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Novel *S*-thiazol-2-yl-furan-2-carbothioate derivatives as potential T3SS inhibitors against *Xanthomonas oryzae* on rice

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ABSTRACT: Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is considered as the most destructive disease of rice. Using bactericides is among the widely used traditional methods to control this destructive disease. The excessive and repeated use of the same bactericides is also becoming the reason behind bactericide resistance development. The widely used method for finding the new anti-microbial agents often involves the bacterial virulence factors as a target without affecting bacterial growth. Type III secretion system (T3SS) is a protein appendage and considered as essential virulence factors in most gram-negative bacteria. Due to the conserved construct, T3SS has been regarded as an important mark for the blooming of novel anti-microbial drugs. Towards the search of new T3SS inhibitors, an alternative series of 1,3-thiazole derivatives were designed and synthesized. Their structures were characterized and confirmed by ^{1}H NMR, ^{13}C NMR, MS, and elemental analysis. All the title compounds inhibited the promoter activity of *hpa1* gene, significantly. Eight of them showed better inhibition than our previous T3SS inhibitor TS006 (*o*-coumaric acid, OCA). The treatment of *Xoo* with eight compounds significantly attenuated HR without affecting bacterial growth. The mRNA levels of some representative genes (*hrp*/*hrc* genes) were reduced up to different extents. *In vivo* bioassay results showed that eight T3SS inhibitors could reduce bacterial leaf blight and bacterial leaf streak symptoms on rice, significantly.

KEYWORDS: thiazole; synthesis; type III secretion system (T3SS); inhibitors

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the most serious rice bacterial disease that causes bacterial leaf blight worldwide.[1] It devastates rice growth and then affects rice production, and ultimately results in huge crop yield and economic losses.[2] Various traditional antibiotics have been used for many years and now they are reported to be restricted in many countries because of their environmental impact and development of disease resistance.[3,4] Therefore, there is always a need for the development of new compounds targeting bacterial virulence factors without affecting their growth.[5] Such compounds which do not affect the bacterial growth little selective pressure, hence, preventing the development of bacterial disease resistance.[6] *Xoo* and many other gram-negative bacteria invade host plant cells by using type three secretion system.[7,8] T3SS is conserved in different species,[7] and the T3SS is encoded by the *hrp*, *hrc* and *hpa* genes.[9,10] The *hrp* genes play a critical role in hypersensitive response (HR) and plant pathogenicity.[7,11] In *Xanthomonas oryzae*, HrpG-HrpX regulatory cascade activates the most *hrp* genes expression.[12] HrpG belongs to the OmpR family, which plays an important role in responding to the environmental stimulation.[13] The expression of *hrpX* was regulated by *hrpG*. HrpX is a member of the AraC-family regulator.[14,15] It is essential for the expression of five operons (*hrpB*, *hrpC*, *hrpD*, *hrpE* and *hrpF*) in the *hrp* gene cluster, while these operons encode the protein products required by the T3SS.[14,15]

Various natural and synthetic compounds have been identified and reported, which are capable of inhibiting T3SS in human or plant pathogens like *Pseudomonas*,[16]

Yersinia,[17] *Salmonella*,[18] and *Xanthomonas*[5], etc. These compounds with different structures (salicylidene acylhydrazide, *p*-coumaric acid, *o*-coumaric acid, *N*-hydroxybenzimidazole, etc.) have shown the great activity *in vitro or in vivo* experiments. The thiazole is a special five-membered heterocyclic ring containing nitrogen and sulfur atoms. Its aromatic structure enables thiazole to be modified into compounds with various biological activities. It has been reported that thiazole derivatives are not only used widely in medical fields (antiviral and anticancer drugs),[19,20] but also plays an indispensable role in agrochemicals (insecticide, herbicides, fungicides).[21-23] Such broad-spectrum biological activities make thiazole derivatives become a hotspot in the design and synthesis of compounds. In our previous work, we have synthesized and screened some thiazole and thiadiazole derivatives as anti-virulence agents through suppressing the T3SS (Scheme 1). It can be concluded that thiazole is a framework with good biological activity. The compounds obtained by substituting thiazole on its rings have great worth in the pharmaceutical and agrochemical industries. Therefore, it is very important to continue the research on the design and synthesis of thiazole-related derivatives.

In this study, we extended our previous work[24-26] to synthesis a new series of 1,3-thiazole derivatives. We have screened all compounds by using flow cytometry analysis. Positive candidate compounds exhibited an excellent ability to control bacterial leaf blight on rice by suppressing T3SS functionality without affecting bacterial growth.

MATERIALS AND METHODS

Instrumental analysis.

Mass spectrum analysis were done by using a Bruker APEX IV spectrometer (Bruker, Fallanden, Switzerland). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DPX400 and Bruker AV600 (Bruker, Fallanden, Switzerland), while tetramethylsilane was used as an internal standard. Chemical shifts δ are given in ppm and coupling constant J are in Hz. signal patterns are indicated as follows: s, singlet; d, doublet; dd, doublet of doublets; dt, doublet of triplets; ddd, doublet of doublets of doublets; t, triplet; m, multiplet; bs, broad singlet, quin., quintet. Melting points were recorded with a Cole-Parmer melting point apparatus (ColeParmer, Vernon Hills, Illinois, USA). Elemental analyses were operated on a Vario EL elemental analyzer. Analytical thin-layer chromatography was carried out on silica gel 60 F254 plates. Technical grade ethyl acetate and hexane used for column chromatography were distilled prior to use. The promoter activity of *hpa1* was checked by a FACS-Caliber flow cytometer (CytoFLEX USA). The growth rates were recorded using a Bioscreen (Bioscreen, Finland). RNA concentration and purity were monitored using the Nanovue UV-Vs spectrophotometer (GE Healthcare Bio-Science, Sweden). The cDNA levels were quantified by real-time PCR using a SYBR Green Master Mix (Thermo, USA).

Synthesis methods of key intermediate I

According to the previous reports,[27,28] by using the method of Meerwein arylation reaction, a series of 5-substituted phenyl-2-furoic acid **I** were synthesized with

117 substituted aniline and furoic acid as starting reagents.

118 **Synthesis methods of key intermediate II**

119 At room temperature, concentrated hydrochloric acid (150 mmol) was added to a
120 round-bottomed flask containing chloracetaldehyde solution (100 mmol). The
121 reaction lasted for two hours. And then the reaction solution was dissolved in 12.12 g
122 water. Under the condition of the water bath, ammonium dithiocarbamate (100 mmol)
123 was added. The mixture was stirred at room temperature for 60 minutes, then the
124 temperature of the reaction system was increased to 74 ^{o}C, and the reaction continued
125 for 3 hours. Upon completion, the reaction was cooled to room temperature. The
126 mixture was extracted three times with ethyl acetate, and the organic phase was dried
127 with anhydrous magnesium sulfate. Concentration and recrystallization of the filtrate
128 were done to obtain thiazole-2-thiol (compounds **II**).

129 **Synthesis methods of title compound III**

130 Compound **I** (2 mmol) and dichlorosulfoxide (5 mL) were stirred and heated in a
131 round bottom flask. Two hours later, the excess dichlorosulfoxide was removed by
132 decompression and distillation. Acetonitrile, triethylamine, 4-dimethylaminopyridine
133 and compound **II** were added to the round bottom flask and stirred overnight at room
134 temperature. When the reaction was completed, the mixture was extracted three times
135 with water and dichloromethane. Later, the organic phase was washed successively
136 with 10% hydrochloric acid, 10% sodium bicarbonate and water, dried with
137 anhydrous magnesium sulfate. Concentration and recrystallization of the filtrate were
138 done to obtain compounds **III**.

S-(thiazol-2-yl) 5-(2-chlorophenyl) furan-2-carbothioate (**III-1**). Pink solid: yield 74%. m.p. 137.8-138.7 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.98 (d, J = 3.4 Hz, 1H, ThiaH), 7.79 (t, J = 1.5 Hz, 1H, PhH), 7.70 (dt, J = 7.1, 1.7 Hz, 1H, PhH), 7.61 (d, J = 3.4 Hz, 1H, ThiaH), 7.45 – 7.36 (m, 3H, PhH + FuH), 6.88 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (101 MHz, DMSO-d_6) ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.18 (C=O), 157.16 (C-2, Thia), 153.64 (C-5, Fu), 148.48 (C-2, Fu), 143.04 (C-4, Thia), 142.89 (C-2, Ph), 135.14 (C-1, Ph), 130.37 (C-4, Ph), 129.68 (C-3, Ph), 125.06 (C-6, Ph), 123.35 (C-5, Ph), 123.15 (C-3, Fu), 119.68 (C-5, Thia), 108.78 (C-4, Fu). ESI-MS:m/e 322.5 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8ClNO_2S_2$: C,52.26; H, 2.51; N, 4.35. Found: C, 52.44;H, 2.30 ; N, 4.60.

S-(thiazol-2-yl) 5-(3-chlorophenyl) furan-2-carbothioate (**III-2**). Pink solid: yield 78%. m.p. 136.8-137.7 °C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.00 (dd, J = 7.9, 1.6 Hz, 1H, PhH), 7.97 (d, J = 3.3 Hz, 1H, ThiaH), 7.60 (d, J = 3.3 Hz, 1H, ThiaH), 7.50 (dd, J = 8.0, 1.2 Hz, 1H, PhH), 7.44 (d, J = 3.8 Hz, 1H, FuH), 7.41 (td, J = 7.7, 1.3 Hz, 1H, PhH), 7.35 (dd, J = 7.5, 1.7 Hz, 1H, , PhH), 7.33 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (151 MHz, $CDCl_3$) δ 175.33 (C=O), 155.02 (C-2, Thia), 153.80 (C-5, Fu), 147.97 (C-2, Fu), 143.04 (C-4, Thia), 131.56 (C-3, Ph), 131.06 (C-1, Ph), 130.26 (C-5, Ph), 129.00 (C-4, Ph), 127.48 (C-2, Ph), 127.31 (C-6, Ph), 123.27 (C-3, Fu), 119.25 (C-5, Thia), 113.42 (C-4, Fu). ESI-MS(*m/e*): 322.6 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8ClNO_2S_2$: C, 52.26; H, 2.51; N, 4.35. Found: C, 52.09; H, 2.75; N, 4.27.

S-(thiazol-2-yl) 5-(4-chlorophenyl) furan-2-carbothioate (**III-3**). Pink solid: yield 73%. m.p. 130.4-134.0 °C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.97 (d, J = 3.3 Hz,

1H, ThiaH), 7.77 – 7.73 (m, 2H, PhH), 7.60 (d, J = 3.4 Hz, 1H, ThiaH), 7.46 – 7.43 (m, 2H, PhH), 7.42 (d, J = 3.8 Hz, 1H, FuH), 6.84 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (151 MHz, $CDCl_3$) δ 175.04 (C=O), 157.75 (C-2, Thia), 153.77 (C-5, Fu), 148.38 (C-2, Fu), 143.05 (C-4, Thia), 135.79 (C-4, Ph), 129.38 (C-1, Ph), 127.31 (C-2, C-6, Ph), 126.36 (C-3, C-5, Ph), 123.31 (C-3, Fu), 119.83 (C-5, Thia), 108.24 (C-4, Fu). ESI-MS(*m/e*): 322.5 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8ClNO_2S_2$: C, 52.26; H, 2.51; N, 4.35. Found: C, 52.38; H, 2.42; N, 4.21.

S-(thiazol-2-yl) 5-(2-fluorophenyl) furan-2-carbothioate (**III-4**). White solid: yield 80%. m.p. 63.7-65.0 °C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.00 (td, J = 7.7, 1.7 Hz, 1H, PhH), 7.97 (d, J = 3.4 Hz, 1H, ThiaH), 7.60 (d, J = 3.4 Hz, 1H, ThiaH), 7.45 (d, J = 3.8 Hz, 1H, FuH), 7.39 (dddd, J = 8.3, 7.0, 5.2, 1.8 Hz, 1H, PhH), 7.29 (td, J = 7.6, 1.1 Hz, 1H, PhH), 7.18 (ddd, J = 11.3, 8.3, 1.1 Hz, 1H, PhH), 7.04 (t, J = 3.6 Hz, 1H, FuH). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 175.18 (C=O), 159.71 (d, $^1J_{C\text{-}F}$ = 253.2 Hz, C-2, Ph), 153.83 (C-2, Thia), 153.08 (d, $^4J_{C\text{-}F}$ = 3.0 Hz, C-4, Fu), 147.88 (C-2, Fu), 143.04(C-5, Thia), 130.97 (d, $^3J_{C\text{-}F}$ = 8.7 Hz, C-4, Ph), 127.07 (d, $^4J_{C\text{-}F}$ = 2.1 Hz, C-5, Ph), 124.79 (d, $^3J_{C\text{-}F}$ = 3.5 Hz, C-6, Ph), 123.27 (C-3, Fu), 119.83 (C-5, Thia), 117.31 (d, $^2J_{C\text{-}F}$ = 11.5 Hz, C-1, Ph), 116.30 (d, $^2J_{C\text{-}F}$ = 21.3 Hz, C-3, Ph), 112.70 (d, $^3J_{C\text{-}F}$ = 12.3 Hz, C-5, Fu). ESI-MS(*m/e*): 306.1 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8FNO_2S_2$: C, 55.07; H, 2.64; N, 4.59. Found: C, 55.22; H, 2.71; N, 4.41.

S-(thiazol-2-yl) 5-(3-fluorophenyl) furan-2-carbothioate (**III-5**). White solid: yield 72%. m.p. 108.6-109.6 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.98 (d, J = 3.4 Hz, 1H, ThiaH), 7.64 – 7.57 (m, 2H, PhH + ThiaH), 7.54 – 7.49 (m, 1H, PhH), 7.48 –

7.41 (m, 2H, PhH + FuH), 7.12 (tdd, J = 8.4, 2.6, 0.9 Hz, 1H, PhH), 6.88 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 175.22 (C=O), 163.07 (d, $^{1}J_{C\text{-}F}$ = 246.8 Hz, C-3, Ph), 157.38 (d, $^{4}J_{C\text{-}F}$ = 3.1 Hz, C-5, Fu), 153.65 (C-2, Thia), 148.43 (C-2, Fu), 143.06 (C-4, Thia), 130.79 (d, $^{3}J_{C\text{-}F}$ = 8.1 Hz, C-5, Ph), 130.67 (d, $^{3}J_{C\text{-}F}$ = 8.1 Hz, C-1, Ph), 123.37 (C-3, Fu), 120.81 (d, $^{4}J_{C\text{-}F}$ = 3.1 Hz, C-6, Ph), 119.68 (C-5, Thia), 116.67 (d, $^{2}J_{C\text{-}F}$ = 21.3 Hz, C-4, Ph), 112.02 (d, $^{2}J_{C\text{-}F}$ = 23.8 Hz, C-2, Ph), 108.77 (C-4, Fu). ESI-MS(*m/e*): 306.3 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8FNO_2S_2$: C, 55.07; H, 2.64; N, 4.59. Found: C, 55.31; H, 2.45; N, 4.70.

S-(thiazol-2-yl) 5-(4-fluorophenyl) furan-2-carbothioate (**III-6**). Gray solid: yield 89%. m.p. 129.7-131.3 ^{o}C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.96 (d, J = 3.3 Hz, 1H, ThiaH), 7.83 – 7.77 (m, 2H, PhH), 7.59 (d, J = 3.4 Hz, 1H, ThiaH), 7.41 (d, J = 3.8 Hz, 1H, FuH), 7.19 – 7.12 (m, 2H, PhH), 6.79 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 175.06 (C=O), 163.66 (d, $^{1}J_{C\text{-}F}$ = 250.9 Hz, C-4, Ph), 158.11 (C-2, Thia), 153.94 (C-5, Fu), 148.30 (C-2, Fu), 143.13 (C-4, Thia), 127.28 (d, $^{3}J_{C\text{-}F}$ = 8.4 Hz, C-2, C-6, Ph), 125.31 (d, $^{4}J_{C\text{-}F}$ = 3.3 Hz, C-1, Ph), 123.39 (C-3, Fu), 120.06 (C-5, Thia), 116.40 (d, $^{2}J_{C\text{-}F}$ = 22.2 Hz, C-3, C-5, Ph), 107.75 (C-4, Fu). ESI-MS(*m/e*): 306.2 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8FNO_2S_2$: C, 55.07; H, 2.64; N, 4.59. Found: C, 54.89; H, 2.84; N, 4.36.

S-(thiazol-2-yl)5-(2,4-difluorophenyl) furan-2-carbothioate (**III-7**). Yellow solid: yield 81%. m.p. 135.9-138.6 ^{o}C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.98 (q, J = 7.1, 6.5 Hz, 2H, ThiaH + PhH), 7.61 (d, J = 2.8 Hz, 1H, FuH), 7.44 (d, J = 3.3 Hz, 1H, ThiaH), 7.04 (t, J = 8.2 Hz, 1H, PhH), 7.00 – 6.87 (m, 2H, FuH + PhH). ^{13}C NMR

(101 MHz, Chloroform-*d*) δ 175.14 (C=O), 163.36 (dd, $^{1}J_{C\text{-}F}$= 253.7, $^{3}J_{C\text{-}F}$ =12.1 Hz, C-4, Ph), 159.93 (dd, ^{1}J = 255.8, $^{3}J_{C\text{-}F}$ =11.9 Hz, C-2, Ph), 153.67 (C-2, Thia), 152.25 (C-5, Fu), 147.81 (C-2, Fu), 143.04 (C-4, Thia), 128.21 (dd, $^{3}J_{C\text{-}F}$= 9.8, $^{3}J_{C\text{-}F}$= 3.9 Hz, C-6, Ph), 123.39(C-3, Fu), 119.94(C-5, Thia), 113.93 (dd, $^{2}J_{C\text{-}F}$ = 11.9, $^{4}J_{C\text{-}F}$=4.0 Hz, C-1, Ph), 112.42 (dd, $^{2}J_{C\text{-}F}$= 21.8, $^{4}J_{C\text{-}F}$=3.6 Hz, C-5, Ph), 112.10 (d, $^{3}J_{C\text{-}F}$= 12.2 Hz, C-4, Fu), 104.91 (dd, $^{2}J_{C\text{-}F}$= 25.5 Hz, $^{2}J_{C\text{-}F}$= 25.5 Hz, C-3, Ph). ESI-MS(*m/e*): 324.1 $[M+H]^{+}$. Anal. Calcd. (%) for $C_{14}H_8F_2NO_2S_2$: C, 52.01; H, 2.18; N, 4.33. Found: C, 52.25; H, 2.36; N, 4.18.

S-(thiazol-2-yl) 5-(2,6-difluorophenyl) furan-2-carbothioate (**III-8**). Yellow solid: yield 70%. m.p. 86.0-86.8 ^{o}C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.97 (d, *J* = 3.3 Hz, 1H, ThiaH), 7.59 (d, *J* = 3.3 Hz, 1H, ThiaH), 7.44 (d, *J* = 3.8 Hz, 1H, FuH), 7.37 (tt, *J* = 8.4, 6.1 Hz, 1H,, PhH), 7.07 – 7.02 (m, 3H, FuH + PhH). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 175.66 (C=O), 159.91 (dd, $^{1}J_{C\text{-}F}$ = 256.5, $^{3}J_{C\text{-}F}$ =6.1 Hz, C-2, C-6, Ph), 153.87 (C-2, Thia), 148.83 (dd, $^{4}J_{C\text{-}F}$ = 2.3 Hz, $^{4}J_{C\text{-}F}$ = 2.3 Hz， C-4, Fu), 148.70 (C-2, Fu), 143.05 (C-4, Thia), 130.84 (t, $^{3}J_{C\text{-}F}$ = 10.7 Hz, C-4, Ph), 123.26 (C-3, Fu), 118.39 (C-5, Thia), 114.82 (dd, $^{3}J_{C\text{-}F}$ = 6.3 Hz, $^{3}J_{C\text{-}F}$ = 6.3 Hz, C-5, Fu), 112.35 (dd, $^{2}J_{C\text{-}F}$ = 21.8, $^{4}J_{C\text{-}F}$ =4.1 Hz, C-3, C-5, Ph), 107.67 (dd, $^{2}J_{C\text{-}F}$= 15.5 Hz, $^{2}J_{C\text{-}F}$= 15.6 Hz, C-1, Ph). ESI-MS(*m/e*): 324.2 $[M+H]^{+}$. Anal. Calcd. (%) for $C_{14}H_8F_2NO_2S_2$: C, 52.01; H, 2.18; N, 4.33. Found: C, 51.96; H,1.96; N, 4.52.

S-(thiazol-2-yl) 5-(2-nitrophenyl) furan-2-carbothioate (**III-9**). Yellow solid: yield 77%. m.p. 110.8-111.9 ^{o}C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.97 (d, *J* = 3.3 Hz, 1H, ThiaH), 7.83 (ddd, *J* = 7.8, 4.1, 1.3 Hz, 2H, PhH), 7.70 (td, *J* = 7.7, 1.3 Hz, 1H,

PhH), 7.63 – 7.56 (m, 2H, ThiaH + PhH), 7.40 (d, J = 3.8 Hz, 1H, FuH), 6.83 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.54 (C=O), 153.26 (C-2, Thia), 152.93 (C-2, Ph), 149.32 (C-5, Fu), 147.95 (C-2, Fu), 143.13 (C-4, Thia), 132.50 (C-5, Ph), 130.46 (C-4, Ph), 129.90 (C-6, Ph), 124.40 (C-1, Ph), 123.54 (C-3, Ph), 122.58 (C-3, Fu), 118.89 (C-5, Thia), 112.27 (C-4, Fu). ESI-MS(*m/e*): 333.2 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8N_2O_4S_2$: C, 50.60; H, 2.43; N, 8.43. Found: C, 50.82; H, 2.66; N, 8.28.

S-(thiazol-2-yl) 5-(3-nitrophenyl) furan-2-carbothioate (**III-10**). Yellow solid: yield 70%. m.p. 236.4-237.9 ^{o}C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.62 (t, J = 1.8 Hz, 1H, PhH), 8.26 (ddd, J = 8.2, 2.2, 0.9 Hz, 1H, PhH), 8.15 (dt, J = 7.8, 1.3 Hz, 1H, PhH), 7.99 (d, J = 3.3 Hz, 1H, ThiaH), 7.68 (t, J = 8.0 Hz, 1H, PhH), 7.62 (d, J = 3.4 Hz, 1H, ThiaH), 7.46 (d, J = 3.8 Hz, 1H, FuH), 7.03 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (151 MHz, $CDCl_3$) δ 175.31 (C=O), 155.84 (C-2, Thia), 153.21 (C-5, Fu), 149.10 (C-3, Ph), 148.83 (C-2, Fu), 143.19 (C-4, Thia), 130.42 (C-6, Ph), 130.40 (C-5, Ph), 130.28 (C-1, Ph), 123.97 (C-4, Ph), 123.52 (C-3, Fu), 119.91 (C-2, Ph), 119.49 (C-5, Thia), 109.71 (C-4, Fu). ESI-MS(*m/e*): 333.3 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8N_2O_4S_2$: C, 50.60; H, 2.43; N, 8.43. Found: C, 50.51; H, 2.37; N, 8.62.

S-(thiazol-2-yl) 5-(4-nitrophenyl) furan-2-carbothioate (**III-11**). Yellow solid: yield 75%. m.p. 229.1-232.4 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (d, J = 8.7 Hz, 2H, PhH), 8.17 (d, J = 8.8 Hz, 2H, PhH), 8.10 (dd, J = 3.3 Hz, 2H, Thia), 7.86 (d, J = 3.9 Hz, 1H, FuH), 7.68 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (151 MHz, DMF) δ 175.07 (C=O), 156.40 (C-2, Thia), 152.47 (C-5, Fu), 149.14 (C-4, Ph), 148.09 (C-2, Fu),

143.57 (C-4, Thia), 134.45 (C-1, Ph), 126.18 (C-2, C-6, Ph), 125.33 (C-3, C-5, Ph), 124.65 (C-3, Fu), 120.91 (C-5, Thia), 112.54 (C-4, Fu). ESI-MS(*m/e*): 331.1 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8N_2O_4S_2$: C, 50.60; H, 2.43; N, 8.43. Found: C, 50.81; H, 2.22; N, 8.31.

S-(thiazol-2-yl) 5-(4-bromophenyl) furan-2-carbothioate (**III-12**). Gray solid: yield 83%. m.p. 150.1-152.0 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.97 (d, J = 3.4 Hz, 1H, Thia), 7.67 (dt, J = 8.5, 1.7 Hz, 2H, PhH), 7.62 – 7.55 (m, 3H, PhH + Thia), 7.40 (dd, J = 3.8, 1.2 Hz, 1H, FuH), 6.85 (dd, J = 3.8, 1.1 Hz, 1H, FuH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.04 (C=O), 157.69 (C-2, Thia), 153.70 (C-5, Fu), 148.27 (C-2, Fu), 142.99 (C-4, Thia), 132.26 (C-3, C-5, Ph), 127.61 (C-1, Ph), 126.48 (C-2, C-6, Ph), 123.99 (C-4, Ph), 123.32 (C-3, Fu), 119.85 (C-5, Thia), 108.34 (C-4, Fu). ESI-MS(*m/e*): 367.2 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_8BrNO_2S_2$: C, 45.91; H, 2.20; N, 3.82. Found: C, 46.12; H, 2.44; N, 3.65.

S-(thiazol-2-yl) 5-(p-tolyl) furan-2-carbothioate (**III-13**). Yellow solid: yield 79%. m.p. 122.9-124.0 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.95 (d, J = 3.4 Hz, 1H, Thia), 7.68 (d, J = 8.2 Hz, 2H, PhH), 7.57 (d, J = 3.4 Hz, 1H, Thia), 7.39 (d, J = 3.8 Hz, 1H, FuH), 7.24 (d, J = 8.0 Hz, 2H, PhH), 6.78 (d, J = 3.8 Hz, 1H, FuH), 2.38 (s, 3H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.77 (C=O), 159.26 (C-2, Thia), 154.12 (C-5, Fu), 147.71 (C-2, Fu), 142.85 (C-4, Thia), 140.08 (C-4, Ph), 129.70 (C-3, C-5, Ph), 126.00 (C-1, Ph), 125.05 (C-2, C-6, Ph), 123.13 (C-3, Fu), 120.09 (C-5, Thia), 107.33 (C-4, Fu), 21.48 (CH_3). ESI-MS(*m/e*): 302.1 $[M+H]^+$. Anal. Calcd. (%) for $C_{15}H_{11}NO_2S_2$: C, 59.78; H, 3.68; N, 4.65. Found: C, 59.92; H, 3.88; N, 4.45.

S-(thiazol-2-yl) 5-(4-methoxyphenyl) furan-2-carbothioate (**III-14**). Yellow solid: yield 84%. m.p. 129.7-130.4 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.96 (d, J = 3.4 Hz, 1H, Thia), 7.78 – 7.74 (m, 2H, PhH), 7.59 (d, J = 3.4 Hz, 1H, Thia), 7.42 (d, J = 3.8 Hz, 1H, FuH), 7.00 – 6.97 (m, 2H, PhH), 6.73 (d, J = 3.8 Hz, 1H, FuH), 3.87 (s, 3H, OCH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.64 (C=O), 160.88 (C-4, Ph), 159.28 (C-2, Thia), 154.27 (C-5, Fu), 147.53 (C-2, Fu), 142.85 (C-4, Thia), 126.79 (C-1, Ph), 123.10 (C-2, C-6, Ph), 121.59 (C-3, Fu), 120.39 (C-5, Thia), 114.47 (C-3, C-5, Ph), 106.57 (C-4, Fu), 55.43 (OCH_3). ESI-MS(*m/e*): 318.2 $[M+H]^+$. Anal. Calcd. (%) for $C_{15}H_{11}NO_3S_2$: C, 56.77; H, 3.49; N, 4.41. Found: C, 56.59; H, 3.31; N, 4.62.

S-(thiazol-2-yl) 5-phenylfuran-2-carbothioate (**III-15**). Yellow solid: yield 77%. m.p. 68.3-70.3 °C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.97 (d, J = 3.3 Hz, 1H, Thia), 7.85 – 7.80 (m, 2H, PhH), 7.59 (d, J = 3.3 Hz, 1H, Thia), 7.49 – 7.45 (m, 2H, PhH), 7.44 – 7.39 (m, 2H, PhH + FuH), 6.86 (d, J = 3.8 Hz, 1H, FuH). ^{13}C NMR (151 MHz, $CDCl_3$) δ 175.05 (C=O), 158.99 (C-2, Thia), 154.02 (C-5, Fu), 148.20 (C-2, Fu), 142.99 (C-4, Thia), 129.79 (C-4, Ph), 129.06 (C-1, Ph), 128.83 (C-3, C-5, Ph), 125.16 (C-2, C-6, Ph), 123.21 (C-3, Fu), 119.88 (C-5, Thia), 107.93 (C-4, Fu). ESI-MS(*m/e*): 288.3 $[M+H]^+$. Anal. Calcd. (%) for $C_{14}H_9NO_2S_2$: C, 58.52; H, 3.16; N, 4.87. Found: C, 58.36 ;H, 2.98 ; N, 4.99.

Bioassays

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. PXO99^A strain (*Xoo* wild-type strain) and other derived strains were grown in rich

293 medium (M210) or on PSA plates at 28 °C.[5,29] XOM2 medium was a culture medium
294 that used to induce the expressions of *hrp* genes. And XOM2 medium was prepared
295 as previous reported.[29] *Escherichia coli* was grown in Luria Bertani (LB) medium at
296 37 °C. Ampicillin (Ap) and cephalexin (Cp) were used at the final concentrations of
297 100 μg/mL and 25 μg/mL. All title compounds were dissolved in dimethyl sulfoxide
298 (DMSO) at a final concentration of 10 μg/mL.

299 **Flow cytometry analysis**

300 pPhpa1 strain (Figure S31) was grown in M210 to OD_{600} ≈ 2.0 at 28 °C and
301 transferred to XOM2 along with the tested compounds. The promoter activity of *hpa1*
302 was tested by a FACS-Caliber flow cytometer (CytoFLEX USA).[16] DMSO was used
303 as a negative control. Three independent experiments were performed with three
304 replications each. The similar method was used to analyze the promoter activities of
305 *hrpG*, *hrpX* and *hrcT*.

306 **Measurement of the growth rate**

307 *Xoo* PXO99^{A} strain was grown in M210 to OD_{600} ≈ 1.0 at 28 °C. The bacterial
308 suspension was transferred to M210 or XOM2 (plus 0.5% sucrose) medium
309 containing 10 μg/mL of tested compounds or DMSO, which started at an OD_{600} of 0.1.
310 The growth rates were recorded every 3 h during the 48 h period using a Bioscreen
311 (Bioscreen, Finland). Three replicates were used each time, and three independent
312 experiments were performed.

313 **HR assay**

314 *Xoo* PXO99^{A} strain was grown in M210 to OD_{600} ≈ 2.0 at 28 °C, and then

315 suspended in sterile distilled water to $OD_{600} \approx 0.5$. *Nicotiana benthamiana* plants were
316 used for HR assays. Each flag leaf was inoculated with bacterial suspensions with
317 15–20 independent individuals, respectively.The HR symptoms were observed at 24
318 hours after inoculation.

319 **RNA extraction and qPCR analysis**

320 *Xoo* $PXO99^A$ strain was grown in M210 to $OD_{600} \approx 2.0$ at 28 °C and subcultured to
321 XOM2 at $OD_{600} \approx 0.6$. The total RNA was extracted from the collected cells using
322 RNAprep Pure Bacteria Kit (Promega, USA). cDNA was synthesized using an
323 HiScriptII Q RT SuperMix Kit (Tiangen, Beijing, China). The cDNA levels were
324 quantified by real-time PCR using a SYBR Green Master Mix (Thermo Scientific,
325 MA, USA). The relative levels of genes expressions were analyzed by the $2^{-\Delta\Delta Ct}$
326 method.[30] Expression values are the means of three biological repeats in each
327 experiment. The Student's t-test was used for statistical analysis.

328 **Pathogenicity assays**

329 *Xoo* $PXO99^A$ strain was grown in M210 to $OD_{600} \approx 2.0$ at 28 °C and then
330 suspended in sterile distilled water to $OD_{600} \approx 0.8$. The rice cultivar IR24 (*Oryza*
331 *sativa* ssp. *indica*) was used for pathogenicity assays. Rice seedlings (2-week-old,
332 using a needleless syringe) and adult plants (2-month-old, by the leaf clipping
333 method)were inoculated with *Xoo*. Plants were scored and the symptoms were
334 observed at three days post-inoculation (dpi) in seedlings, and at 14 dpi for lesion
335 lengths (the length from the tip to the leading edge of the grayish symptom) in adult
336 rice plants. And *X. oryzae* pv. *oryzicola* (*Xoc*) used similar experimental methods with

Xoo.

***In vivo* protection activity test**

The protection activity of title compounds against rice bacterial leaf blight in potted plants was conducted under greenhouse conditions. The experiment was conducted following a previous study.[31]

Other virulence factors assay

For analyzing extracellular cellulase activity, PSA plates containing 0.5% carboxymethyl cellulose were used. *Xoo* was grown in M210 to $OD_{600} \approx 0.5$ at 28 °C, and were inoculated into the PSA plates. After incubating at 28 °C for 24 h, the plates were dyed with 0.1% Congo red for 20 min and washed twice with 1.0 M NaCl; cellulase-positive colonies are supposed to show pale-yellow clear zones against a red background.[32]

For analyzing the production of extracellular xylanase activity, PSA plates containing 0.2% RBB-xylan were used to analyze xylanase activity. *Xoo* was grown in M210 to $OD_{600} \approx 0.5$ at 28 °C, and they were inoculated on the PSA plates. After incubating at 28 °C for 48 h, the plates appeared as the white clear zones among a blue background.[32]

For analyzing exopolysaccharides (EPS) production, *Xoo* was grown in M210 to $OD_{600} \approx 2.0$ at 28 °C. 100 mL bacterial culture was centrifuged at 12,000 rpm for 10 min, then the supernatants of bacterial culture were collected. The supernatants were added two volumes of absolute ethanol and kept at –20 °C for at least 15 h. The mixtures were centrifuged, and the precipitates of EPS were dried at 50 °C overnight

before determination of dry weight.[33,34]

Biofilm formation was performed according to reported studies.[35] Bacterial suspension (2 mL) was placed in each glass tube with 10 μg/mL III-15 compounds and incubated at 28 °C for 72 h. The culture medium was poured out, and attached bacterial cells were gently washed three times with distilled water. The cells were then stained with 0.1% crystal violet (2 mL) for 15 min. Unbound crystal violet was poured out, and the glass tubes were washed three times with distilled water. The crystal violet-stained cells were solubilized in DMSO (2 mL).

RESULTS AND DISCUSSION

Synthesis

Figure 1 demonstrated the synthetic route to compound III. Following the reported procedure via Meerwein arylation, key intermediate **I** was synthesized using substituted aniline and furoic acid as starting substrate.[27,28] This 5-substituted phenyl-2-furancarboxylic acid **I** and thionyl chloride were refluxed in anhydrous toluene for 3h to produce 5-phenyl-2-furancarbonyl chloride, which was reacted with thiazole-2-thiol, triethylamine and 4-dimethylaminopyridine in acetonitrile to afford compounds **III** in moderate to good yields. Title compounds were characterized and confirmed by ^{1}H NMR, ^{13}C NMR, and elemental analyses.

Screening compounds that suppress *hpa1* transcription

To detect potential T3SS inhibitors, we used a strain containing a gene named *hpa1* promoter into the pPROBE-AT vector, which exhibited a green fluorescence protein (GFP) reporter gene without a promoter. The *hpa1* gene in *Xoo* encodes a harpin

protein that could cause HR on non-host plants like tobacco, and its expression is regulated by HrpX protein. The strain was grown in an induced medium called XOM2 that could induce *hpa1* gene expression. *Hpa1* promoter activity was measured 15 hours after incubation with each of the compounds at 10 μg/mL by fluorescence-activated cell sorting (FACS) system. The mean fluorescence intensity (MFI) was listed in Table 1, which showed the promoter activity of *hpa1*. %DMSO was calculated by the ratio of MFI after incubation with each compound to that of the DMSO (dimethyl sulfoxide) control. All compounds showed significant inhibition of *hap1* promoter activity, and the inhibition rate of **III-1**, **III-2**, **III-3**, **III-4**, **III-6**, **III-8**, **III-9** and **III-15** were higher than our previous published T3SS inhibitor called TS006 (Table 1). Among all the compounds, eight were selected as positive candidates for further experiments.

Effects of positive candidates on bacterial growth

T3SS inhibitors should target T3SS without affecting bacterial growth. Thus, we tested bacterial growth treated with compounds **III-1**, **III-2**, **III-3**, **III-4**, **III-6**, **III-8**, **III-9** and **III-15** compounds in 48 hours at different stages. It was measured in M210 (rich medium) and XOM2 (hrp-inducing medium and supplement 0.5% sucrose to sustain bacterial growth). In comparison with the wild type and the DMSO solvent control, *Xoo* treated with eight different compounds showed no significant difference. As these compounds met the requirements of potential T3SS inhibitors (Figure 2), we continued further study using these eight compounds.

Hypersensitive response inhibition by positive candidates

In *Xoo*, Hpa1 could trigger HR on tobacco and secrete by T3SS. Therefore, we checked the HR-inducing ability of *Xoo* on tobacco to know whether eight compounds influence the T3SS in *Xoo*. All of the eight compounds inhibited HR, significantly (Figure 3).

Positive candidates inhibit T3SS expression at *hrpG* and *hrpX* level

HrpG and HrpX are two key regulatory proteins in *Xanthomonas*, and their *hrp* genes expressions are mainly regulated by HrpX-HrpG pathway. Thus, it is important to verify whether positive candidates affect the expression of *hrpG*/*hrpX*. We also used a strain that contained *hrpG* promoter into the pPROBE-AT vector, and the *hrpG* promoter activity was measured by FACS system. As shown in Figure 4A, the mRNA level of *hrpG* was reduced by eight compounds in comparison with the solvent control DMSO. We also used the same way to construct an expression vector to test *hrpX* promoter activity, the results showed that the mRNA level of *hrpX* was also reduced at different extent (Figure 4B). **III-2**, **III-4**, **III-8**, **III-9**, **III-15** reduced the mRNA level of *hrpX* more significantly than the other three compounds (Figure 4B).

Meanwhile, HrpX activates the transcription of *hrp* genes (*hrpB* to *hrpF*) and T3SS effector genes. The promoter region regulated by HrpX usually has a conserved sequence containing PIP-box (TTCGC-N15-TTCGC), like that of *hpa1*. Therefore, we selected *hrpT* as a representative gene containing a complete PIP-box in its promoter region to identify the eight positive candidates could affect the transcription of T3SS genes in *Xoo* (Figure S32). The activity of PhrcT was observed by FACS assays, and the results showed that all of the eight compounds truly affected the *hrcT*

425 in mRNA level (Figure 4C). These results along with statistical analysis of the *hpa1*
426 promoter may infer that these potential T3SS inhibitors target the promoters with a
427 PIP-box. And it also revealed that HrpX has played important role in regulating the
428 inhibitory function.

429 **Positive candidates inhibit representative *hrp/hrc* genes expression**

430 The above results showed that eight compounds inhibited the T3SS genes of *Xoo*
431 without affecting bacterial growth. Indicating that these compounds may work on the
432 regulatory pathway to affect the expression of T3SS genes. We selected some special
433 *hrp*/*hrc* gene to check the effect of eight compounds for influencing the expression of
434 T3SS genes by doing qRT-PCR experiment. *HrpE* gene is used to encode the *hrp*
435 pilus protein, and the other *hrp* gene named *hrpF* encodes a putative translocon
436 protein. *HrpC* encodes the outer-membrane secretin, while *hrcU* is export apparatus
437 gene like *hrcT*.[36] The *hrp*/*hrc* genes in mRNA levels were reduced significantly as
438 compared to DMSO control (Figure 5).

439 Our results showed that eight potential T3SS inhibitors reduced the expression of
440 *hrp*/*hrc* gene probably by HrpX-HrpG pathway. In this study, six genes were used
441 (*hpa1*, *hrpE*, *hrpF*, *hrcC*, *hrcT* and *hrcU*) to identify the expression of T3SS in *Xoo*.
442 Three of the six genes (*hpa1*, *hrcT,* and *hrcU)* contained the PIP-box in their promoter
443 region. Interestingly, except the *hrcC* gene, five genes were reduced significantly by
444 our compounds in mRNA levels. So, more works still need to be done in the future to
445 find out the exact mechanism involved in making these eight T3SS inhibitors as
446 potential candidates.

Positive candidates reduce bacterial leaf blight symptoms on rice

This study aimed to find out the most potential T3SS inhibitors, which could control the virulence phenotypes of *Xoo* in planta. In *Xanthomonads*, water soaking is induced by the TAL (transcription activator-like) effectors.[37] There are several TAL effectors in *Xoo* strain PXO99^{A}, which confer gene-specific resistant to host plants.[38] We used the seedlings of susceptible rice (cultivar IR24) to estimate the symptoms of water-soaked lesions induced by *Xoo*. After treatment of *Xoo* with the compounds, the water soaked symptoms on seedlings IR24 plants (Figure 6A) and the yellowish disease symptoms on adult (Figure 6B) were reduced significantly as compared to control. The effect of **III-2**, **III-4**, **III-6**, **III-9**, and **III-15** compounds on rice was better than our previous T3SS inhibitors TS006 (Figure 6B).

Xanthomonas oryzae pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xoc*) belong to *Xanthomonas oryzae*. They are closely related that have similar physiological properties and T3SS regulatory pathways.[5] So, we wanted to check the compounds whether had the same effect to control the *Xoc*. The results showed that some compounds could reduce the disease symptoms caused by *Xoc* in different extent (Figure 6C). Those helped to enhance the broad spectrum of the compounds.

As mentioned earlier, T3SS invades plant cells by secreting TAL effectors. So we selected compound III-15 which showed the most significant control effect on rice to test the transcription levels of two TAL effectors named *avrXa10* and *pthXo6*. *AvrXa10* could activate the *Xa10* (the host resistance gene) to confer the immune or HR in IRRB10.[39,40] *PthXo6* could induce the virulence of PXO99^{A} and ectopic the

469 bZIP transcription factor (OsTFX1) expression that conduces to host susceptibility.[41]
470 The compound III-15 suppressed the expression of the *avrXa10* and *pthXo6* (Figure
471 6D), so we guessed the compound not only inhibited the T3SS but reduced the
472 effectors' transcription to control the virulence of the bacteria.

473 In vivo, protection activity was evaluated and the results showed that title
474 compound III-15 exhibited the best protective activity of 63.77% (Table 2). In
475 greenhouse test, compounds III-2, III-4, III-6, and III-9 gave better protective activity
476 (54.51%, 54.63%, 54.63%, and 54.74%) than that of compounds III-1, III-3, III-8 and
477 TS006 (45.71%, 45.49%, 45.60%, and 45.60%) against rice bacterial leaf blight as
478 compared with commercial drugs bismerthiazol (49.66%) and thiodiazole copper
479 (39.39%). Meanwhile, the tested compounds were also found safe to the plants.

480 **Other virulence factors assay**

481 In search of finding the best T3SS inhibitors without affecting other virulence
482 factors, we investigated whether our T3SS inhibitors affect some other representative
483 virulence factors, such as exopolysaccharides (EPS), extracellular cellulase,
484 extracellular xylanase, and biofilm.[23,35] We also selected compound III-15 to do the
485 phenotypic experiments. As shown in Figure 7A and 7B, no significant difference was
486 observed as compared to the negative control indicating that **III-15** may not affect the
487 expression of extracellular cellulase and extracellular xylanase. Similarly, **III-15**
488 caused no significant effect on the production of EPS and surface morphology (Figure
489 7C). Crystal violet assay was used to monitor the biofilm formation by *Xoo*. The
490 crystal violet stained adherent biofilms retained on glass test tubes (Figure 7D). As

expected, **III-15** compound was not found to restrict the production of biofilms by *Xoo*.

CONCLUSION

A series of structurally novel *S*-thiazol-2-yl-furan-2-carbothioate derivatives were designed and synthesized. Bioassay results demonstrated that eight of title compounds showed better bioactivities than our previous published T3SS inhibitor named TS006. Compounds **III-2**, **III-4**, **III-6**, **III-9**, and **III-15** could control bacterial leaf blight on rice, significantly. These findings provide a new series of compounds, which may help to increase rice production by effectively controlling *Xoo*.

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637 **Figure Captions**

638 Scheme 1. The designed strategy for the title compounds

639 Figure 1. Synthesis routes of target compounds III.

640 Figure 2. Effects of eight compounds on bacterial growth rates.

641 Figure 3. Effects of eight compounds on the HR induced by *Xoo* on tobacco leaves.

642 Figure 4. The effects of the eight compounds on the promoter activity of *hrpG* (A),

643 *hrpX* (B) and *hrcT* (C) in *Xoo* grown in XOM2 medium supplement with 10 μg/mL of

644 tested compounds.

645 Figure 5. Relative mRNA levels of representative genes in the *hrp* cluster in *Xoo*

646 PXO99^{A} incubated with eight compound were measured by qRT-PCR.

647 Figure 6. Significant reduction in bacterial blight disease by eight compounds.

648 Figure 7. Other virulence factors analysis.

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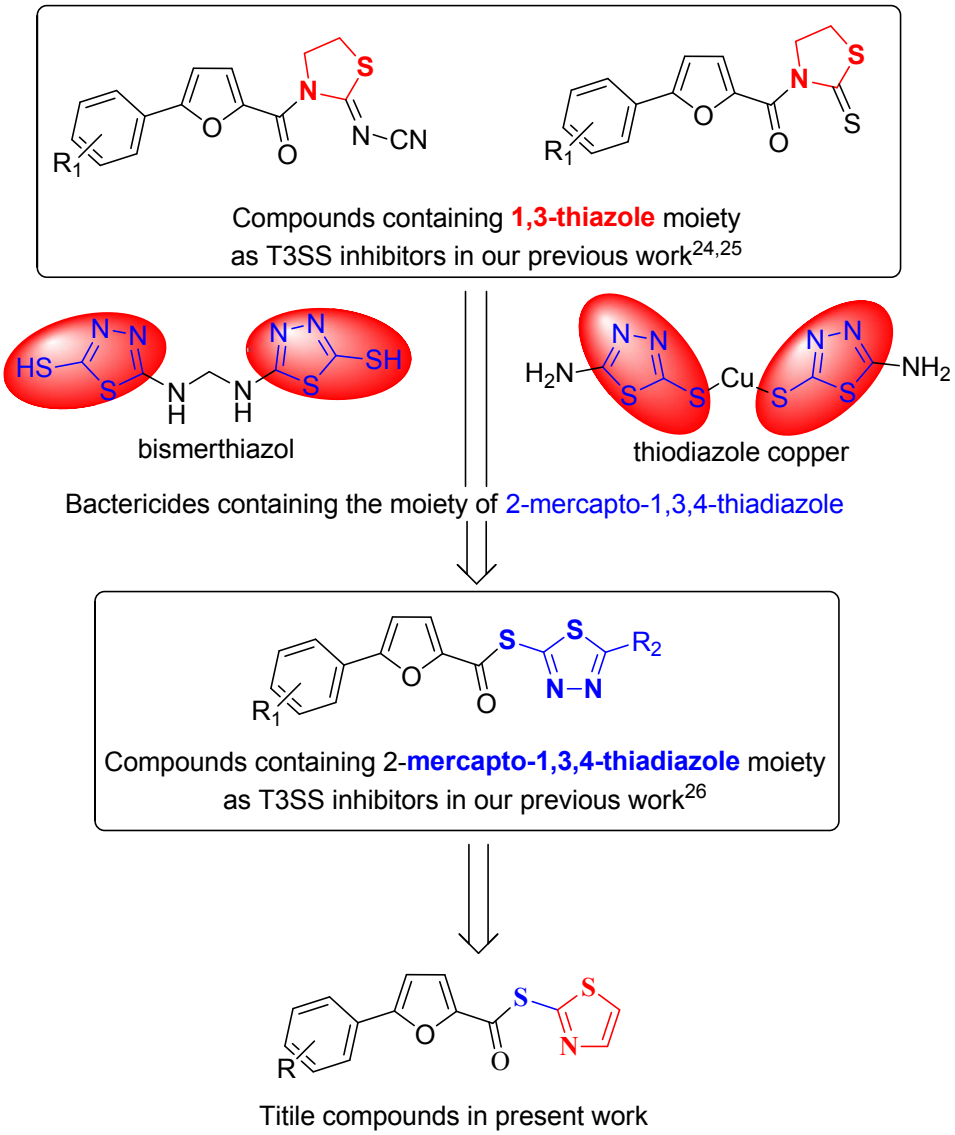

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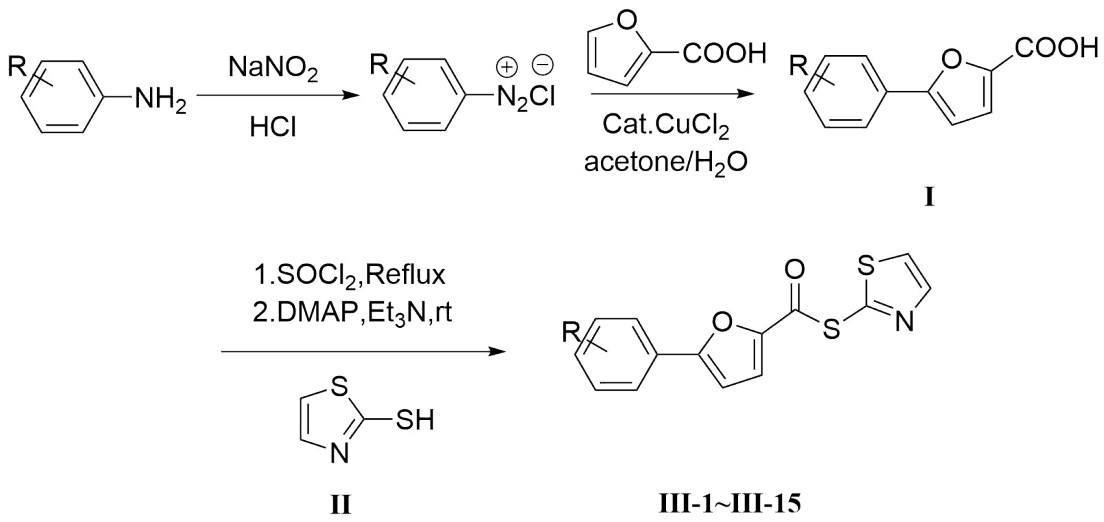

Figure 1. Synthesis routes of target compounds **III**. **III-1**:R = 2-Cl, **III-2**: R = 3-Cl, **III-3**: R = 4-Cl, **III-4**: R = 2-F, **III-5**: R = 3-F, **III-6**: R = 4-F, **III-7**: R = 2,4-di-F, **III-8**: R = 2,6-di-F, **III-9**: R = 2-NO_2, **III-10**: R = 3-NO_2, **III-11**: R = 4-NO_2, **III-12**: R = 4-Br, **III-13**: R = 4-CH_3, **III-14**: R = 4-OCH_3, **III-15**:R = H.

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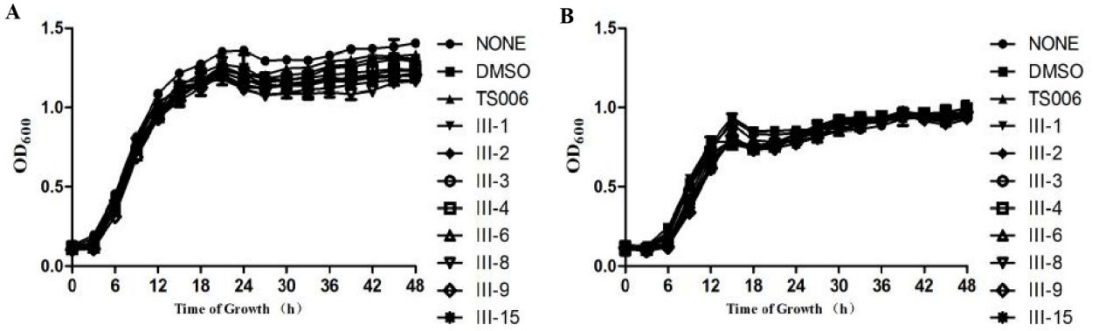

663

664 **Figure 2.** Effects of eight compounds on bacterial growth rates. (A) The growth rate
665 of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) PXO99^{A} in rich medium (M210)
666 supplemented with DMSO (dimethylsulfoxide) or 10 μg/mL of each compound. (B)
667 The growth rate of *Xoo* PXO99^{A} in hrp-inducing medium (XOM2 plus 0.5% sucrose)
668 supplemented with DMSO or 10 μg/mL of each compound. The optical density at 600
669 nm (OD_{600}) of the culture suspensions was recorded every 3 h during the 48 h period.

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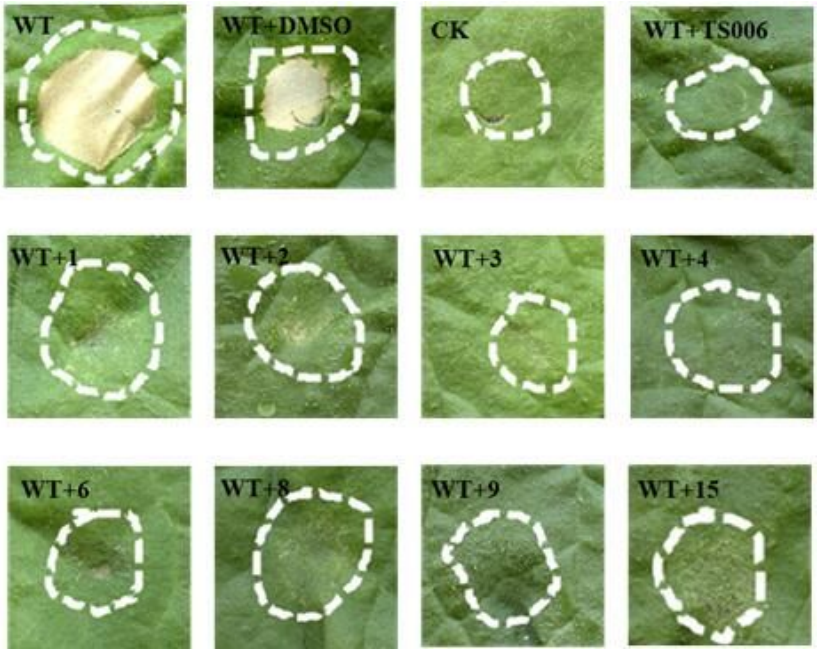

672

673 **Figure 3.** Effects of eight compounds on the HR induced by *Xoo* on tobacco leaves.

674 eight compounds suppressed HR induced by *Xoo*. TS006 was used as a positive

675 control. The similar results are representative of at least three independent

676 experiments.

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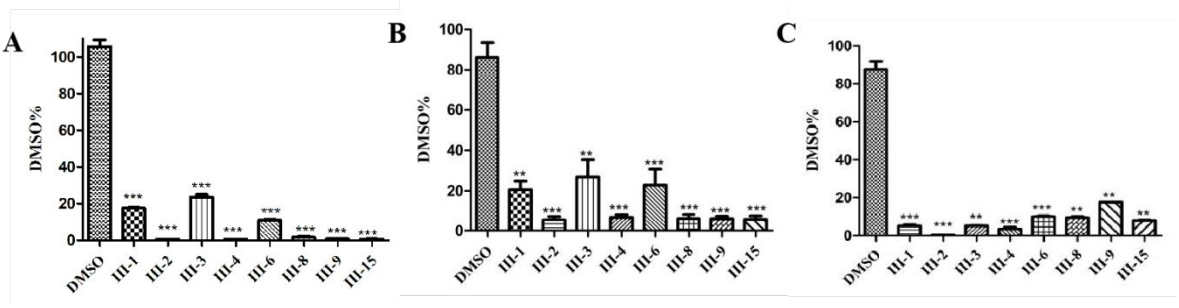


679

680 **Figure 4.** The effects of the eight compounds on the promoter activity of *hrpG* (A),
681 *hrpX* (B) and *hrcT* (C) in *Xoo* grown in XOM2 medium supplement with 10 μg/mL of
682 tested compounds. DMSO was used as a negative control. GFP mean fluorescence
683 intensity (MFI) was determined by flow cytometry. The calculated method was
684 following the formula: DMSO% = 100×MFI (XOM2 with tested compounds)/MFI
685 (XOM2 with DMSO). Each experiment had three replicates. **P < 0.01, ***P <
686 0.0001.

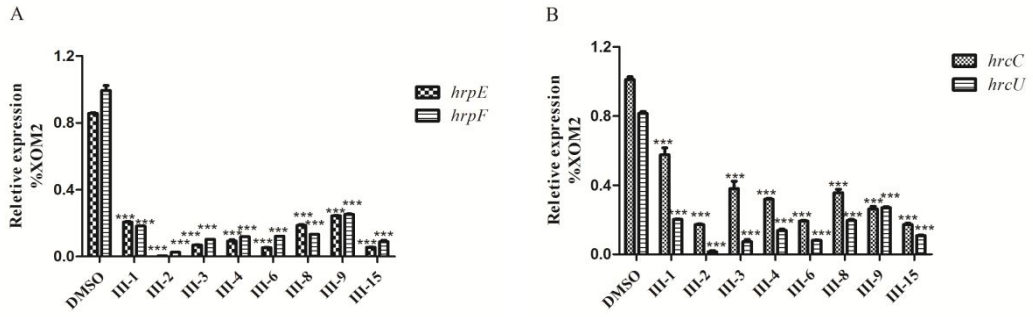


Figure 5. Relative mRNA levels of representative genes in the *hrp* cluster in *Xoo* PXO99^{A} incubated with eight compound were measured by qRT-PCR. (A) Compared with the DMSO control, the mRNA levels of *hrpE* and *hrpF* genes were reduced significantly after treatment with these inhibitors. (B) Compared with the DMSO control, the mRNA levels of two *hrc* genes (*hrcC* and *hrcU*) were also reduced in different extents after treatment with these inhibitors. The DNA gyrase subunit B (*gyrB*) gene was used as the internal control for data analysis. Each experiment had three replicates.

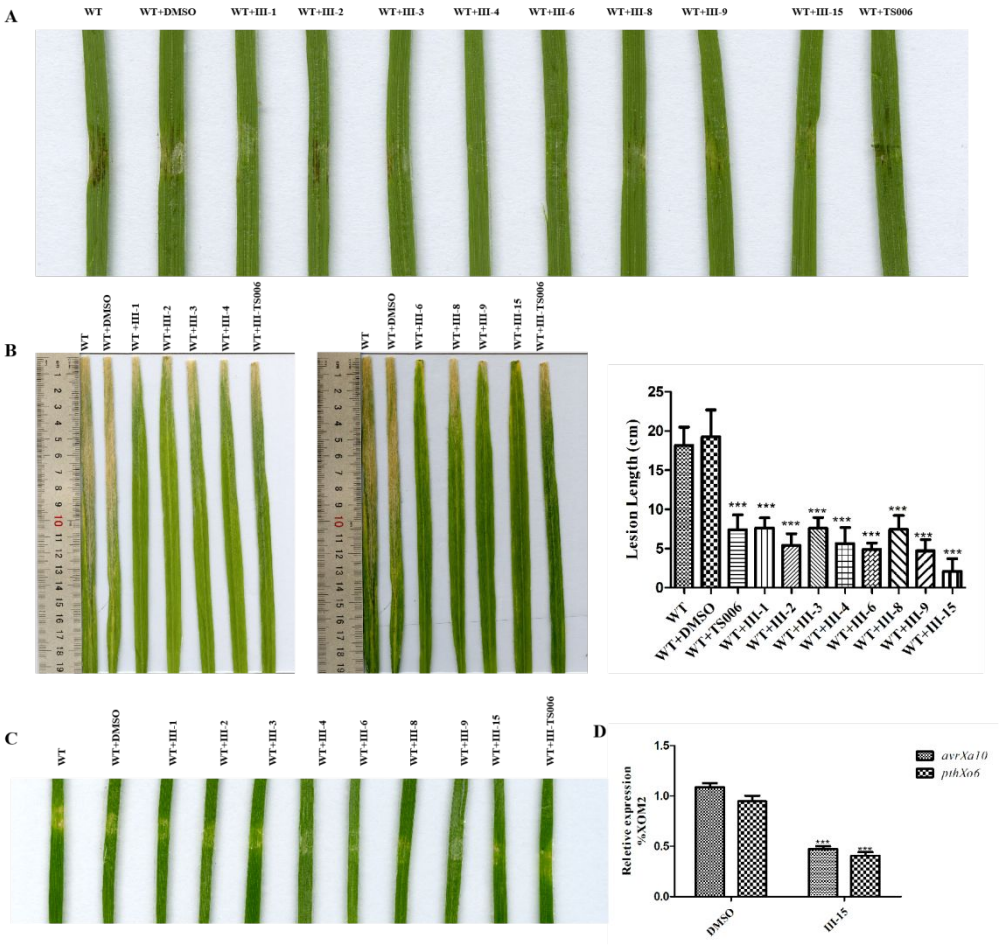
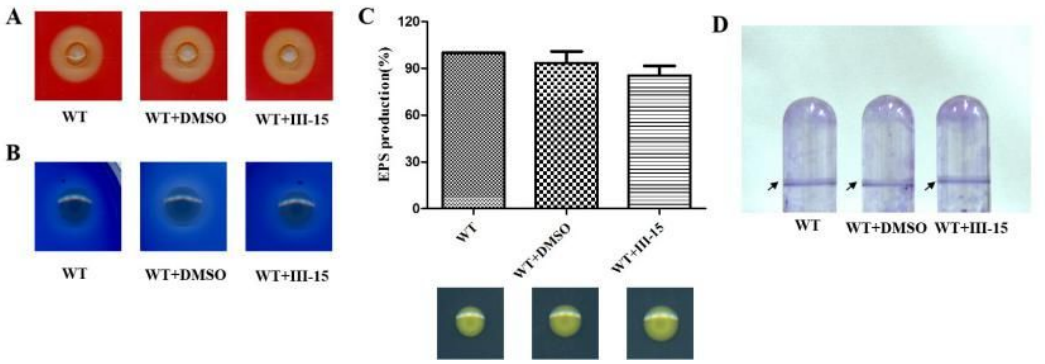


Figure 6. Significant reduction in bacterial blight disease by eight compounds. (A) The effect of eight compounds on the water-soaking symptoms caused by *Xoo* wild-type on IR24 seedling. The disease symptoms (B) and lesion lengths of *Xoo* wild-type on adult plants of rice cultivar IR24 were reduced after supplementing with 10 μg/mL of tested compounds. (C) Eight compounds reduced the disease symptoms caused by *Xoc* in different extent. (D) Compared with the DMSO control, the mRNA levels of *avrXa10* and *pthXo6* genes were reduced after treatment with compound III-15. At least three experiment tests had similar results. Asterisks indicate statistically significant differences. ***P < 0.0001.



709

710 **Figure 7.** Other virulence factors analysis. (A) Detection of cellulase secreted by *Xoo*
711 grown in PSA containing 0.5% carboxymethyl cellulose for 24 h. Pale-yellow clear
712 zones showed no difference between *Xoo* wild-type and that of treatment with **III-15**
713 compounds. (B) Detection of xylanase secreted by *Xoo* grown in PSA 0.2%
714 RBB-xylan for 48 h. Both *Xoo* wild-type and that of treatment with **III-15** compounds
715 appeared as the white clear zones among a blue background. (C) Secretion of
716 exopolysaccharide (EPS) in *Xoo* wild-type and that of treatment with **III-15**
717 compounds had no significant difference. From left to right, it provided WT,
718 WT+DMSO, and WT+III-15. (D) 2 mL of a bacterial suspension was placed in each
719 glass tube with 10 μg/mL **III-15** compounds and incubated at 28 ^{0}C after 72 h. The
720 crystal violet stained adherent biofilms on glass test tubes.

722

723 **Table 1**. Screening for inhibitors of *Xoo* T3SS by fluorescence-activated cell sorting
724 assays.

Compound	Avg MFI ± SD[a]	DMSO%[b]	Inhibition rate% (100% - DMSO%)
DMSO	5142.50±149.83		
III-1	66.33±21.69	1.29	98.71
III-2	82.33±16.86	1.60	98.40
III-3	134.50±48.26	2.62	97.38
III-4	69.77±19.88	1.36	98.64
III-5	210.47±57.95	4.09	95.91
III-6	90.43±19.65	1.76	98.24
III-7	231.70±137.20	4.51	95.49
III-8	77.80±27.50	1.51	98.49
III-9	86.20±25.69	1.62	98.38
III-10	158.87±83.88	3.09	96.91
III-11	281.73±156.06	5.48	94.52
III-12	546.57±85.99	10.63	89.37
III-13	837.80±103.56	16.29	83.71
III-14	538.03±49.65	10.46	89.54
III-15	64.43±14.43	1.25	98.75
TS006	139.03±96.28	2.70	97.30

725 [a] Green fluorescent protein (GFP) and mean fluorescence intensity (MFI) were measured for gated
726 populations of bacterial cells by flow cytometry (bacterial cells cultured in XOM2 with DMSO or
727 XOM2 added with 10 μg/mL of each compound). Values are representative of at least three
728 independent experiments, and three replicates were done for each experiment. [b] The calculated
729 method was following the formula: DMSO% = 100×MFI (XOM2 with tested compounds)/MFI
730 (XOM2 with DMSO). TS006 was used as a positive control.

732
733

734 **Table 2.** Protection activity of compounds against rice bacterial leaf blight under

735 greenhouse conditions at 200 μg/mL

Treatment	14 days after inoculation		
	Morbidity (%)	Disease index (%)	Control efficiency (%)
III-1	100	48.10	45.71[d]
III-2	100	40.30	54.51[b]
III-3	100	48.30	45.49[d]
III-4	100	40.20	54.63[b]
III-6	100	40.20	54.63[b]
III-8	100	48.20	45.60[d]
III-9	100	40.10	54.74 [b]
III-15	100	32.10	63.77 [a]
TS006	100	48.20	45.60 [d]
Bismerthiazol	100	44.60	49.66 [c]
Thiodiazole copper	100	53.70	39.39 [e]
CK	100	88.60	-

736 The letters a-e denoted the results of difference significance analysis. The different letter indicates
737 the values of activity with significant difference among different treatment groups ($P < 0.05$,
738 Fisher's LSD multiple comparison test).
739

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Table of Contents Graphic

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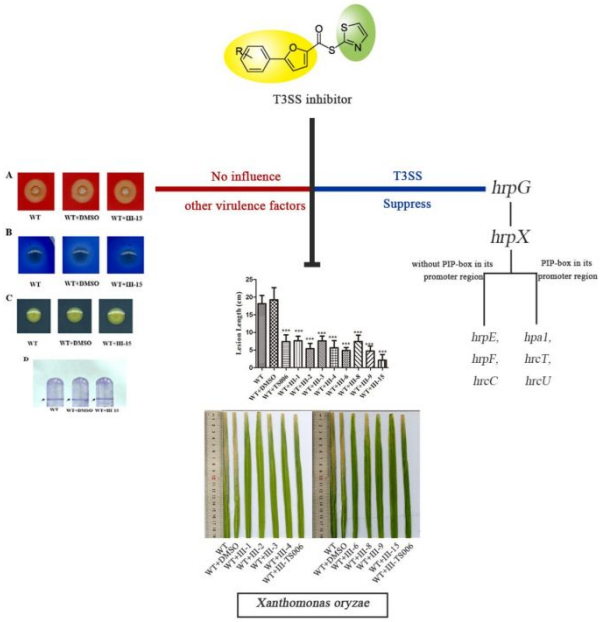

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