

Cite this: *RSC Adv.*, 2018, 8, 39593

# Design, synthesis and fungicidal activity of isothiazole–thiazole derivatives†

Qi-Fan Wu,<sup>‡</sup> Bin Zhao,<sup>‡</sup> Zhi-Jin Fan,<sup>\*a</sup> Jia-Bao Zhao,<sup>a</sup> Xiao-Feng Guo,<sup>a</sup> Dong-Yan Yang,<sup>\*a</sup> Nai-Lou Zhang,<sup>a</sup> Bin Yu,<sup>a</sup> Tatiana Kalinina<sup>\*b</sup> and Tatiana Glukhareva<sup>b</sup>

3,4-Dichloroisothiazoles can induce systemic acquired resistance (SAR) to enhance plant resistance against a subsequent pathogen attack, and oxathiapiprolin exhibits excellent anti-fungal activity against oomycetes targeting at the oxysterol-binding protein. To discover novel chemicals with systemic acquired resistance and fungicidal activity, 21 novel isothiazole–thiazole derivatives were designed, synthesized and characterized according to the active compound derivatization method. Compound **6u**, with EC<sub>50</sub> values of 0.046 mg L<sup>-1</sup> and 0.20 mg L<sup>-1</sup> against *Pseudoperonospora cubensis* (Berk. et Curt.) Rostov and *Phytophthora infestans in vivo*, might act at the same target as oxysterol binding protein (PcORP1) of oxathiapiprolin; this result was validated by cross-resistance and molecular docking studies. The expression of the systemic acquired resistance gene *pr1* was significantly up-regulated after treating with compound **6u** for 24 h (43-fold) and 48 h (122-fold). These results can help the development of isothiazole–thiazole-based novel fungicides.

Received 13th September 2018

Accepted 8th November 2018

DOI: 10.1039/c8ra07619g

rsc.li/rsc-advances

## 1 Introduction

Crops are often infected by various pathogens, which results in yield reduction. Oomycetes are filamentous eukaryotic microorganisms<sup>1</sup> that attack a large number of plants<sup>2</sup> and animals<sup>3</sup> and therefore, they can sometimes threaten agricultural ecosystems. Some pathogens cause humanitarian disasters; for example, the Great Famine was caused by oomycetes attacking potato fields.<sup>4</sup> The oomycetes classified as *Phytophthora* are one of the most serious pathogenic threats all over the world.<sup>5</sup> Only *Phytophthora infestans* (Mont.) de Bary caused the failure of the potato crop protection in 1840s and decreased the population of Ireland nearly by 25%.<sup>6</sup>

Heterocyclic compounds are widely used in novel agrochemical researches and developments.<sup>7–10</sup> Thiazoles have attracted the interest of pharmaceutical and agrochemical anti-fungal research since the development of thiabendazole as a fungicide in 1962 by Merck.<sup>11</sup> Oxathiapiprolin was

developed by DuPont in 2007 as a piperidinyl thiazole isoxazoline fungicide targeting at oxysterol binding protein (PcORP1) against *Peronospora belbahrii*, *Phytophthora parasitica* var. *Nicotianae* (Breda de Hean) Tucker, *Phytophthora capsici* Leonian and downy mildew.<sup>12–16</sup> However, fungicides acting at a single site of action have a high level of resistance risk.<sup>12</sup>

As a derivative of 1,3-thiazole, isotianil has a wide range of biological activities<sup>17–19</sup> including fungicidal, insecticidal, herbicidal and antiviral activities *via* activating the plant induced systemic resistance and affecting multiple links in the life cycle of pathogens. Particularly, 3,4-dichloroisothiazoles not only have antifungal activity, but also show good systemic acquired resistance.<sup>17</sup> Novel fungicide development is one of the important directions and measures for fungicide resistance management.<sup>20</sup>

On the basis of our former findings,<sup>4,5,21–23</sup> to continue our aim of finding novel highly anti-fungal active compounds with novel modes of action and without resistance risks, 4 types of novel target molecules were designed and synthesized for fungicidal activity and systemic acquired resistance evaluation based on PcORP1 as the target and N and S containing five-membered heterocycles as bioactive substructures by the combination of bioactive substructures of plant elicitor isotianil and fungicide oxathiapiprolin according to the active compound derivatization method<sup>24</sup> (Fig. 1 and Table 1). The mode of action of the active compound was validated by cross resistance, molecular biology and molecular docking studies.

<sup>a</sup>State Key Laboratory of Elemento-Organic Chemistry, College of Chemistry, Nankai University, Tianjin 300071, P. R. China. E-mail: fanzj@nankai.edu.cn; yangdy@nankai.edu.cn; Fax: +86-22-23503620; Tel: +86-23499464

<sup>b</sup>The Ural Federal University named after the First President of Russia B. N. Yeltsin, 19 Mira Str., Ekaterinburg 620002, Russia. E-mail: t.a.kalinina@urfu.ru

† Electronic supplementary information (ESI) available: Crystal data of **6j** (CCDC 1867013), antifungal activities of target compounds *in vivo* and *in vitro* and the physico-chemical data of the title compound. CCDC 1867013. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c8ra07619g

‡ Qifan Wu and Bin Zhao contributed equally to this work.



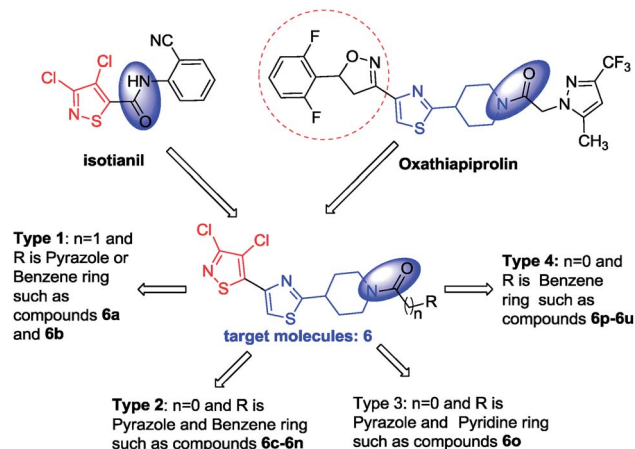


Fig. 1 Design of target isothiazole-thiazoles.

## 2 Results and discussion

### 2.1 Chemistry

The synthesis route of isothiazole-thiazole derivatives is designed and shown in Scheme 1. Compound **1** was constructed according to reported procedures.<sup>17</sup> 3,4-Dichloro-*N*-methoxy-*N*-methylisothiazole-5-carboxamide was obtained by the reaction between 3,4-dichloroisothiazole-5-carboxylic acid and *N*,*O*-dimethylhydroxylamine hydrochloride to produce the Weinreb amide, which was then reacted with methyl magnesium bromide to obtain 1-(3,4-dichloroisothiazol-5-yl)ethan-1-

one **1** with a good yield. Compound **2** could be prepared in one step from compound **1** by a substitution reaction with pyridinium tribromide. Compound **4** was synthesized by condensation between intermediate **2** and *tert*-butyl 4-carbamothioylpiperidine-1-carboxylate **3**. After removal of protection group at *N*-Boc of compound **4** under trifluoroacetic acid, intermediate **5** was obtained. By the activation of the corresponding acid using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride and 1-hydroxybenzotriazole and the succeeding condensation with compound **5**, isothiazole-thiazole derivatives **6** were effectively afforded. A crystal of the representative compound **6j** was obtained from the mixture of dichloromethane and ethyl acetate for X-ray diffraction (Fig. 2).

### 2.2 Compound 6u has the same potent target as oxathiapiroline

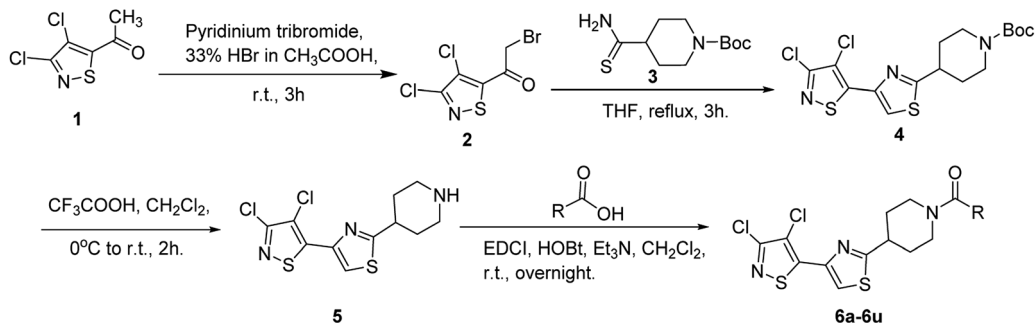
**2.2.1 Fungicidal spectrum *in vitro*.** The median effective concentration ( $EC_{50}$ ) values of compounds **6a-6u** with inhibition rates greater than 70% at  $50 \text{ mg L}^{-1}$  are listed in Table 2.

Both compounds **6o** and **6s** showed higher activities, with  $EC_{50}$  values of  $8.92 \text{ mg L}^{-1}$  and  $7.84 \text{ mg L}^{-1}$ , respectively, than oxathiapiroline ( $EC_{50} = 296.60 \text{ mg L}^{-1}$ ) and azoxystrobin ( $EC_{50} = 185.42 \text{ mg L}^{-1}$ ) against *Alternaria solani*. The  $EC_{50}$  values of compounds **6b** ( $EC_{50} = 0.22 \text{ mg L}^{-1}$ ) and **6c** ( $EC_{50} = 0.53 \text{ mg L}^{-1}$ ) were about 1/20 and 1/10, respectively, as compared to that of commercial fungicides oxathiapiroline ( $EC_{50} = 5.98 \text{ mg L}^{-1}$ ) and azoxystrobin ( $EC_{50} = 4.04 \text{ mg L}^{-1}$ ) against *Sclerotinia sclerotiorum*.

Table 1 The structures of the target compounds **6a-6u**

Compound	$n$	R	R <sup>1</sup>	Compound	$n$	R	R <sup>1</sup>
<b>6a</b>	1		—	<b>6k</b>	—		
<b>6b</b>	1		—	<b>6l</b>	—		
<b>6c</b>	—	—		<b>6m</b>	0		
<b>6d</b>	—	—		<b>6n</b>	—	—	
<b>6e</b>	0			<b>6o</b>	0		—
<b>6f</b>	—	—		<b>6p</b>	0		—
<b>6g</b>	—	—		<b>6q</b>	0		—
<b>6h</b>	—	—		<b>6r</b>	0		—
<b>6i</b>	0			<b>6s</b>	0		—
<b>6j</b>	—	—		<b>6t</b>	0		—
				<b>6u</b>	0		—





Scheme 1 Synthesis route of isothiazole–thiazole derivatives.

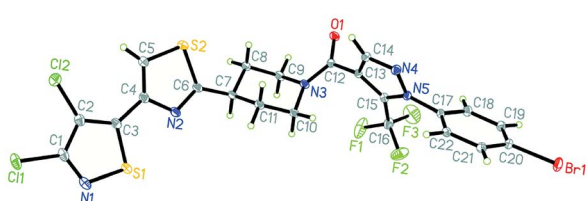


Fig. 2 X-ray single crystal structure of compound 6j.

**2.2.2 Result of cross-resistance.** The cross-resistance between oxathiapiprolin and **6u** against *P. capsici* was detected. As shown in Table 3, the mutants LP3-m and LP3-h exhibited significant resistance to **6u** (the inhibition rates were 12% and 7%, respectively, whereas the inhibition rate of LP3 was 32% at 10 mg L<sup>-1</sup>). These results indicated that the oxysterol binding protein PcORP1, *i.e.*, the target site of oxathiapiprolin was the potent target of **6u**. These tentative results were further validated by docking studies.

**2.2.3 Docking analysis.** Molecular docking is the commonly used method to validate the potential target of pesticides. To elucidate the possible binding modes and affinities between the newly designed inhibitors and the potent target PcORP1, detailed interactions of oxathiapiprolin and

compound **6u** were docked into the active center of PsORP1 (Fig. 3). The conformation of PsORP1 in the complex with oxathiapiprolin was used as the control for molecular docking, and the docked complexes were shown by Pymol and LigPlot, which displayed potential bonding interactions for a hypothetical binding mode of oxathiapiprolin and PcORP1. The F atoms in trifluoromethyl of oxathiapiprolin could form a hydrogen bond with a NH of adjacent Asn765 in very excellent interaction. The thiazole ring of **6u** could interact with Trp762 through  $\pi$ - $\pi$  interaction. In the docked complexes between **6u** and PsORP1, no hydrogen bond appeared. This might be the cause of poor activity of **6u** towards PsORP1. However,  $\pi$ - $\pi$  interaction between the piperidine ring of **6u** and Trp762 appeared to be possible.

### 2.3 Results of compounds 6a–6u against oomycetes *in vivo*

The fungicidal activities of **6a–6u** against oomycetes of *P. cubensis* and *P. infestans* at 100 mg L<sup>-1</sup> *in vivo* are listed in Table 4. Most of the designed isothiazole–thiazole derivatives displayed excellent *in vivo* anti-oomycete activity (100%) against *P. cubensis* and *P. infestans* at 100 mg L<sup>-1</sup>, and the EC<sub>50</sub> values of the compounds with inhibition over 90% at 100 mg L<sup>-1</sup> against *P. cubensis* (Table 5) and *P. infestans* (Table 6) *in vivo* were determined.

Table 2 Toxicities (EC<sub>50</sub> values) of the target compounds *in vitro*

Fungi	Compd	Regression equation	R <sup>2</sup>	EC <sub>50</sub> /mg L <sup>-1</sup>
<i>A. solani</i>	<b>6o</b>	$y = 4.2714 + 0.7903x$	0.9642	8.92
	<b>6s</b>	$y = 4.3901 + 0.6928x$	0.9839	7.84
	Oxathiapiprolin	$y = 2.3920 + 1.0678x$	0.9857	296.60
	Azoxystrobin <sup>a</sup>	$y = 3.7648 + 0.5446x$	0.9251	185.42
<i>G. zeae</i>	<b>6a</b>	$y = 3.5238 + 1.2423x$	0.9917	15.03
	<b>6f</b>	$y = 4.2432 + 0.8867x$	0.9828	7.67
	<b>6o</b>	$y = 3.8997 + 0.9485x$	0.9858	15.60
	Oxathiapiprolin	$y = 4.8606 + 0.8936x$	0.9944	1.47
	Azoxystrobin <sup>b</sup>	$y = 4.3881 + 0.7278x$	0.9777	6.92
<i>S. sclerotiorum</i>	<b>6b</b>	$y = 6.8128 + 2.7830x$	0.9298	0.22
	<b>6c</b>	$y = 5.1267 + 0.4377x$	0.9284	0.53
	<b>6l</b>	$y = 4.5982 + 0.7369x$	0.9611	3.83
	<b>6r</b>	$y = 3.1831 + 1.4723x$	0.9582	17.36
	Oxathiapiprolin	$y = 3.9644 + 1.3662x$	0.9855	5.98
	Azoxystrobin <sup>a</sup>	$y = 4.6795 + 0.5283x$	0.8375	4.04

<sup>a</sup> Azoxystrobin, the data cited from the ref. 21. <sup>b</sup> Azoxystrobin, the data cited from the ref. 22.



Table 3 Oomycete sensitivity in wild-type and oxathiapiprolin-resistant strains of *P. capsici*

Strains	Inhibition ratio (mean $\pm$ SD)		
	6u (10 mg L <sup>-1</sup> )	Oxathiapiprolin (10 mg L <sup>-1</sup> )	Oxathiapiprolin (1 mg L <sup>-1</sup> )
LP3 (wild-type)	32 $\pm$ 3%	100%	100%
LP3-m (oxathiapiprolin-resistant)	12 $\pm$ 2%	78 $\pm$ 2%	26 $\pm$ 3%
LP3-h (oxathiapiprolin-resistant)	7 $\pm$ 2%	71 $\pm$ 5%	17 $\pm$ 5%

All compounds except **6a**, **6f**, **6g**, **6i**, **6j**, **6o** and **6q** exhibited good to excellent inhibitory activity against *P. cubensis* and *P. infestans* with EC<sub>50</sub> values of 0.046–12.49 mg L<sup>-1</sup> and 0.20–13.00 mg L<sup>-1</sup>, respectively. For *P. cubensis*, compounds **6k**, **6l**, **6n** and **6s** displayed the same level of fungicidal activity as that of isotianil (EC<sub>50</sub> = 1.01 mg L<sup>-1</sup>) with EC<sub>50</sub> from 1.05 mg L<sup>-1</sup> to 2.13 mg L<sup>-1</sup>, whereas compounds **6b**, **6m**, **6p** and **6u** with EC<sub>50</sub> of less than 1.00 mg L<sup>-1</sup> exhibited higher activity than the positive control of isotianil against *P. cubensis*. The anti-oomycete activity of compound **6u** (EC<sub>50</sub> = 0.046 mg L<sup>-1</sup>) was 10 times less effective than that of the positive control oxathiapiprolin (EC<sub>50</sub> = 0.0046 mg L<sup>-1</sup>) against *P. cubensis*; however, it was 20 times better than that of the positive control isotianil (EC<sub>50</sub> = 1.01 mg L<sup>-1</sup>). It is worth noting that compounds **6b**, **6m**, **6p** and **6u** also displayed excellent inhibitory activities against *P. infestans* with EC<sub>50</sub> values less than 1.00 mg L<sup>-1</sup>. Moreover, EC<sub>50</sub> of compound **6n** was 0.75 mg L<sup>-1</sup>. Ultimately, compound **6u** exhibited the best fungicidal activity against *P. infestans* with EC<sub>50</sub> of 0.20 mg L<sup>-1</sup> among all the isothiazole-thiazoles designed and synthesized in this study.

## 2.4 Compound 6u can activate SAR in plants

**2.4.1 Defense gene expression was induced in the SA pathway.** Systemic acquired resistance (SAR) is a predominant

form of inducing disease resistance in plant defence systems. SAR promotes plant protection against pathogens through induced salicylic acid (SA) biosynthesis, activated SA signalling pathway and enhanced expression of pathogenesis-related proteins (PRs).<sup>25</sup> In particular, the expressed *pr1* is a marker gene for SAR activation<sup>26</sup> and NPR1 is an SA receptor that leads to *pr1* activation.<sup>27</sup>

“\*” and “\*\*\*” indicate significant difference between treated and mock at  $P < 0.05$  and  $P < 0.01$ , respectively.

The expressions of *npr1* and *pr1* were analyzed in *A. thaliana*. The *pr1* expression was significantly up-regulated by 64-fold and 143-fold when treated with **6u** for 24 hours and 48 hours, respectively (Fig. 4). The *npr1* expression was also significantly up-regulated by 4.7-fold after 48 hours of treatment, but the change was not very significant when treated for 24 hours. Isotianil, discovered by Bayer CropScience AG in 1997 and developed jointly with Sumitomo Chemical Co., Ltd, is used as a crop protectant against rice blast and rice leaf blight. The difference in gene expressions identified after isotianil treatment for 24 hours and 48 hours in *Oryza sativa* by microarray

Table 4 Fungicidal activity of piperidinyl-thiazoles at 100 mg L<sup>-1</sup> *in vivo*

Compd	Inhibition rate (%)	
	<i>P. cubensis</i>	<i>P. infestans</i>
6a	0	0
6b	100	100
6c	100	100
6d	100	100
6e	100	100
6f	30 $\pm$ 2	0
6g	0	0
6h	100	0
6i	40 $\pm$ 1	100
6j	0	0
6k	100	100
6l	100	100
6m	100	100
6n	100	100
6o	100	60 $\pm$ 4
6p	100	100
6q	100	0
6r	100	100
6s	100	100
6t	80 $\pm$ 4	100
6u	100	100
Isotianil	100	100
Oxathiapiprolin	100	100

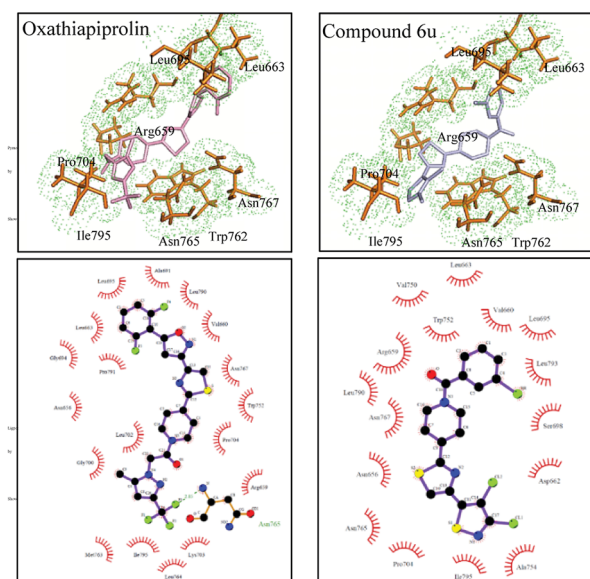


Fig. 3 Molecular docking comparison results of oxathiapiprolin and compound **6u** with PsORP1.



Table 5 The EC<sub>50</sub> values of 6a–6u against *P. cubensis* in vivo

Compd	Regression equation	R <sup>2</sup>	EC <sub>50</sub> (mg L <sup>-1</sup> )	95% CI <sup>a</sup> of EC <sub>50</sub>
6a	— <sup>b</sup>	—	>100	—
6b	y = 5.7565 + 0.7424x	0.9808	0.10	0.01–0.70
6c	y = 2.9999 + 3.1494x	0.9330	4.61	0.97–22.46
6d	y = 2.9744 + 3.1505x	0.9273	4.71	0.91–23.79
6e	y = 0.4131 + 4.9556x	0.9901	12.49	7.59–22.36
6f	—	—	>100	—
6g	—	—	>100	—
6h	y = 0.4131 + 4.9556x	0.9901	12.49	7.59–22.36
6i	—	—	>100	—
6j	—	—	>100	—
6k	y = 4.7655 + 1.5431x	0.9665	1.42	0.35–6.39
6l	y = 4.7479 + 0.8326x	1.0000	2.02	1.99–2.18
6m	y = 5.1520 + 2.2378x	0.9609	0.91	0.16–4.94
6n	y = 4.9881 + 1.0722x	0.9998	1.05	0.93–1.24
6o	y = 0.4131 + 4.9556x	0.9901	12.49	7.59–22.36
6p	y = 7.2490 + 2.3175x	0.9788	0.11	0.05–0.24
6q	y = 0.4710 + 4.9817x	0.9858	13.58	7.23–24.05
6r	y = 3.4658 + 2.9562x	0.9253	3.49	0.62–19.46
6s	y = 4.1499 + 2.9208x	1.0000	2.13	2.06–2.11
6t	—	—	—	—
6u	y = 9.1621 + 3.0757x	0.9621	0.046	0.016–0.13
Oxathiapiprolin	y = 8.7940 + 1.6011x	0.9997	0.0046	0.0042–0.0051
Isotianil	y = 5.1578 + 5.0000x	0.9985	1.01	0.78–1.22

<sup>a</sup> 95% confidence interval. <sup>b</sup> Not determined.

analysis showed that 17 *PR* genes including *pr1a*, *pbz*, *npr1* and *PR* involved in antifungal activities such as chitinase and β-glucanase were upregulated.<sup>28</sup> Based on these results, it is possible that the activation of *pr1* can induce SAR and increase the fungicidal activity of compound 6u in vivo.

## 3 Experimental

### 3.1 General information

All solvents were of analytical grade unless otherwise noted. 1-Substituted phenyl-5-trifluoromethyl-4-pyrazole carboxylic acid and substituted phenyl-5-difluoromethyl-4-pyrazole carboxylic

Table 6 The EC<sub>50</sub> values of 6a–6u against *P. infestans* in vivo

Compd	Regression equation	R <sup>2</sup>	EC <sub>50</sub> (mg L <sup>-1</sup> )	95% CI <sup>a</sup> of EC <sub>50</sub>
6a	— <sup>b</sup>	—	>100	—
6b	y = 5.3634 + 1.9964x	0.9634	0.68	0.21–2.10
6c	y = 3.0649 + 3.1028x	0.9704	4.44	1.73–11.03
6d	y = 0.2101 + 4.8366x	0.9889	13.00	7.16–22.02
6e	y = 0.2101 + 4.8366x	0.9889	13.00	7.16–22.02
6f	—	—	>100	—
6g	—	—	>100	—
6h	—	—	>100	—
6i	y = 3.0513 + 3.1078x	0.9662	4.39	1.67–11.19
6j	—	—	>100	—
6k	y = 4.7053 + 1.0010x	0.9969	2.14	1.61–2.86
6l	y = 3.2938 + 3.0991x	0.9770	3.66	1.76–8.40
6m	y = 5.1537 + 1.0510x	0.9921	0.74	0.48–1.25
6n	y = 5.2728 + 1.0510x	0.9352	0.75	0.17–3.47
6o	—	—	>100	—
6p	y = 6.3442 + 2.9208x	0.9167	0.38	0.06–2.31
6q	—	—	>100	—
6r	y = 3.0513 + 3.1078x	0.9662	4.39	1.67–11.19
6s	y = 5.1960 + 1.7904x	0.9555	0.81	0.26–2.67
6t	y = 4.7813 + 2.0405x	0.9050	1.32	0.22–7.80
6u	y = 6.8798 + 2.6878x	0.9651	0.20	0.06–0.75
Oxathiapiprolin	—	—	<0.10	—
Isotianil	y = 5.4639 + 2.0486x	0.9749	0.61	0.23–1.71

<sup>a</sup> 95% confidence interval. <sup>b</sup> Not determined.



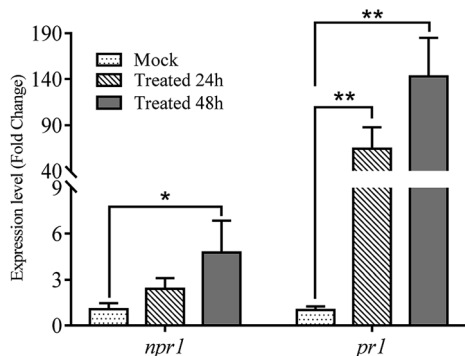


Fig. 4 The expressions of *pr1* and *npr1* after treating with **6u**.

acid were provided by Jia-xing Huang of China Agricultural University (Beijing, China). Melting points (temperature uncorrected) were recorded on an X-4 binocular microscope.  $^1\text{H}$  and  $^{13}\text{C}$  Nuclear Magnetic Resonance (NMR) spectra in  $\text{CDCl}_3$  or dimethyl sulfoxide- $d_6$  were recorded on a Bruker AV400 spectrometer with tetramethylsilane as the internal standard. Mass spectrometry was conducted using Agilent 6520 Q-TOF LC/MS. The single crystal diffraction was carried out on a Rigaku 007HF XtaLAB P200 diffractometer. Fungicidal activities *in vitro* and *in vivo* were tested at Nankai University (Tianjin, China) and Shenyang Research Institute of Chemical Industry (Shenyang, China), respectively.

## 3.2 Synthesis

**3.2.1 Synthesis and characterization of compound 2.** To a solution of compound **1** (15.3 mmol) in 5 mL of 33% HBr and  $\text{CH}_3\text{COOH}$ , pyridinium tribromide (18.8 mmol) was added. The mixture was stirred at room temperature for 3 h. After stopping the reaction, 50 mL of water was added into the reaction solution and the pH was adjusted to 4–5 with sodium bicarbonate. The aqueous layer was extracted using ethyl acetate ( $2 \times 100$  mL). The combined organic layers were washed with saturated brine (100 mL) and then dried over anhydrous  $\text{Na}_2\text{SO}_4$ . After removing the solvent *via* vacuum, the residue was purified by column chromatography on a silica gel using ethyl acetate and petroleum ether (60–90 °C) with *v/v* of 1 : 50 to obtain **2** as a white solid (15.3 mmol, 100%). Mp 52–53 °C;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  4.47 (d,  $J = 2.9$  Hz, 2H,  $\text{CH}_2$ ). HRMS (ESI)  $[\text{M} - \text{H}]^-$  calcd For  $\text{C}_5\text{H}_2\text{BrCl}_2\text{NOS}$  ( $\text{M} - \text{H}$ ): 271.8418, found: 271.8344

**3.2.2 Synthesis and characterization of compound 4.** A solution of *tert*-butyl 4-carbamothioylpiperidine-1-carboxylate **3** (8.25 mmol), which can be prepared from piperidine-4-carboxamide,<sup>29</sup> in tetrahydrofuran (50 mL) was added to the intermediate **2** (9.07 mmol) while stirring and refluxing for 3 h. The solvent was evaporated, and the residue was dissolved using ethyl acetate (50 mL). The organic layer was washed with sodium hydroxide solution (1 mol  $\text{L}^{-1}$ , 50 mL) and saturated brine (50 mL) successively and then dried over anhydrous sodium sulfate. After the solvent was removed under reduced pressure, the residue was purified by column chromatography on a silica gel using ethyl acetate and petroleum ether (60–90

°C) with *v/v* of 1 : 16 to obtain **4** as a white solid (3.48 mmol, 42%). Mp 153–155 °C;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.04 (s, 1H, thiazole-CH), 4.21 (t,  $J = 10.7$  Hz, 2H, piperidine- $\text{CH}_2$ ), 3.19 (m, 1H, piperidine-CH), 2.93 (t,  $J = 12.1$  Hz, 2H, piperidine- $\text{CH}_2$ ), 2.13 (m, 2H, piperidine- $\text{CH}_2$ ), 1.77 (m, 2H, piperidine- $\text{CH}_2$ ), 1.46 (s, 9H,  $\text{CH}_3$ ). HRMS (ESI)  $(\text{M} + \text{H})^+$  calcd for  $\text{C}_{16}\text{H}_{19}\text{Cl}_2\text{N}_3\text{O}_2\text{S}_2$ : 420.0296, found: 420.0345; HRMS (ESI)  $(\text{M} + \text{Na})^+$  calcd for  $\text{C}_{16}\text{H}_{19}\text{Cl}_2\text{N}_3\text{O}_2\text{S}_2$ : 442.0296, found: 442.0183.

**3.2.3 Synthesis and characterization of compound 5.** To an ice-cooled solution of **4** (3.48 mmol) in dry dichloromethane (30 mL), trifluoroacetic acid (87 mmol) was added, and the reaction mixture was stirred at room temperature for 2 h. After completion of the conversion, dichloromethane (50 mL) was added again to the mixture, and the pH was adjusted to 8 with sodium hydroxide solution (1 mol  $\text{L}^{-1}$ ). Then, the organic layer was extracted and washed using saturated brine (50 mL). After drying the organic layer over anhydrous sodium sulfate, the solvent dichloromethane (50 mL) was added again and the pH was adjusted to 8 with sodium hydroxide solution (1 mol  $\text{L}^{-1}$ ). Then, the organic layer was extracted and washed using saturated brine (50 mL). After the organic layer was dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure and the residue was purified by column chromatography on a silica gel using dichloromethane, methanol and triethylamine with *v/v/v* of 6 : 1 : 0.001 to obtain **5** as a white solid (3.19 mmol, 92%). Mp 97–99 °C;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.04 (s, 1H, thiazole-CH), 5.31 (s, 1H, NH), 3.22 (t,  $J = 11.6$  Hz, 2H, piperidine- $\text{CH}_2$ ), 3.17 (s, 1H, piperidine-CH), 2.78 (t,  $J = 11.7$  Hz, 2H, piperidine- $\text{CH}_2$ ), 2.14 (m, 2H, piperidine- $\text{CH}_2$ ), 1.76 (m, 2H, piperidine- $\text{CH}_2$ ). HRMS (ESI)  $[\text{M} + \text{H}]^+$  calcd for  $\text{C}_{11}\text{H}_{11}\text{Cl}_2\text{N}_3\text{S}_2$ : 319.9771, found: 319.9850.

**3.2.4 General procedure for the preparation of title compounds.** The solution of 2-(5-methyl-3-(trifluoromethyl)-1H-pyrazol-1-yl)acetic acid (0.395 mmol), *N*-(3-dimethylamino-propyl)-*N'*-ethylcarbodiimide hydrochloride (0.451 mmol) and 1-hydroxybenzotriazole (0.395 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (10 mL) was cooled to 0 °C, and the mixture was stirred for 1 h under nitrogen atmosphere. Then, the mixture of compound **5** (0.376 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (10 mL) and  $\text{Et}_3\text{N}$  (0.451 mmol) were added. The reaction mixture was stirred overnight at room temperature. After stopping the reaction, the mixture was washed with saturated  $\text{NaHCO}_3$  (20 mL) and brine (20 mL). The organic layer was dried (sodium sulfate anhydrous) and concentrated, and the residue was purified by column chromatography on a silica gel using ethyl acetate and petroleum ether (60–90 °C) with *v/v* of 1 : 9–1 : 3 to obtain target compound **6a**.

Compounds **6b** to **6u** were synthesized according to a similar procedure as that used for **6a** by changing the raw materials, as shown in Scheme 1.

**Data for 6a.** White solid, mp > 200 °C, yield 60%,  $^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  8.08 (s, 1H, thiazole-CH), 6.35 (s, 1H, pyrazole-CH), 5.01 (s, 2H,  $\text{CH}_2$ ), 4.58 (d,  $J = 13.3$  Hz, 1H, piperidine- $\text{CH}_2$ ), 4.08 (d,  $J = 12.5$  Hz, 1H, piperidine- $\text{CH}_2$ ), 3.33 (s, 2H, piperidine- $\text{CH}_2$ ), 2.93 (m, 1H, piperidine-CH), 2.34 (s, 3H,  $\text{CH}_3$ ), 2.22 (m, 2H, piperidine- $\text{CH}_2$ ), 1.91–1.68 (m, 2H, piperidine- $\text{CH}_2$ ). HRMS (ESI)  $[\text{M} + \text{H}]^+$  calcd for



$C_{18}H_{16}Cl_2F_3N_5OS_2$ : 510.0125, found: 510.0203. Anal. calcd for  $C_{18}H_{16}Cl_2F_3N_5OS_2$ : C, 42.36; H, 3.16; N, 13.72. Found: C, 42.15; H, 3.69; N, 13.74.

**Data for 6c.** White solid, mp 125–127 °C, yield 95%,  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  8.10 (s, 1H, thiazole-CH), 7.78 (s, 1H, pyrazole-CH), 7.54 (s, 5H, Ph-H), 6.93 (t,  $J = 53.0$  Hz, 1H,  $CHF_2$ ), 4.76 (s, 1H, piperidine- $CH_2$ ), 4.24 (d,  $J = 64.7$  Hz, 1H, piperidine- $CH_2$ ), 3.38 (s, 2H, piperidine- $CH_2$ ), 3.14 (s, 1H, piperidine-CH), 2.27 (m, 2H, piperidine- $CH_2$ ), 1.91 (m, 2H, piperidine- $CH_2$ ). HRMS (ESI)  $[M + H]^+$  calcd for  $C_{22}H_{17}Cl_2F_2N_5OS_2$ : 540.0220, found: 540.0299. Anal. calcd for  $C_{22}H_{17}Cl_2F_2N_5OS_2$ : C, 48.89; H, 3.17; N, 12.96. Found: C, 48.23; H, 3.30; N, 13.08.

**Data for 6g.** White solid, mp 181–183 °C, yield 92%,  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  8.09 (s, 1H, thiazole-CH), 7.76 (s, 1H, pyrazole-CH), 7.50 (d,  $J = 8.2$  Hz, 5H, Ph-H), 4.79 (d,  $J = 12.8$  Hz, 1H, piperidine- $CH_2$ ), 3.91 (d,  $J = 12.7$  Hz, 1H, piperidine- $CH_2$ ), 3.34 (t,  $J = 14.6$  Hz, 2H, piperidine- $CH_2$ ), 3.10 (m, 1H, piperidine-CH), 2.26 (m, 2H, piperidine- $CH_2$ ), 1.87 (m, 2H, piperidine- $CH_2$ ). HRMS (ESI)  $[M + H]^+$  calcd for  $C_{22}H_{16}Cl_2F_3N_5OS_2$ : 558.0125, found: 558.0190. Anal. calcd for  $C_{22}H_{16}Cl_2F_3N_5OS_2$ : C, 47.32; H, 2.89; N, 12.54. Found: C, 47.14; H, 3.41; N, 12.88.

**Data for 6k.** White solid, mp 167–169 °C, yield 90%,  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  7.86 (d,  $J = 74.7$  Hz, 1H, thiazole-CH, pyrazole-CH), 7.50–7.04 (m, 5H, Ph-H), 4.60 (s, 1H, piperidine- $CH_2$ ), 3.59 (d,  $J = 64.5$  Hz, 1H, piperidine- $CH_2$ ), 3.12 (t,  $J = 64.9$  Hz, 2H, piperidine- $CH_2$ ), 2.99–2.75 (m, 1H, piperidine-CH), 2.12 (m, 2H, piperidine- $CH_2$ ), 1.71 (m, 2H, piperidine- $CH_2$ ). HRMS (ESI)  $[M + H]^+$  calcd for  $C_{22}H_{16}Cl_2F_3N_5OS_2$ : 591.9736, found: 591.9812. Anal. calcd for  $C_{22}H_{16}Cl_2F_3N_5OS_2$ : C, 44.57; H, 2.55; N, 11.81. Found: C, 44.42; H, 2.68; N, 12.28.

**Data for 6p.** White crystals, mp 163–164 °C, yield 100%,  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  7.95 (s, 1H, thiazole-CH), 7.33 (s, 5H, Ph-H), 4.68 (s, 1H, piperidine- $CH_2$ ), 3.81 (s, 1H, piperidine- $CH_2$ ), 3.23 (m, 1H, piperidine-CH), 3.04 (t,  $J = 55.1$  Hz, 2H, piperidine- $CH_2$ ), 2.10 (m, 2H, piperidine- $CH_2$ ), 1.74 (m, 2H, piperidine- $CH_2$ ). HRMS (ESI)  $[M + H]^+$  calcd for  $C_{18}H_{15}Cl_2N_3OS_2$ : 424.0034, found: 424.0109. Anal. calcd for  $C_{18}H_{15}Cl_2N_3OS_2$ : C, 50.95; H, 3.56; N, 9.90. Found: C, 50.33; H, 3.36; N, 9.87.

### 3.3 *In vitro* antifungal activity test

The fungicidal activities of title compounds against *Cercospora arachidicola* (C. A), *Alternaria solani* (A. S), *Botrytis cinerea* (B. C), *Gibberella zeae* (G. Z), *Physalospora piricola* (P. P), *Sclerotinia sclerotiorum* (S. S), *Rhizoctonia cerealis* (R. C) and *Pellicularia sasakii* (P. S) were tested at 50 mg L<sup>-1</sup> *in vitro* by the mycelium growth rate method according to our previous publications.<sup>30,31</sup> Commercial fungicides oxathiapiprolin, isotianil and azoxystrobin were chosen as positive controls. The EC<sub>50</sub> values of the compounds with fungi growth inhibition greater than 70% at 50 mg L<sup>-1</sup> were measured.

### 3.4 Oomycete sensitivity in wild-type and oxathiapiprolin-resistant strains of *P. capsici*

The *P. capsici* strains of the oxathiapiprolin-resistant mutants LP3-m, LP3-h and wild-type LP3 (ref. 32) were used for the

effectiveness tests of compound **6u** and oxathiapiprolin by measuring their colony diameters after dark-incubation at 25 °C for 5 days. Each strain and concentration were measured in three plates and the experiments were performed for three times.

### 3.5 Molecular modelling studies and docking analysis of PsORP1

For molecular modelling, the structure of PsORP1 (ref. 32) (Protein ID: 558498) in *Phytophthora capsici* was generated using the homology model application within the YASARA program<sup>33</sup> with default parameters. The binding center of PsORP1 was identified by aligning to the template structure in Autodock Tools.<sup>34,35</sup> The coordinate of the binding center in PsORP1 was identified by the model structures of 4B2Z and 5H2D. The structures of the ligands were optimized with the ligand minimization protocol. The molecular docking analysis was performed by Autodock Tools and ten random conformations were generated for each ligand. The rest of the parameters were set to default values. The optimal structure of the complex was selected based on visual inspection and the docking score. The structures of the complexes were shown by Pymol<sup>36</sup> and LigPlot.<sup>37</sup>

### 3.6 *In vivo* antifungal activity test

The protective activities in potted plants of the title compounds were determined by the procedures described below:<sup>31</sup> compounds **6a–6u** (10.0 mg) and positive controls oxathiapiprolin and isotianil were dissolved in 0.5 mL DMF solutions. Then, they were diluted using 95.5 mL of distilled water (containing 0.1% Tween 80) to obtain the working solutions of 100 mg L<sup>-1</sup>. The working solutions were sprayed on to the host plant using a sprayer when the plants were grown to 1 to 3 leaf stages. After 24 h, the leaves treated by working solution were inoculated by the fungi of *P. cubensis* and *P. infestans*; 7 days later, the inhibitory activities *in vivo* of all the compounds were assessed. Two concentrations of 10 mg L<sup>-1</sup> and 1 mg L<sup>-1</sup> were further tested for the compounds with activity over 90% against *P. cubensis* at 100 mg L<sup>-1</sup>. Then, the compounds with more than 90% inhibition against *P. cubensis* at 1 mg L<sup>-1</sup> were investigated at 0.1 mg L<sup>-1</sup>, 0.01 mg L<sup>-1</sup> and 0.001 mg L<sup>-1</sup>. The compounds with 100% inhibition rate against *P. infestans* were tested for 3 concentrations of 10 mg L<sup>-1</sup>, 1 mg L<sup>-1</sup> and 0.1 mg L<sup>-1</sup>. An inhibition rate of 100% represents complete control of the fungi growth and an inhibition rate of 0% represents no control of the fungi growth.

### 3.7 RNA extraction and Q-PCR analysis of the expression of defence genes in the salicylic acid pathway

Based on the difference between anti-oomycete activities *in vivo* and *in vitro*, we proposed that compound **6u** might be able to induce systemic acquired resistance of plants. The RNAs of *Arabidopsis thaliana*, which was treated with **6u** (10 mg L<sup>-1</sup>) and DMF, were isolated by using E.Z.N.A.® Plant RNA Kit (Omega, USA) and the mRNA was transcribed into cDNA reversely. Q-PCR was performed using the 2<sup>- $\Delta\Delta C_T$</sup>  method<sup>38</sup> with TransStart Top



Table 7 Primers used for Q-PCR

Gene ID	Gene name	Primer-F	Primer-R
AT3G41768	<i>18s</i>	tgtttgatggtaactactactc	gaatgatgcctcgccagcacaga
AT1G64280	<i>npr1</i>	acgaagagaacatcacceggg	cgggaagaatcgtttcccca
AT2G14610	<i>pr1</i>	attttactggctattctcgatt	agttgcctcttagttgtctgcg

Green Q-PCR Super Mix (K31102, TransStart, China). The expressions of defense genes *pr1* and *npr1* in the salicylic acid pathway were analyzed and the primers used for Q-PCR are shown in Table 7.

## 4 Conclusions

A series of novel isothiazole–thiazole derivatives were designed, synthesized and rationally characterized. The novel compounds were screened for antifungal activity *in vitro* and anti-oomycete activity *in vivo*. Compound **6u** exhibited excellent anti-oomycete activity *in vivo* with EC<sub>50</sub> values of 0.046 mg L<sup>-1</sup> and 0.27 mg L<sup>-1</sup> against *P. cubensis* and *P. infestans*, respectively. Although **6u** was not as effective as oxathiapiprolin, it was much better than isotianil, a compound which can induce plant systemic acquired resistance. Therefore, isothiazole–thiazole derivatives are worthy of further research. Since the docking experiments indicated that oxathiapiprolin can form a hydrogen bond with Asn 765 of PsORP1, analogues with appropriately placed N–H, O–H and F–H units in a target molecule will be designed for further improvement of anti-fungal activity of compound **6u**.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 31571991 and No. 31872007), the Tianjin Natural Science Foundation (No. 18JCZDJC33500), the China Postdoctoral Science Foundation (No. 2017M611156) and the International Science & Technology Cooperation Program of China (No. 2014DFR41030). The authors also thank Dr Jiaying Huang for providing pyrazole compounds (synthesized in the Key Technologies R&D program of China, 2015BAK45B01, CAU) for model reaction. The authors are grateful to the research team of Professor Xi-Li Liu at CAU for their help of determination of the active compounds against oxathiapiprolin-resistant fungi. Kalinina T. A. thanks the Russian Foundation for Basic Research (Grant No. 18-316-20018).

## Notes and references

- M. Larousse and E. Galiana, *PLoS Pathog.*, 2017, **13**, e1006028.
- S. Kamoun, O. Furzer, J. D. G. Jones, H. S. Judelson, G. S. Ali, R. J. D. Dalio, S. G. Roy, L. Schena, A. Zambounis and F. Panabières, *Mol. Plant Pathol.*, 2015, **16**, 413–434.

- P. V. West and G. W. Beakes, *Fungal Biol.*, 2014, **118**, 525–526.
- Y. J. Zhu, J. Q. Huo, Z. J. Fan, Q. F. Wu, X. Li, S. Zhou, L. X. Xiong, T. Kalinina and T. Glukhareva, *Org. Biomol. Chem.*, 2018, **16**, 1163–1166.
- Z. J. Fan, Z. K. Yang, H. K. Zhang, M. Na, H. Wang, C. Fei, X. Zuo, Q. X. Zheng and H. B. Song, *J. Agric. Food Chem.*, 2010, **58**, 2630–2636.
- W. Fry, *Mol. Plant Pathol.*, 2008, **9**, 385–402.
- M. A. Castro, A. M. Gamito, V. Tangarife-Castano, V. Roa-Linares, M. D. C. J. Ma, A. C. Mesa-Arango, L. Betancur-Galvis, A. M. Francesch and A. S. Feliciano, *RSC Adv.*, 2015, **46**, 1244–1261.
- B. L. Wang, H. W. Zhu, Z. M. Li, L. Z. Wang, X. Zhang, L. X. Xiong and H. B. Song, *Pest Manage. Sci.*, 2018, **74**, 726–736.
- S. K. Li, K. U. Huang, B. A. Cao, J. W. Zhang, W. J. Wu and X. M. Zhang, *Angew. Chem., Int. Ed.*, 2012, **51**, 8573–8576.
- L. Zhang, W. Li, T. F. Xiao, Z. H. Song, R. Csuk and S. K. Li, *J. Agric. Food Chem.*, 2018, **66**, 8957–8965.
- H. D. Brown, A. R. Matzuk, I. R. Ilves, L. H. Peterson, S. A. Harris, L. H. Sarett, J. R. Egerton, J. J. Yakstis, W. C. Campbell and A. C. Cuckler, *J. Am. Chem. Soc.*, 1961, **83**, 174–175.
- R. J. Pasteris and M. A. Hanagan, *US Pat.*, WO/2013/0261154, 2014.
- J. Q. Miao, X. Dong, D. Lin, Q. S. Wang, P. F. Liu, F. R. Chen, Y. X. Du and X. L. Liu, *Pest Manage. Sci.*, 2016, **72**, 1572–1577.
- P. Ji, A. S. Csinos, L. L. Hickman and U. Hargett, *Plant Dis.*, 2014, **98**, 1551–1554.
- P. Ji and A. S. Csinos, *Ann. Appl. Biol.*, 2015, **166**, 229–235.
- Y. Cohen, *PLoS One*, 2015, **10**, e0140015.
- L. Chen, X. F. Guo, Z. J. Fan, N. L. Zhang, Y. J. Zhu, Z. M. Zhang, I. Khazhieva, M. Y. Yurievich, N. P. Belskaya and V. A. Bakulev, *RSC Adv.*, 2017, **7**, 3145–3151.
- J. G. Samaritoni, J. M. Babcock, M. L. Schlenz and G. W. Johnson, *J. Agric. Food Chem.*, 1999, **47**, 3381–3388.
- X. Y. Chen, L. Y. Dai, Y. D. Li, W. T. Mao, Z. Fang, J. J. Li, D. Wang, K. Tatiana and Z. J. Fan, *Chin. J. Pestic. Sci.*, 2013, **15**, 140–144.
- D. Worrall, G. H. Holroyd, J. P. Moore, M. Glowacz, P. Croft, J. E. Taylor, N. D. Paul and M. R. Roberts, *New Phytol.*, 2012, **193**, 770–778.
- L. Chen, B. Zhao, Z. J. Fan, X. M. Liu, Q. F. Wu, H. P. Li and H. X. Wang, *J. Agric. Food Chem.*, 2018, **66**, 7319–7327.
- L. Chen, Y. J. Zhu, Z. J. Fan, X. F. Guo, Z. M. Zhang, J. H. Xu, Y. Q. Song, Y. Y. Morzherin, N. P. Belskaya and V. A. Bakulev, *J. Agric. Food Chem.*, 2017, **65**, 745–751.





- 23 B. Zhao, S. J. Fan, Z. J. Fan, H. X. Wang, N. L. Zhang, X. F. Guo, D. Y. Yang, Q. F. Wu, B. Yu and S. Zhou, *J. Agric. Food Chem.*, 2018, **66**, 12439–12452.
- 24 A. Y. Guan, C. L. Liu, X. P. Yang and M. Dekeyser, *Chem. Rev.*, 2014, **114**, 7079–7107.
- 25 F. Sun, P. Y. Zhang, M. R. Guo, W. Q. Yu and K. S. Chen, *Food Chem.*, 2013, **138**, 539–546.
- 26 L. C. Van Loon, M. Rep and C. M. Pieterse, *Annu. Rev. Phytopathol.*, 2006, **44**, 135–162.
- 27 Y. Wu, D. Zhang, J. Y. Chu, P. Boyle, Y. Wang, I. D. Brindle, L. V. De and C. Després, *Cell Rep.*, 2012, **1**, 639–647.
- 28 V. Toquin, C. Sirven, L. Assmann and H. Sawada, in *Modern Crop Protection Compounds*, 2012, ed. W. Krämer, U. Schirmer, P. Jeschke and M. Witschel, Wiley-VCH, Weinheim, Germany, pp. 739–748.
- 29 H. Kim, S. J. Cho, M. Yoo, S. K. Kang, K. R. Kim, H. H. Lee, J. S. Song, S. D. Rhee, W. H. Jung and J. H. Ahn, *Bioorg. Med. Chem. Lett.*, 2017, **27**, 5213–5220.
- 30 Z. J. Fan, Z. G. Shi, H. K. Zhang, X. F. Liu, L. L. Bao, L. Ma, X. Zuo, Q. X. Zheng and N. Mi, *J. Agric. Food Chem.*, 2009, **57**, 4279–4286.
- 31 F. Y. Li, X. F. Guo, Z. J. Fan, Y. Q. Zhang, G. N. Zong, X. L. Qian, L. Y. Ma, L. Chen, Y. J. Zhu and K. Tatiana, *Chin. Chem. Lett.*, 2015, **26**, 1315–1318.
- 32 J. Q. Miao, C. Meng, D. Xue, L. Li, L. Dong, C. Zhang, Z. L. Pang and X. L. Liu, *Front. Microbiol.*, 2016, **7**, 615.
- 33 H. Land and M. S. Humble, *Methods Mol. Biol.*, 2018, **1685**, 43–67.
- 34 E. D. Muzio, D. Toti and F. Polticelli, *J. Comput.-Aided Mol. Des.*, 2017, **31**, 1–6.
- 35 G. M. Morris, D. S. Goodsell, R. Huey and A. J. Olson, *J. Mol. Recognit.*, 1996, **10**, 293–304.
- 36 D. Seeliger and B. L. D. Groot, *J. Comput.-Aided Mol. Des.*, 2010, **24**, 417–422.
- 37 A. C. Wallace, R. A. Laskowski and J. M. Thornton, *Protein Eng.*, 1995, **8**, 127–134.
- 38 K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402–408.

