CHEMISTRY & BIODIVERSITY

Accepted Article

Title: N-(2-(3-Chlorophenylamino)phenyl)-3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide: Synthesis, crystal structure, molecular docking and biological activities

Authors: Aigui Zhang, Yihua Yang, Ying Yue, Ke Tao, Taiping Hou, and Hong Jin

To be cited as: *Chem. Biodiversity* 10.1002/cbdv.201900118

Link to VoR: http://dx.doi.org/10.1002/cbdv.201900118

Chem. Biodiversity

N-(2-(3-Chlorophenylamino)phenyl)-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide: Synthesis, crystal structure, molecular docking and biological activities

Aigui Zhang, Yihua Yang, Ying Yue, Ke Tao, Taiping Hou* and Hong Jin*

Key Laboratory of Bio-Resource and Eco-Environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610065, Sichuan, P. R. China

e-mail:jinhong@scu.edu.cn; houtplab@scu.edu.cn

ABSTRACT: In continuation of our previous research on the development of novel pyrazole-4-carboxamide with potential antifungal activity, compound **SCU2028**, namely *N*-(2-(3-chlorophenylamino)phenyl)-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide, was synthesized by new method, structurally characterized by IR, ESI-HRMS, ^{1}H and ^{13}C NMR spectra and further identified by single-crystal X-ray diffraction. In pot tests compound **SCU2028** showed good *in vivo* antifungal activity against *Rhizoctonia solani* (*R. solani*) and IC_{50} value of it was 7.48 mg L^{-1}. And in field trials control efficacy of compound **SCU2028** at 200 g. a.i ha^{-1} was 42.30 % on the 7th day after the first spraying and 68.10 % on the 14th day after the second spraying, only slightly lower than that of thifluzamide (57.20 % and 71.40 %, respectively). Further *in vitro* inhibitory activity showed inhibitory ability of compound **SCU2028** was 45-fold higher than that of bixafen and molecular docking of compound **SCU2028** to SDH predicted its binding orientation in the active site of the target protein SDH. These results suggested that compound **SCU2028** was a potential fungicide for control of rice sheath blight.

Keywords: pyrazole-4-carboxamide • crystal structure • molecular docking • biological activities • succinate dehydrogenase.

Introduction

Plant diseases caused by fungal pathogens led to detrimental agricultural crop losses.[1] Among the plant diseases rice sheath blight is one of the most important diseases worldwide and can cause heavy losses in rice yield.[2-4] At present agrochemicals are still the better choice for controlling rice sheath blight and in China the succinate dehydrogenase inhibitor (SDHI) thifluzamide had been registered for rice sheath blight and exhibited excellent efficacy in controlling this disease.[5] Besides it in SDHI fungicides pyrazole carboxamides were also a class of important SDHIs and widely used, especially CF_2H substituted pyrazole

carboxamides such as fluxapyroxad, bixafen, sedaxane, isopyrazam and isoflucypram (Figure 1).[6] However, the extensively application of a single chemical for a long time might increase the risk of resistance development of certain fungi.[7] Therefore, development and application of a new fungicide is one of the most important strategies.[8]

Small molecules containing the diarylamine moieties displayed excellent agricultural activities such as antifungal, insecticidal and herbicidal activities.[9-11] In previous studies we discovered a novel fenfuram-diarylamine hybrid by replacing the phenyl group in fenfuram with the diarylamine moieties, which had exhibited the better antifungal activities than fenfuram.[12] Inspired by this result, bixafen was choose as a lead compound, the diarylamine moieties were introduced to replace the biphenyl group and pyrazole carboxamide with diarylamine-modified scaffold were synthesized. Among them, compound **SCU2028**, namely *N*-(2-(3-chlorophenylamino)phenyl)-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide showed good *in vitro* antifungal activities against *Rhizoctonia solani* (*R. solani*) and IC_{50} value of it reached 0.015 mg L^{-1} (Figure 1).[13]

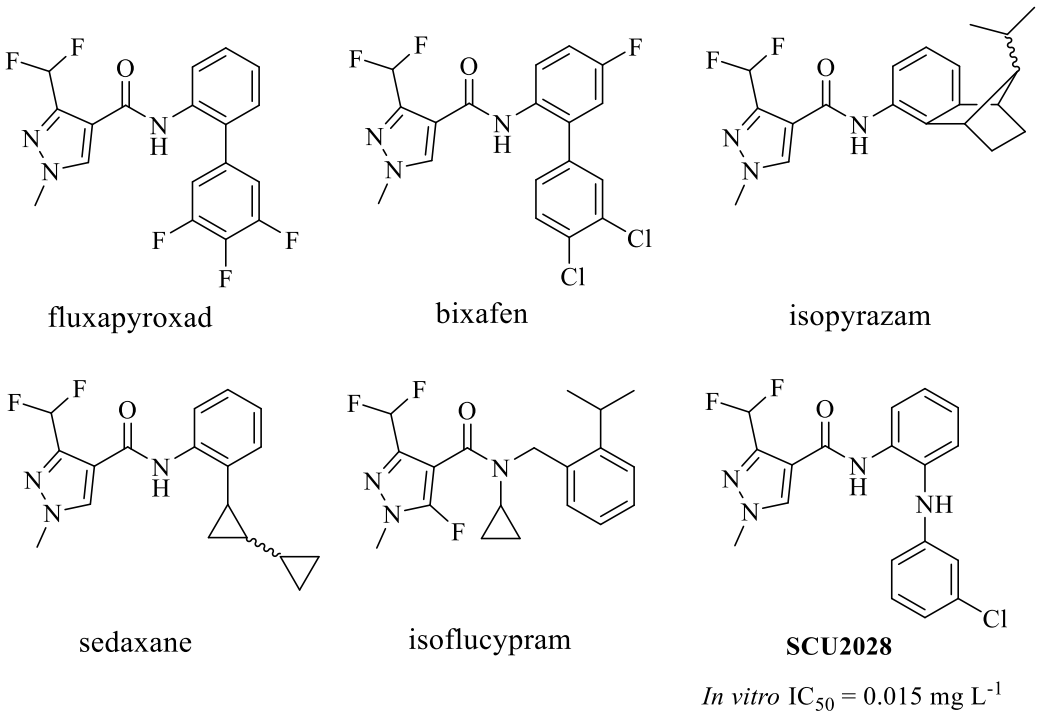


Figure 1. Structures of the commercial pyrazole-4-carboxamide fungicides and compound **SCU2028**.

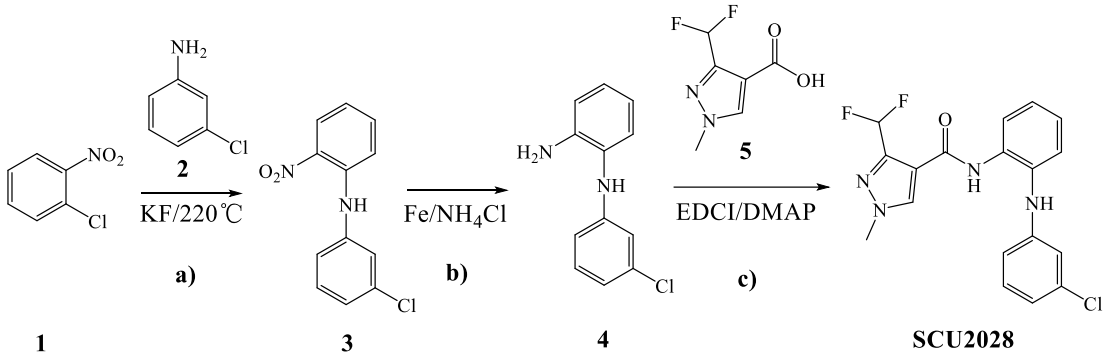
Thus, as our continuous work on studying compound **SCU2028** in depth, in paper compound **SCU2028** was synthesized by new synthesis method and its structure was further identified by single-crystal X-ray diffraction. In addition, to further evaluate its biological activities, its *in vivo* antifungal activities in pot tests and field trials and *in vitro* inhibitory

activity against SDH were evaluated. Furthermore, molecular docking study predicted the binding pose of it to SDH.

Results and Discussion

Chemistry

Scheme 1 detailed the synthesis and chemical structure of compound **SCU2028**. Initially, Intermediate **3** was obtained by condensation reaction and then transformed into the corresponding compound **4** through reduction reaction. Then the key pyrazole acid **5** directly reacted with compound **4** under EDCI and DMAP immediately to afford compound **SCU2028.**[14] And this method was different from previous method.[13] At last, the structure of compound **SCU2028** was confirmed by ^{1}H NMR, ^{13}C NMR, IR and ESI-HRMS spectroscopic data.



Scheme 1. Synthetic route of compound **SCU202**8.

X-ray crystallography

The crystallographic data and refinement information of compound **SCU2028** is summarized in Table 1. CCDC 1870000 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033).

Table 1. Crystal data for compound **SCU2028**.

Parameter	**SCU2028**
Empirical formula	$C_{18}H_{15}ClF_2N_4O$
Formula weight	376.79
Temperature/K	293.01(10)
Crystal system	triclinic
Space group	P-1

a/Å	7.0253(4)
b/Å	9.0047(4)
c/Å	13.6853(7)
α/°	89.605(4)
β/°	82.635(4)
γ/°	88.091(4)
Volume/Å3	858.12(8)
Z	2
ρcalcg/cm3	1.458
μ/mm-1	2.298
F (000)	388.0
Crystal size/mm3	0.65 × 0.45 × 0.25
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/ °	9.828 to 145.098
Index ranges	$-8 \leq h \leq 8$, $-9 \leq k \leq 11$, $-16 \leq l \leq 16$
Reflections collected	9409
Independent reflections	3329 [Rint = 0.0256, Rsigma = 0.0223]
Data/restraints/parameters	3329/0/236
Goodness-of-fit on F2	1.049
Final R indexes [I>=2σ (I)]	R1 = 0.0534, wR2 = 0.1467
Final R indexes [all data]	R1 = 0.0568, wR2 = 0.1509
Largest diff. peak/hole / e Å-3	0.36/-0.55

The molecular structure of compound **SCU2028** was illustrated in Figure 2. Crystal data and the selected bond lengths and angles of **SCU2028** were listed in Tables 1 and 2. **SCU2028** was composed of a pyrazole ring moiety, two phenyl moieties, a chlorine atom, a CF_2 methyl group, a methyl group and an amide group. For CF_2 methyl group, bond length of C5-F_2 is 1.35 Å and the bond angle of corresponding F2-C5-C4 is 109.82 °. In pyrazole ring moiety, bond length of N2-C4 is 1.33 Å, shorter than that of C2-C3 (1.38 Å). In the amide group, bond length of the C6-O1 is 1.22 Å, and bond length of the C6-N3 is 1.36 Å. The bond angle of C3-C6-N3 is 115.53 °, while the bond angle of C6-N3-C7 is 124.97 °. From the molecular structure, it can be seen that the bond angle of C12-N4-C13 is 127.78 ° and the bond length of the C17-Cl1 is 1.75 Å. The dihedral angle between the two benzene rings is

41.55 °, which indicates that the two rings are not coplanar in the molecular structure.

Table 2. Selected bond lengths (Å) and angles (℃) for compound **SCU2028**.

Bond Lengths/angles	Calculated Values	Bond Lengths/angles	Calculated Values
C5- F2	1.35 Å (2)	C2-C3	1.38 Å (3)
C6-O1	1.22 Å (2)	C6-N3	1.36 Å (2)
N2-C4	1.33 Å (2)	C17-Cl1	1.75 Å (2)
C6-N3-C7	124.97 ℃(16)	C12-N4-C13	127.78 ℃(17)
C3-C6 -N3	115.53 ℃(16)	F2-C5-C4	109.82 ℃(17)

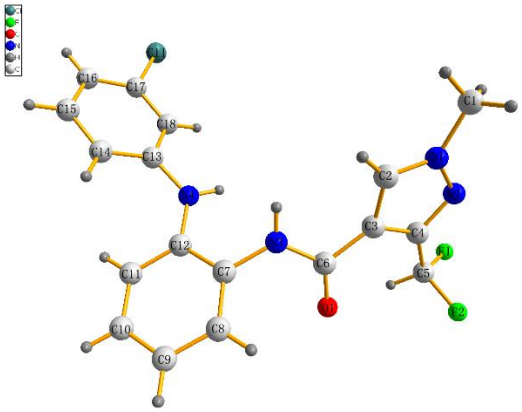


Figure 2. Molecular structure of compound **SCU2028**.

Crystal data for compound **SCU2028**: $C_{18}H_{15}ClF_2N_4O$ (M =376.79 g/mol): triclinic, space group P-1 (no. 2), a = 7.0253 (4) Å, b = 9.0047(4) Å, c = 13.6853 (7) Å, α = 89.605 (4)°, β = 82.635 (4)°, γ = 88.091 (4)°, V = 858.12 (8) Å3, Z = 2, T = 293.01 (10) K, μ(CuKα) = 2.298 mm-1, Dcalc = 1.458 g/cm3, 9409 reflections measured (9.828° ≤ 2Θ ≤ 145.098°), 3329 unique (Rint = 0.0256, Rsigma = 0.0223) which were used in all calculations. The final R1 was 0.0534 (I > 2σ(I)) and wR2 was 0.1509 (all data).

Biological activities

Pot tests

To further assess biological activities of compound **SCU2028**, the *in vivo* antifungal activities in pot tests and field trials and inhibitory activity against SDH were evaluated. Firstly the *in vivo* antifungal activities of compound **SCU2028** against *R. solani* in pot tests were evaluated in a greenhouse environment according to the previously reported procedure and thifluzamide was selected as positive control in Table 3.[15] As shown in Table 3, compound **SCU2028** displayed promising antifungal activity (90 %) against *R. solani* at a dosage of 80 mg L^{-1}.

Even at a dosage of 20 mg L^{-1}, it still showed antifungal activity of 80 %. At a lower dosage of 10 mg L^{-1}, it displayed 60 % antifungal activity against *R. solani*. When its concentration was reduced to 5 mg L^{-1}, the effect was still 35 %. Although IC_{50} value of compound **SCU2028** was 7.48 mg L^{-1} and higher than that of thifluzamide (IC_{50} = 3.12 mg L^{-1}), the novel fungicide **SCU2028** with diarylamine scaffold had potentially antifungal activity against *R. solani*. So, the *in vivo* antifungal activities of compound **SCU2028** in field trials were carried out in the next trials.[15-17]

Table 3. *In vivo* antifungal activities against *R. solani* of compound **SCU2028** in pot tests.

Compd	Dosage (mg L^{-1})	Antifungal activities (%)	IC_{50} (mg L^{-1})
SCU2028	80	90	7.48
	40	85	
	20	80	
	10	60	
	5	35	
	2.5	15	
thifluzamide	80	100	3.12
	40	100	
	20	95	
	10	85	
	5	80	
	2.5	55	
	1.25	15	

Field trials

The *in vivo* antifungal activities of compound **SCU2028** against rice sheath blight in field trials were evaluated in according to the previously reported procedure and thifluzamide was selected as positive control in Table 4.[15-16] As shown in Table 4, applications of compounds **SCU2028** and thifluzamide at 200 g.ai ha^{-1} recorded protection effects with 42.30 % and 57.20 % on the 7th day after the first spraying. Obviously, the effect of thifluzamide was slightly better than that of compound **SCU2028** at 200 g.ai ha^{-1} after the first spraying. Furthermore, the control effects would respectively go up to 68.10 % and 71.40 % on the 14th day after the second spraying for compound **SCU2028** and thifluzamide. When the treatment concentration of compounds decreased to 100 g.ai ha^{-1}, compound **SCU2028** displayed a

slightly lower efficacy after the two sprayings, 39.50 % and 62.10 % respectively. At the same time control efficacy of thifluzamide also slightly dropped at 100 g.ai ha^{-1}. Obviously, although the effect of thifluzamide was slightly better than that of compound **SCU2028**, the difference between the control effects of the two compounds was weak at 200 g.ai ha^{-1}. This suggested that compound **SCU2028** could be a potential antifungal candidate.

Table 4. *In vivo* control efficacy of compound **SCU2028** against rice sheath blight in field trials.

treatment	CTa (g. ai ha^{-1})	7^{th} after the first spraying		14^{th} after the second spraying	
		Disease index	Control effects (%)	Disease index	Control effects (%)
SCU2028	200	1.24	42.30	0.96	68.10
	100	1.30	39.50	1.14	62.10
	50	1.46	32.10	1.26	58.10
thifluzamide	200	0.92	57.20	0.86	71.40
	100	0.94	56.10	0.92	69.40
	50	1.18	45.10	1.01	66.40
control	0	2.15	--	3.01	--

a: concentration

SDH inhibition

In order to investigate whether the SDH is a potential target enzyme of compound **SCU2028** or not, the fungal SDH inhibition assay was performed.[12] Compounds **SCU2028** and bixafen (a commercial SDH inhibitor) were selected to test *in vitro* inhibitory activity against SDH from mitochondria of *R. solani*. As demonstrated in Table 5, compound **SCU2028** (IC_{50} = 0.0392 mg L^{-1}) showed higher inhibitory activity against SDH than bixafen (IC_{50} = 1.766 mg L^{-1}), which indicated that the inhibitory ability of compound **SCU2028** was 45-fold higher than that of bixafen. It proved that the SDH is one of the important action targets of compound **SCU2028**.

Table 5. *In vitro* inhibitory activity of compound **SCU2028** against SDH.

Compd	IC_{50} (mg L^{-1})*
SCU2028	0.0392 ± 0.020

bixafen	1.766 ±0.105

*Data are means ± SE, n=3

Molecular docking studies

In addition, in order to theoretically illuminate the mechanism of action of compound **SCU2028** against SDH, molecular docking of compound **SCU2028** into the binding site of the SDH was performed according to previous method.[18-20] The theoretical binding mode between compound **SCU2028** and SDH was shown in Figure 3. Compound **SCU2028** fit in the gap composed of subunit B, C and D of SDH. The phenyl group linking amide bond in the middle of compound **SCU2028** was occupied the hydrophobic pocket composed of the residues B/Pro-202, B/Ile-251, C/Ile-77 and C/Trp-73, while the 4-methylpyrazole scaffold of compound **SCU2028** located at another hydrophobic pocket, surrounded by the residues B/Trp-205, B/Trp-206, C/Phe-64 and C/Trp-73, forming a stable hydrophobic binding. Detailed analysis showed that a π-π stacking interaction was observed between the phenyl group linking amide bond of compound **SCU2028** and sidechain of the residue C/Trp-73. In addition, 4-methylpyrazole group of compound **SCU2028** formed CH-π interaction with the residue C/Trp-73. Moreover, the 3-chlorophenyl group of **SCU2028** formed cation-π interaction C/Arg-80 (Figure 3). All these interactions helped compound **SCU2028** anchor in the binding site of SDH. The above molecular simulations gave us rational explanation of the interactions between compound **SCU2028** and SDH, which also provided valuable information for further discovery of the SDHIs.

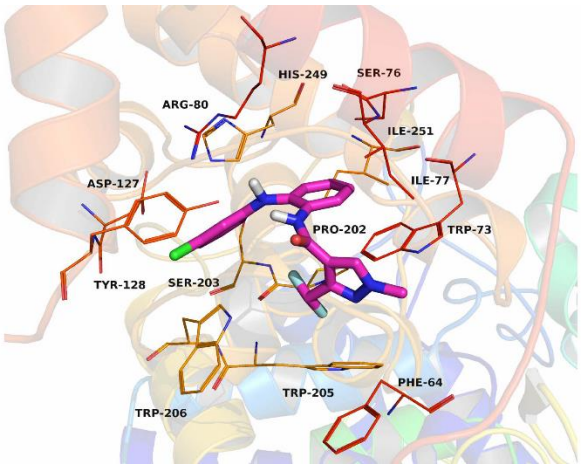


Figure 3. The putative binding mode between **SCU2028** and SDH.

Conclusions

In summary, compound **SCU2028** was synthesized by new method and evaluated as a new fungicide candidate. Its structure was confirmed by using different spectroscopic techniques and its configuration was determined via its single crystal X-ray analysis. By pot tests, it showed good promising *in vivo* antifungal activity against *R. solani* with IC_{50} value of 7.48 mg L^{-1}. Moreover, the field trials showed that the control effect of compound **SCU2028** was only slightly lower than that of thifluzamide against rice sheath blight at 200 g.ai ha^{-1}. Further *in vitro* inhibitory activity showed inhibitory ability of compound **SCU2028** was 45-fold higher than that of bixafen and molecular docking study showed the possible binding mode of compound **SCU2028** to the SDH. It was believed that compound **SCU2028** could be further utilized as a potential fungicide to control rice sheath blight. Further synthesis optimization and toxicological studies are currently in progress.

Experimental Section

Reagents and Materials

All chemicals and reagents were purchased from commercial sources and were used as received. All reactions were monitored by thin layer chromatography (TLC) using pre-coated silica gel GF254 plates. Column chromatography purification was performed over silica gel (300-400 mesh, Qingdao Marine Chemical Ltd., P.R. China). Melting points (uncorrected) were determined on a SPSIC WRS-1B digital melting point apparatus. IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer (KBr presser method). ESI-HRMS spectra were recorded on a Bruker Daltonics ESI-BioTOF-Q High Definition Mass Spectrometer. ^{1}H NMR and ^{13}C NMR spectra were collected on a Bruker AVII-400 NMR spectrometer (Bruker Company, Germany) using DMSO-d_6 as solvent. Chemical shifts (δ) are reported in parts per million (ppm) with reference to internal TMS, and coupling constants (J) are provided in Hertz (Hz). Single crystal X-Ray diffraction measurements were conducted on a Bruker Smart Apex II diffractometer.

General Procedure

Synthesis

A reaction mixture containing 1-chloro-2-nitrobenzene 1 (20 mmol), 3-chlorobenzenamine 2 (30 mmol) and KF (30 mmol) was stirred at 220 ℃ and were monitored by TLC. After reaction completion, the desired intermediate 3 was obtained *via* column chromatographic purification. Secondly, pure compound 3 (20 mmol), reductive iron powder (60 mmol),

ammonium chloride (60 mmol) and ethanol aqueous solution (75 %, 50 mL) were mixed and refluxed for 2 h and the key intermediate 4 was obtained. At last, 3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxylic acid 5 (30 mmol), EDCI (30 mmol), DMAP (0.6 mmol) and compound 4 (25 mmol) were dissolved in DCM (CH_2Cl_2, 50 mL). The mixture was stirred at room temperature for 6 h and the target compound **SCU2028** was gained and purified *via* recrystallization. Recrystallisation from anhydrous methanol afforded a white powder: yield, 70 %; mp, 128-129 ℃; ^{1}HNMR (400 MHz, DMSO-d_6) δ 3.93 (s, 3H, N-CH_3), 6.76 (dd, 1H, J = 7.6, 1.6 Hz, Ph-H), 6.82-6.86 (m, 2H, Ph-H), 7.07 (td, 1H, J = 7.7, 1.3 Hz, Ph-H), 7.16 (t, 1H, J = 8.0 Hz, Ph-H), 7.19 (td, 1H, J = 7.6, 1.5 Hz, Ph-H), 7.31 (t, 1H, JH-F= 54.0 Hz, CHF_2), 7.33 (s, 1H, Ph-H), 7.55 (d, 1H, J = 7.6 Hz, Ph-H), 7.73 (s, 1H, Ph-NH-Ph), 8.37 (s, 1H, Py-H), 9.51 (s, 1H, CO-NH); ^{13}C NMR (100MHz, DMSO-d_6) δ 39.42 (-NMe-C), 109.74 (t, J_{C-F} = 232.9 Hz, -CF_2H-), 114.07 (Ph-C), 114.94 (Ph-C), 116.06 (t, J_{C-F} = 3.5 Hz, Py-C), 118.42 (Ph-C), 121.45 (Ph-C), 122.76 (Ph-C), 126.08 (Ph-C), 126.49 (Ph-C), 129.61 (Ph-C), 130.58 (Ph-C), 133.15 (Py-C), 133.54 (Ph-C), 135.51 (Ph-C), 144.91 (t, J_{C-F} = 23.1 Hz, Py-C), 146.15 (Ph-C), 160.23 (-CONH-); IR (KBr, cm^{-1}): 1663.86 (C = O); ESI-HRMS: m/z $[M+H]^+$ calcd. for $C_{18}H_{16}ClF_2N_4O$ 377.0981, found 377.0973.

X-ray analysis

Condition on crystal growing of compound **SCU2028**: **SCU2028** (1.0 g) was dissolved in 60 ml anhydrous methanol, heated to 50 ℃ and stirred until the mixture approaching saturated solution. Subsequently, the hot solution was filtered immediately and solvent slowly was evaporated at room temperature and colorless crystal suitable for XRD was obtained.

Single crystal X-ray diffraction measurements were conducted on a Bruker APEX-II diffractometer using Cu-Kα radiation (λ = 1.54184 Å) with a graphite monochromator at 293.01(10) K in scan mode. Collected data and cell refinement were reduced using Olex2[21] and the structure was solved with the ShelXT[22] structure solution program using Direct Methods and refined with the ShelXL[23] refinement package using Least Squares minimisation.

Biologal activities

*Pot tests for compound **SCU2028** against R. solani*

In vivo fungicidal activity of compound **SCU2028** against *R. solani* was tested according to the procedure described previously.[13] Rice plants were grown under greenhouse conditions (Relative Humidity = 70-80 %, Temperature = 24-26 °C, Light Intensity ≥ 2000 lux) in vinyl

planting pots. Compound **SCU2028** dissolved in acetone and distilled water containing Tween-80 (0.05 %) at the given concentration was sprayed over the plant and subsequently cultivated for 24 h. The blank control groups and the treated rice seedlings at the third-leaf stage were inoculated with strain *R. solani*, then the symptoms were examined 5 days later. Pots were arranged as a randomized complete block with three replicates per treatment. The inhibition percentage was expressed as the mean of values obtained in three independent experiments. Commercial fungicide thifluzamide was used as a control.

*Field trials for compound SCU**2028** in controlling rice sheath blight*

A rice field naturally infected by *R. solani* was selected to perform the field trials by using the standard method.[17] Field trials were conducted in Chengdu, Sichuan Province, China, to determine the potential control of rice sheath blight in summer rice in 2018. The field was divided into 21 plots, and the size of each plot was 5 × 6 m. Each of the plots was separated by a 50 cm interval with untreated rice plants. The concentrations used were 0, 50, 100, 200 g.ai ha^{-1} for compound **SCU2028** and thifluzamide treatments. All field trials were arranged in a block design and each treatment on a given test was replicated a minimum of three times throughout the field. Foliar application of fungicides was made by using a hand sprayer after the emergence of disease. Each treatment took up in three plots and was distributed randomly in each field. Fungicides were sprayed upon initiation of disease on August 4, 2018 for the first time and the second application was carried out seven days later. The relative control effects were assessed at the 7^{th} and 14^{th} day of foliar application of compound **SCU2028** and thifluzamide.

The disease severity was scored by using the following scale: [15-17]

0: no symptoms;

1: lesions limited to lower 1/4 of leaf sheath area;

3: lesions present on lower 1/2 of leaf sheath area;

5: lesions present on more than 1/2 of leaf sheath area, with slight infection on lower (3^{rd} or 4^{th}) leaves;

7: lesions present on more than 3/4 of leaf sheath, with severe infection on upper leaves (flag and 2nd leaf);

9: lesions reaching top of tillers, with severe infection on all leaves and some plants killed.

Disease severity and the control control efficacy were calculated as follows:

Disease severity = [Σ (The number of diseased plants in this index × Disease index)/(Total number of plants investigated × The highest disease index)] ×100 %.

Control efficacy = [(Disease severity of control−Disease severity of treated group)/Disease severity of control] ×100 %.

*In vitro enzyme assay for compound **SCU2028** against SDH*

Isolation of R. solani

Fungus mitochondrial was isolated according to a previously reported method.[12] Cultures were inoculated at 0.05 OD_{600} nm and grown on a reciprocal shaker (180 rpm, 25 ℃) for 5 days in Sabouraud maltose broth (SMB) medium. Cells were harvested by vacuum filtration and disrupted in liquid nitrogen using a mortar and pestle. The resultant powder was resuspended to 10 % w/v in mitochondrial extraction buffer (10 mM KH_2PO_4, pH 7.2, 10 mM KCl, 10 mM $MgCl_2$, 0.5 M sucrose, 0.2 mM EDTA, 2 mM PMSF). The extract was clarified by centrifugation (5000 g, 4 ℃ for 10 min, 2 times), and intact mitochondria were then pelleted at 10000 g for 20 min at 4 ℃ and resuspended in the same buffer. Mitochondrial suspensions were brought to a concentration of 10 mg/mL and stored at −80 °C until use. SDH activity was found to remain stable for months.

Succinate:Ubiquinone/DCPIP activity inhibition

Mitochondrial suspensions were diluted 1/20 in extraction buffer and preactivated at 30 ℃ for 30 min in the presence of 10 mM succinate. Succinate:ubiquinone/DCPIP activity inhibition measurements were performed by adding 10 μL of preactivated mitochondria to 200 μL of assay buffer (50mM phosphate-sodium, pH 7.2, 250mM sucrose, 3mM NaN3, 10 mM succinate) supplemented with 140 μM dichloro- phenolindophenol (DCPIP) and 1 mM 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0). Inhibitor concentrations ranged between 0.0050 and 15 μg/mL, with uniform 5× dilution factor steps (six inhibitor concentrations + DMSO control). A total of 96 well plates were pre-equilibrated at reaction temperature (30 ℃) for 10 min before the reactions were started by the addition of 10 μL of preactivated *R. solani* mitochondrial suspension. DCPIP reduction was conducted at 30 ℃ and monitored at 595 nm. Calculated absorbance slopes (OD/h) were used for half-inhibitory concentration (IC_{50}) calculations using GraphPad Prism 5.0 software.

Homology modeling and molecular docking

Homology modeling

The NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/) was used to search the SDH amino acid sequence of *R. solani*. The employed protein sequence was CUA72490.1, CUA71217.1, CUA73421.1 and CUA73959.1 reported by Wibberg. The BLAST server

(http://blast.ncbi.nlm.nih.gov) was used to search a template for the chain. We applied SDH from avian (PDB ID:1YQ3) as the template, and the homology of amino acid sequence was aligned. Homology modeling of SDH from R. solani was carried out using MODELER 9.15 (http://salilab. org/modeller/).

Molecular docking

Molecular docking studies were performed to investigate the binding mode of the compound to the SDH using Autodock vina 1.1.2.[18-19] The 3D structure of compound **SCU2028** was drawn by ChemBioDraw Ultra 14.0 and ChemBio3D Ultra 14.0 softwares. The AutoDockTools 1.5.6 package [20] was employed to generate the docking input files. The ligand was prepared for docking by merging non-polar hydrogen atoms and defining rotatable bonds. The search grid of SDH was identified as center_x: 86.459, center_y: 65.6, and center_z: 85.537 with dimensions size_x: 15, size_y: 15, and size_z: 15. The value of exhaustiveness was set to 20. For Vina docking, the default parameters were used if it was not mentioned. The best-scoring pose as judged by the Vina docking score was chosen and visually analyzed using PyMoL 1.7.6 software (http://www.pymol.org/).

Acknowledgements

This study was supported by the National Key Research and Development Program of China (2016YFC0502004, 2017YFD0200503) for the financial support.

Author Contribution Statement

H. Jin designed and managed the project. A. G. Zhang contributed to synthetic work. Y. Yue and Y. H. Yang contributed to activity evaluation. K. Tao and T. P. Hou conducted the optical experiments. Y. H. Yang worked on the molecular docking studies.

References

[1] M. Schirra, S. D'Aquino, P. Cabras, A. Angioni, 'Control of Postharvest Diseases of Fruit by Heat and Fungicides: Efficacy, Residue Levels, and Residue Persistence. A Review', *J. Agric. Food Chem.* **2011**, *59*, 8531-8542.

[2] D. E. Groth, 'Azoxystrobin Rate and Timing Effects on Rice Sheath Blight Incidence and Severity and Rice Grain and Milling Yields', *Plant Dis.* **2005**, *89*, 1171-1174.

[3] B. Prasad, G. C. Eizenga, 'Rice Sheath Blight Disease Resistance Identified in Oryza spp. Accessions', *Plant Dis.* **2008**, *92*, 1503-1509.

[4] C. Zhang, Y. Liu, X. Ma, Z. Feng, Z. Ma, 'Characterization of Sensitivity of Rhizoctonia Solani, Causing Rice Sheath Blight, to Mepronil and Boscalid', *Crop Prot.* **2009**, *28*, 381-386.

[5] H. Sierotzki, G. Scalliet, 'A Review of Current Knowledge of Resistance Aspects for the Next-Generation Succinate Dehydrogenase Inhibitor Fungicides', *Phytopathology* **2013**, *103*, 880-887.

[6] Z. Ma, T. Michailides, 'Advances in Understanding Molecular Mechanisms of Fungicide Resistance and Molecular Detection of Resistant Genotypes in Phytopathogenic Fungi', *Crop Prot.* **2005**, *24*, 853-863.

[7] Y. Chen, A. Zhang, W. Wang, Y. Zhang, T. Gao, 'Baseline sensitivity and efficacy of thifluzamide in Rhizoctonia solani', *Ann. Appl. Biol.* **2012**, *161*, 247-254.

[8] L. Xiong, Y. Shen, L. Jiang, X. Zhu, W. Yang, W. Huang, G. Yang, Succinate Dehydrogenase: An Ideal Target for Fungicide Discovery. In *Discovery and Synthesis of Crop Protection Products, Vol. 1204*, American Chemical Society, 2015, pp. 175-194.

[9] B. Dreikorn, K. Kramer, US. Pat. 4407820A, 1983.

[10] B. Dreikorn, K. Kramer, US. Pat. 4670596A, 1987.

[11] B. Dreikorn, K. Kramer, D. Berard, R. Harper, E. Tao, L. Thompson, J. Mollet, 'Synthesis and Structure-Activity Relationship Development of Novel *N*-(substituted-phenyl)-*N*-Alkyl-2-(Trifluoromethyl)-4,6-Dinitrobenzenamines Leading to a Potent Miticide (EL-462)', *ACS Symposium Series, Amer. Chem. Soc.* **1992**, 336-341.

[12] H. Jin, J. Zhou, T. Pu, A. Zhang, X. Gao, K. Tao, T. Hou, 'Synthesis of Novel Fenfuram-Diarylether Hybrids as Potent Succinate Dehydrogenase Inhibitors', *Bioorg. Chem.* **2017**, *73*, 76-82.

[13] X. Zhang, H. Jin, Y. Deng, X. Gao, Y. Li, Y. Zhao, K. Tao, T. Hou, 'Synthesis and biological Evaluation of Novel Pyrazole Carboxamide with Diarylamine-Modified Scaffold as Potent Antifungal Agents', *Chin. Chem. Lett.* **2017**, *28*, 1731-1736.

[14] S. Han, Y. Kim, 'Recent Development of Peptide Coupling Reagents in Organic Synthesis', *Tetrahedron* **2004**, *60*, 2447-2467.

[15] Y. Chen, J. Yao, X. Yang, A. Zhang, T. Gao, 'Sensitivity of Rhizoctonia Solani Causing Rice Sheath Blight to Fluxapyroxad in China', *Eur. J. Plant Pathol.* **2014**, *140*, 419-428.

[16] D. Yang, B. Wang, J. Wang, Y. Chen, M. Zhou, 'Activity and Efficacy of Bacillus Subtilis Strain NJ-18 Against Rice Sheath Blight and Sclerotinia Stem Rot of Rape', *Biol. Control* **2009**, *51*, 61-65.

[17] B. Gu, N. Liu, X. Wu, Q. Zhu, ' Pesticide-Guidelines for the Field Efficacy Trials(I)-Fungicides Against Sheath Blight of Rice', *National Standards of China* **2000**, *GB/T 17980. 34-2000*.

[18] O. Trott, A. J. Olson, 'Software News and Update AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading', *J. Comput. Chem.* **2010**, *31*, 455-461.

[19] M. F. Sanner, 'Python: A Programming Language for Software Integration and Development', *J. Mol. Graph. Model.* **1999**, *17*, 57-61.

[20] G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, 'AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility', *J. Comput. Chem.* **2009**, *30*, 2785-2791.

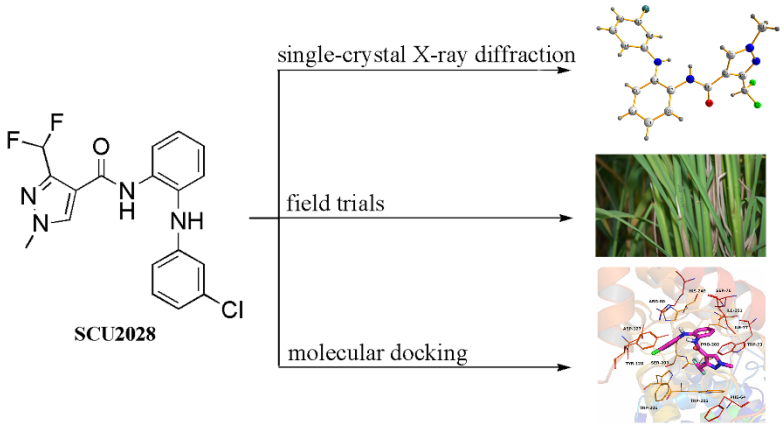
[21] O.V. Dolomanov, L. J. Bourhis, R. J Gildea, J. A. K. Howard, H. Puschmann, 'OLEX2: A Complete Structure Solution, Refinement and Analysis Program', *J. Appl. Cryst.* **2009**, *42*, 339-341.

[22] G. M. Sheldrick, 'SHELXT - Integrated Space-Group and Crystal-Structure Determination', *Acta Cryst.* **2015**. *A71*, 3-8.

[23] G. M. Sheldrick, 'Crystal Structure Refinement with SHELXL', *Acta Cryst.* **2015**, *C71*, 3-8.

Chem. Biodiversity

Entry for the Graphical Illustration



Twitter Text

In continuation of the development of pyrazolecarboxamide, *N*-(2-(3-chlorophenylamino)phenyl)-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide showed good *in vivo* antifungal activity against *Rhizoctonia solani*.