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

Qi-Fan Wu, † Bin Zhao, † Zhi-Jin Fan, † Jia-Bao Zhao, † Xiao-Feng Guo, † Dong-Yan Yang, † Nai-Lou Zhang, † Bin Yu, † Tatiana Glukhareva †b

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
1 designing the active compound binding protein. To discover new chemical entities with systemic acquired resistance and fungicidal activity, 21 novel isothiazole–thiazole derivatives were designed, synthesized and
categorized by the active compound derivatization method. Compound 6u, with EC50 values of 0.046 mg \( L^{-1} \) and 0.20 mg \( L^{-1} \) against \( Pseudoperonospora cubensis \) (Berk. et Curt.) Rostov and
2 Phytophthora infestans in vivo, might act at the same target as oxysterol binding protein (PcORP1) of
3 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
4 compound 6u for 24 h (43-fold) and 48 h (122-fold). These results can help the development of
5 isothiazole–thiazole-based novel fungicides.

1 Introduction

Crops are often infected by various pathogens, which results in
yield reduction. Oomycetes are filamentous eukaryotic microorganisms1 that attack a large number of plants2 and
animals3 and therefore, they can sometimes threaten agricultural ecosystems. Some pathogens cause humanitarian disasters; for example, the Great Famine was caused by oomycetes
4 attacking potato fields.5 The oomycetes classified as Phytophthora
5 are one of the most serious pathogenic threats all over the world.6 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%.6
6 Heterocyclic compounds are widely used in novel agrochemical research and developments.7–9 Thiazoles have
7 attracted the interest of pharmaceutical and agrochemical anti-fungal research since the development of thiabendazole
8 as a fungicide in 1962 by Merck.10 Oxathiapiprolin was
developed by DuPont in 2007 as a piperidinyl thiazole iso-
xazoline fungicide targeting at oxysterol binding protein
(PcORP1) against \( Pseudoperonospora belbahrii, Phytophthora para-
stica \) var. \( Nicotianae \) (Breda de Hean) Tucker, \( Phytophthora capsici \) Leonian and downy mildew.11,12 However, fungicides
9 acting at a single site of action have a high level of resistance risk.12

As a derivative of 1,3-thiazole, isolatinil has a wide range of
10 biological activities17–19 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
11 only have antifungal activity, but also show good systemic
acquired resistance.17 Novel fungicide development is one of the
important directions and measures for fungicide resistance management.20

On the basis of our former findings,4,5,21–23 to continue our
aim of finding novel highly anti-fungal active compounds with
novel modes of action and without resistance risks, 4 types of
12 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 iso-
latinil and fungicide oxathiapiprolin according to the active
compound derivatization method24 (Fig. 1 and Table 1). The
mode of action of the active compound was validated by cross
resistance, molecular biology and molecular docking studies.

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

‡ Electronic supplementary information (ESI) available: Crystal data of 6j (CCDC 186701), antifungal activities of target compounds in vivo and 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

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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.\textsuperscript{17} 3,4-Dichloro-N-methoxy-N-methylisothiazole-5-carboxamide was obtained by the reaction between 3,4-dichloroisothiazole-5-carboxylic acid and N,N-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 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. 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 oxathiapiprolin

2.2.1 Fungicidal spectrum \textit{in vitro}. The median effective concentration (EC\textsubscript{50}) values of compounds 6a–6u with inhibition rates greater than 70% at 50 mg L\textsuperscript{-1} are listed in Table 2. Both compounds 6o and 6s showed higher activities, with EC\textsubscript{50} values of 8.92 mg L\textsuperscript{-1} and 7.84 mg L\textsuperscript{-1}, respectively, than oxathiapiprolin (EC\textsubscript{50} = 296.60 mg L\textsuperscript{-1}) and azoxystrobin (EC\textsubscript{50} = 185.42 mg L\textsuperscript{-1}) against \textit{Alternaria solani}. The EC\textsubscript{50} values of compounds 6b (EC\textsubscript{50} = 0.22 mg L\textsuperscript{-1}) and 6c (EC\textsubscript{50} = 0.53 mg L\textsuperscript{-1}) were about 1/20 and 1/10, respectively, as compared to that of commercial fungicides oxathiapiprolin (EC\textsubscript{50} = 5.98 mg L\textsuperscript{-1}) and azoxystrobin (EC\textsubscript{50} = 4.04 mg L\textsuperscript{-1}) against \textit{Sclerotinia sclerotiorum}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>R</th>
<th>R'</th>
<th>Compound</th>
<th>n</th>
<th>R</th>
<th>R'</th>
</tr>
</thead>
<tbody>
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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⁻¹). 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 trifuluormethyl 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 π–π 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, π–π 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⁻¹ 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⁻¹, and the EC₅₀ values of the compounds with inhibition over 90% at 100 mg L⁻¹ against *P. cubensis* (Table 5) and *P. infestans* (Table 6) in vivo were determined.
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). In particular, the expressed pr1 is a marker gene for SAR activation and NPR1 is an SA receptor that leads to pr1 activation.

"**" 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.
The activation of \( pr1 \) can induce SAR and increase the fungicidal activity of compound \( 6u \) \textit{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>
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<tr>
<th>Compd</th>
<th>Regression equation</th>
<th>( R^2 )</th>
<th>( EC_{50} ) (mg L(^{-1}))</th>
<th>95% CI(^a) of ( EC_{50} )</th>
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<td>6a</td>
<td>— (^b)</td>
<td>—</td>
<td>&gt;100</td>
<td>—</td>
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<tr>
<td>6b</td>
<td>( y = 5.7565 + 0.7424x )</td>
<td>0.9808</td>
<td>0.10</td>
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<td>6c</td>
<td>( y = 2.9999 + 3.1494x )</td>
<td>0.9330</td>
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<tr>
<td>6d</td>
<td>( y = 2.9744 + 3.1505x )</td>
<td>0.9273</td>
<td>4.71</td>
<td>0.91–23.79</td>
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<td>6e</td>
<td>( y = 0.4131 + 4.9556x )</td>
<td>0.9901</td>
<td>12.49</td>
<td>7.59–22.36</td>
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<td>6f</td>
<td>—</td>
<td>—</td>
<td>&gt;100</td>
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<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>6h</td>
<td>( y = 0.4131 + 4.9556x )</td>
<td>0.9901</td>
<td>12.49</td>
<td>7.59–22.36</td>
</tr>
<tr>
<td>6i</td>
<td>—</td>
<td>—</td>
<td>&gt;100</td>
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<td>—</td>
</tr>
<tr>
<td>6k</td>
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<td>6m</td>
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<tr>
<td>6n</td>
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<td>1.05</td>
<td>0.93–1.24</td>
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<tr>
<td>6o</td>
<td>( y = 0.4131 + 4.9556x )</td>
<td>0.9901</td>
<td>12.49</td>
<td>7.59–22.36</td>
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<td>6p</td>
<td>( y = 7.2490 + 2.3175x )</td>
<td>0.9788</td>
<td>0.11</td>
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<td>6q</td>
<td>( y = 0.4710 + 4.9817x )</td>
<td>0.9858</td>
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<td>6r</td>
<td>( y = 3.4658 + 2.9562x )</td>
<td>0.9253</td>
<td>3.49</td>
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<td>6s</td>
<td>( y = 4.1499 + 2.9208x )</td>
<td>1.0000</td>
<td>2.13</td>
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<tr>
<td>6t</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6u</td>
<td>( y = 9.1621 + 3.0757x )</td>
<td>0.9621</td>
<td>0.046</td>
<td>0.016–0.13</td>
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<tr>
<td>Oxathiapiprolin</td>
<td>( y = 8.7940 + 1.6011x )</td>
<td>0.9997</td>
<td>0.0046</td>
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<tr>
<td>Isotianil</td>
<td>( y = 5.1578 + 5.0000x )</td>
<td>0.9985</td>
<td>1.01</td>
<td>0.78–1.22</td>
</tr>
</tbody>
</table>

\(^a\) 95% confidence interval. \(^b\) Not determined.

Table 5 The \( EC_{50} \) values of \( 6a–6u \) \textit{against} \( P. \) \textit{cubensis \textit{in vivo}}

Table 6 The \( EC_{50} \) values of \( 6a–6u \) \textit{against} \( P. \) \textit{infestans \textit{in vivo}}
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. ¹H and ¹³C Nuclear Magnetic Resonance (NMR) spectra in CDCl₃ or dimethyl sulfoxide-d6 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 CH₃COOH, 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 × 100 mL). The combined organic layers were washed with saturated brine (100 mL) and then dried over anhydrous Na₂SO₄. After removing the solvent via vacuum, the residue was purified by column chromatography on silica gel using petroleum ether (60–90 °C) with v/v of 1 : 5 to obtain 2 as a white solid. Mp 52–53 °C; ¹H NMR (400 MHz, CDCl₃) δ 4.47 (d, J = 2.9 Hz, 2H, CH₂), 3.33 (s, 2H, piperidine-CH₂), 2.93 (t, J = 11.6 Hz, 2H, piperidine-CH₂), 2.14 (m, 2H, piperidine-CH₂). HRMS (ESI) [M + Na]⁺ calced for C₁₆H₁₅Cl₂N₃O₂S: 420.0345; found: 420.0345.

3.2.2 Synthesis and characterization of compound 4. 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 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 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; ¹H NMR (400 MHz, CDCl₃) δ 8.04 (s, 1H, thiazole-CH), 5.31 (s, 1H, NH), 3.22 (t, J = 11.6 Hz, 2H, piperidine-CH₂), 3.17 (s, 1H, piperidine-CH₂), 2.78 (t, J = 11.7 Hz, 2H, piperidine-CH₂), 2.14 (m, 2H, piperidine-CH₂), 1.76 (m, 2H, piperidine-CH₂) HRMS (ESI) [M + H]⁺ calced for C₁₁H₁₄Cl₂N₃S₂: 319.9771; found: 319.9850.

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 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 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; ¹H NMR (400 MHz, CDCl₃) δ 8.04 (s, 1H, thiazole-CH), 5.31 (s, 1H, NH), 3.22 (t, J = 11.6 Hz, 2H, piperidine-CH₂), 3.17 (s, 1H, piperidine-CH₂), 2.78 (t, J = 11.7 Hz, 2H, piperidine-CH₂), 2.14 (m, 2H, piperidine-CH₂), 1.76 (m, 2H, piperidine-CH₂) HRMS (ESI) [M + H]⁺ calced for C₁₁H₁₄Cl₂N₃S₂: 319.9771; found: 319.9850.
NMR (400 MHz, CDCl₃) δ 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, CHF₂), 4.76 (s, 1H, piperidine-CH₂), 4.24 (d, J = 64.7 Hz, 1H, piperidine-CH₃), 3.38 (s, 2H, piperidine-CH₂), 3.14 (s, 1H, piperidine-CH), 2.27 (m, 2H, piperidine-CH₂), 1.91 (m, 2H, piperidine-CH₂). HRMS (ESI) [M + H]+ calcd for C₂₂H₁₇Cl₂F₂N₅OS₂: 540.0220, found: 540.0299. Anal. calcd for C₂₂H₁₇Cl₂F₂N₅OS₂: C, 48.89; H, 3.69; N, 9.90. Found: C, 50.33; H, 3.36; N, 9.87.

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 with default parameters. The binding center of PsORP1 was identified by aligning to the template structure in Autodock Tools. The coordinate of the binding center in PsORP1 was identified by the model structures of 4BZ 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 then visualized by Pymol and LigPlot.

3.6 In vivo antifungal activity test

The protective activities in potted plants of the title compounds were determined by the procedures described below: compounds 6a-6u (10.0 mg) and positive controls oxathiapiprolin and isolianil 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⁻¹. 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⁻¹ and 1 mg L⁻¹ were further tested for the compounds with activity over 90% against P. cubensis at 100 mg L⁻¹. Then, the compounds with more than 90% inhibition against P. cubensis at 1 mg L⁻¹ were investigated at 0.1 mg L⁻¹, 0.01 mg L⁻¹ and 0.001 mg L⁻¹. The compounds with 100% inhibition rate against P. infestans were tested for 3 concentrations of 10 mg L⁻¹, 1 mg L⁻¹ and 0.1 mg L⁻¹. An inhibition rate of 100% represents complete control of the fungal growth and an inhibition rate of 0% represents no control of the fungal 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⁻¹) 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⁻ΔΔCt method with TransStart Top
Green Q-PCR Super Mix (K31102, TransStart, China). The expressions of defense genes \textit{prt} and \textit{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 \textit{in vitro} and anti-oomycete activity \textit{in vivo}. Compound 6u exhibited excellent anti-oomycete activity \textit{in vivo} with EC_{50} values of 0.046 mg L^{-1} and 0.27 mg L^{-1} against \textit{P. cubensis} and \textit{P. infestans}, respectively. Although 6u was not as effective as oxathiapiprolin, it was much better than isothianil, 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.

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### Notes and references