mp 230–231 °C; ^{1}H NMR (400 MHz, DMSO): δ 4.05 (s, 3H), 7.05 (d, 1H, J = 7.2 Hz), 7.12 (d, 2H, J = 8.0 Hz), 7.41 (t, 1H, J = 8.0 Hz), 9.24 (d, 1H, J = 7.2 Hz), 10.75 (br s, 1H). ESI-MS: m/z (%) 342 ([M+1]$^+$), 340 ([M−1]$^+$); Anal. Calcd for $C_{12}H_9F_2N_5O_3S$: C, 42.23; H, 2.66; N, 20.52. Found: C, 42.12; H, 2.57; N, 20.36.

4.7. X-ray diffraction analysis

Colorless blocks of **Y6610** (0.40 mm × 0.04 mm × 0.02 mm) were counted on a quartz fiber with protection oil. Cell dimensions and intensities were measured at 298 K on a Bruker SMART CCD area detector diffractometer with graphite monochromated Mo Kα radiation (λ = 0.71073 Å); θ_{max} = 25.99; 14,210 measured reflections; 2723 independent reflections (R_{int} = 0.1417) of which 1251 had $I > 2\sigma(I)$. Data were corrected for Lorentz and polarization effects and for absorption (T_{min} = 0.8959; T_{max} = 0.9944). The structure was solved by direct methods using SHELXS-2001;[17] all other calculations were performed with Bruker SAINT System and Bruker SMART programs.[18] Full-matrix least-squares refinement based on F^2 using the weight of $1/[\sigma^2(F_o^2) + (0.0849P)^2 + 0.0555P]$ gave final values of R = 0.0506, ωR = 0.1152, and GOF(F) = 0.897. Hydrogen atoms were observed and refined with a fixed value of their isotropic displacement parameter.

4.8. Methods of computational simulations

The X-ray crystal structures of *A. thaliana* AHAS in complex with herbicide molecules have been reported.[19–22] To save computational time we constructed a dimmer model of *A. thaliana* AHAS for further study since the active site is formed at the interface between two monomers. The initial monomer coordinates were chosen from the X-ray crystal structure of *A. thaliana* AHAS in complex with Chlorsulfuron (PDB code: 1YHZ).[22] The crystal structure of the dimmer AHAS from yeast (PDB code: 1NOH) was used as the template. After the herbicide molecule was removed, flumetsulam and **Y6610** were docked into the active site of AHAS with FlexX. The residues within 6.5 Å around the ligand were defined as the active site and the final models were selected on the basis of total score. The MD simulations were performed using the Sander module of the AMBER 8 program. The partial atomic charges of ligands and cofactors ThDP and flavin adenine dinucleotide (FAD) were calculated using the RESP protocol after electrostatic potential calculations at the Hartree-Fock (HF) level with 6-31G* basis set using the GAUSSIAN 03 program. Parm99 force field was used to establish

the potentials of proteins, and general AMBER force field (gaff) was used to establish the potentials of inhibitors and cofactors. The Counterions Na^+ was added to neutralize the system using LEAP module. And then the AHAS complexes were solvated in a rectangular box of TIP3P water molecules with a minimum solute–wall distance of 8 Å. In molecular minimization and molecular dynamics simulations, particle mesh Ewald (PME) was employed to treat the long-range electrostatic interactions. To equilibrate the solvated system, two steps of minimization were carried out. First, the protein systems were frozen and the solvent molecules with counterions were allowed to move; then, only the added hydrogen atoms of protein systems were energy-minimized; finally, all atoms were permitted to move freely. The three minimization stages consisted of 5000 steps in which the first 1000 were steepest descent (SD) and the last 4000 conjugate gradient (CG). The systems were gradually heated from 0 K to 300 K over 50 ps and the equilibrating calculation were executed at 1 atm and at 300 K using NPT ensemble. The time step was set to 1.0 fs with a cutoff of 10 Å for the non-bonded interaction, and the SHAKE procedure was employed to constrain all hydrogen atoms. The convergence of energies, temperatures, and pressures of the systems, and the atomic root-mean-square deviation of the enzyme and the inhibitor (RMSD), were used to verify the stability of the systems. The final structures of the complexes were obtained as the average of the last 200 ps of MD minimized with CG method until a convergence of 0.05 kcal mol^{-1} $Å^{-1}$. The binding free energies were calculated by using the molecular mechanics-Poisson–Boltzmann surface area (MM-PBSA) binding free energy calculation method. The entropic contribution to the binding free energy was calculated according to our modified method.[11]

4.9. Herbicidal activities of Y6610

The herbicidal activities of compound **Y6610** against *C.* album (CA), Cassia tora (CT), *B.* juncea (BJ), *A.* retroflexus (AR), *E.* prostrate (EP), *C.* arvense (CAR), *P.* oleracea (PO), *A.* theophrasti (AT), *C.* hirsute L. (CHL), *E.* crusgalli (EC), *D.* sanguinalis (DS), and *S.* faberii (SF) were evaluated according to a previously reported procedure,[23–26] **FS** was selected as a control. All test compounds were formulated as a 100 g/L emulsified concentrates by using DMF as solvent and TW-80 as emulsification reagent. The concentrates were diluted with water to the required concentration and applied to pot-grown plants in a greenhouse. The soil used was a clay soil, pH 6.5, 1.6% organic matter, 37.3% clay particles, and CEC 12.1 mol/kg. The rate of application (g ai/ha) was calculated by the total amount of active ingredient in the formulation divided by the surface area of the pot. Plastic pots with a diameter of 9.5 cm were filled with soil to a depth of 8 cm. Approximately 20 seeds of *C. album,* C. tora, *B. juncea, A. retroflexus, E. prostrate, C. arvense, P. oleracea, A. theophrasti, C. hirsute* L., *E. crusgalli, D. sanguinalis,* and *S. faberii* were sown in the soil at the depth of 1–3 cm and grown at 15–30 °C in a greenhouse. The diluted formulation solutions were applied for post-emergence treatment, dicotyledon weeds were treated at the 2-leaf stage and monocotyledon weeds were treated at the 1-leaf stage, respectively. The post-emergence application rate was 150 g ai/ha. Untreated seedlings were used as the control group and the solvent (DMF) treated seedlings were used as the solvent control group. Herbicidal activity was evaluated visually 15 days post-treatment. The results of herbicidal activities were shown in Table 2.

4.10. Crop selectivity[26,27]

The conventional rice, soybean, cotton, wheat, rape, and maize were respectively planted in plots (diameter = 12 cm) containing test soil and grown in a greenhouse at 20–25 °C. After the plants had reached the 4-leaf stage, the spraying treatment was conducted at different dosages by diluting the formulation of compound **Y6610** with water. The visual injury and growth state of the individual plant were observed at regular intervals. The final evaluation for crop safety of compound **Y6610** was conducted by visual observation in 30 days after treatment on the 0–100 scale.

4.11. HPLC determination of the half-lives of Y6610 and FS in soil[28]

Methanol was of HPLC grade purchased from J&KCHEMICA Co., and dichloromethane was of analytical grade. Other reagents were of analytical grade. The water used was ultrapure water (Millipore Simplicity 185, Corporation, Billerica, MA, USA). Stock solutions of **Y6610** and flumetsulam were prepared by dissolving 10.0 mg of the analytes in 100 mL of Methanol. Standard solutions of different concentrations (0.01–5 μg/mL) were obtained by diluting the stock solutions with ultrapure water. All solutions prepared were stored at 4 °C. An Agilent 1100 liquid chromatograph (Agilent Technologies Inc.) equipped with a variable wavelength detector (VWD), a quatpump, an analytical ChemStation, and a 20 μL injection loop was used for all analyses. The analytes were carried out on a Venusil, XBP C18 column (250 mm × 4.6 mm i. d., 5 μm). The mobile phase was a methanol/phosphate buffer solution (50:50, v/v; pH 3.0). The flow rate was kept at 0.8 mL/min. The detection wavelength was 225 nm, and the column temperature was 25 °C.

4.12. Preparation of soil samples

Soil sample (pH 5.31, organic matter, 2.84 g/kg) was spiked with **Y6610** and **FS** at the level of 5 mg kg^{-1} by spraying of a standard solution of the two analytes in methanol. The soil was air-dried and left outdoors for periods of time that vary between 1 day and 2 months to evaluate the half-life of the pesticides in the soil. Any analyte–matrix interactions were assumed to have occurred over the weathering period and to an extent similar to that in actual contaminated soil of similar properties.

4.13. Extraction procedure

Soil samples collected were lyophilized, smashed and sieved to a 40-mesh sieve. In this study, 1 g of soil was weighed and added into 50 mL conical vessels. It was extracted with a sequence of two 5 mL aliquots of methanol and water (9:1, v/v) in a shaking extraction (HQ45 shaking apparatus, Wuhan, China) for 30 min. Afterwards, the sample were filtrated with a 0.45 μm millipore filter. All of the filtrates were poured into a pear-shaped flask, 5 mL of 0.1 mol/L KCl and 2 mL of 0.2 mol/L hydrochloric acid were added. Then the target substances in soil samples were extracted using 5 mL of dichloromethane in duplicate. The organic solvent phase was then separated and evaporated to near dryness under a stream of nitrogen in automated nitrogen evaporator (AutoScience Co., Tianjin, China). The residue was re-dissolved in exactly 1 mL of methanol–water (1:1, v/v) mixture, and 20 μL was subsequently injected into the HPLC system for analysis.

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