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New Axially Chiral Molecular Scaffolds with Antibacterial Activities against *Xanthomonas oryzae* pv. *oryzae* for Protection of Rice

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ABSTRACT: A new class of axially chiral thiazine molecules were constructed and showed promising antibacterial activities against the plant pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The axial chiralities of these compounds (*R*- or *S*-atropisomer) showed clear impacts on the in vitro inhibitory activities against *Xoo*. An optimal molecule of this class with the (*S*)-axially chiral configuration was identified to exhibit inhibitory activity against *Xoo* with an EC_{50} value of 4.18 μg/mL. This inhibition efficiency is superior to that of two commercial antibacterial agrochemicals, thiodiazole-copper and bismerthiazol, as the positive controls. This hit compound also performed better than the controls in our in vivo studies. Preliminary mechanistic studies via scanning electron microscopy images showed that our hit compound at a concentration of 10 μg/mL destroyed the bacterial integrity of *Xoo*. Label-free quantitative proteomics analysis indicated that a total of 366 differentially expressed proteins of the rice plants were significantly influenced in the presence of our hit molecule.

KEYWORDS: *axial chirality, thiazine, atropisomer, antibacterial, Xoo*

INTRODUCTION

The protection of agricultural plants from diseases caused by bacterial infections is an endless battle of both fundamental and practical significance. The problems are often complicated involving many disciplines; and solutions based on physical, biological, and chemical strategies are all used.[1,2] Each of these strategies has its own merits and limitations, and therefore, advancements in all these three domains continue to be critical. In particular, the use of chemicals constitutes a sizable share in modern plant disease management. Many organic molecules and metal compounds, such as azoxystrobin,[3] kasugamycin,[4] and Bordeaux mixture,[5] have been widely used to treat fungal and/or bacterial infections on various plants.

Among these bacterial-related plant diseases, rice bacterial leaf blight caused by *Xanthomonas oryzae* pv. oryzae (*Xoo*) remains the most difficult one with potentially big damages.[6] To date, management of rice bacterial leaf blight is primarily achieved through integration of planting techniques or with the use of disease-resistant rice plants as the most effective method.[7,8] Antimicrobial chemical agents, such as bismerthiazol (BT)[9] and zinc thiazole,[10,11] have also been used to control *Xoo* infections. Somewhat unfortunately, the efficacies of these metal or organic chemical agents are largely unsatisfactory as of today.[12,13] There is a strong need to search for effective chemical entities to cure or prevent *Xoo* infections on rice plants.

Our entry to this problem was directed toward designing new chiral molecules[14] for agricultural applications. Agrochemicals containing one or multiple stereocenters (chiral centers) show an increasing presence in the markets.[14−16] The enantiomers/stereoisomers often have different efficacies and/or environmental impacts. Typically, one of the two enantiomers (in the case of a racemate) performs better in terms of both efficiency and side effects. Most of these chiral agrochemicals are still used as a mixture of stereoisomers (e.g., the racemate of two enantiomers or a mixture of multiple diastereomers) to date. On the other hand, with the fast development of chiral synthesis techniques,[17−22] one can expect to see commercialization of a bigger number of optically enriched single stereoisomer agrochemicals. It has now been well accepted that the development of chiral agrochemicals can significantly contribute to new and greener plant protection solutions. Of the numerous chiral agrochemicals being commercialized or under development, the vast majorities are central-chiral molecules (e.g., molecules with an atom as the stereogenic center) (Figure 1a). In contrast, other types of chiral molecules, such as those based on axial chirality (Figure 1b), are barely explored as pesticides for plant protection.[23−27] Axial chirality is a common phenomenon observed in living systems, including natural products isolated from plants.[28−31] Multiple human medicines approved by FDA (U.S. Food and Drug Administration) are racemizing atropisomeric (axially chiral) molecules,[29] such as dabrafenib,[32] eszopiclone,[33,34] and afatinib.[35,36] A much bigger number of axially chiral molecules as potential drug candidates are under active studies at different stages of development.[29,37−40] The relatively rapid development of axially chiral molecules as human medicines does not seem to influence much of the landscape of the

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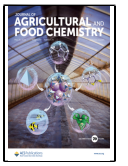

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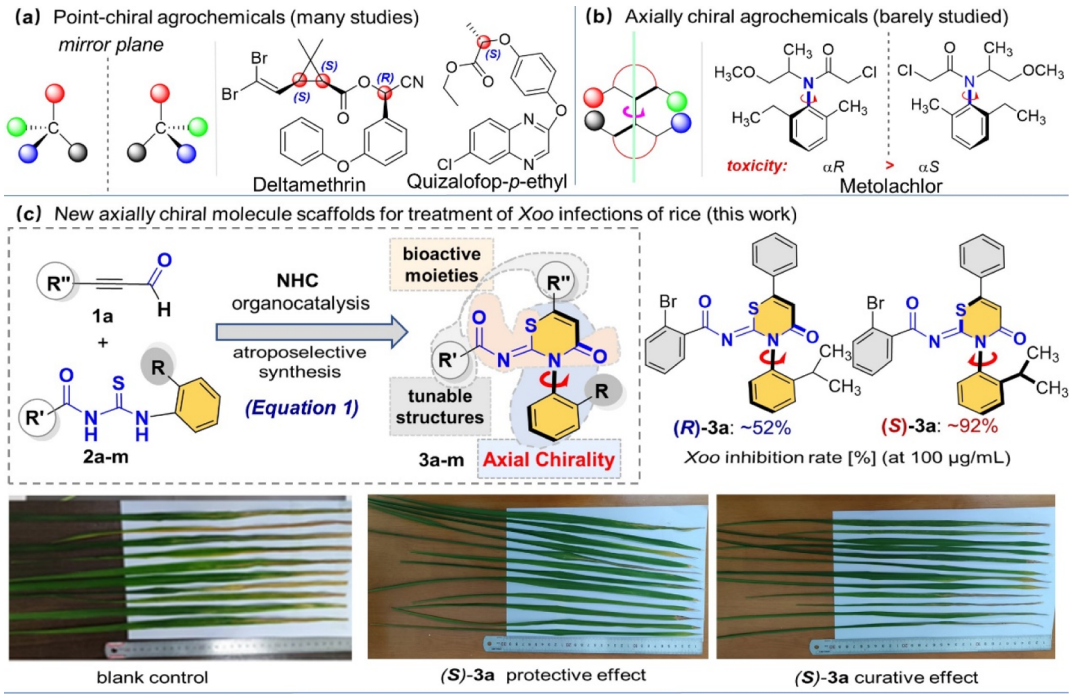

Figure 1. Designing new axially chiral molecule scaffolds for potential treatment of rice plant *Xoo* infections.

pesticide research field. A representative example of the rather limited studies of axially chiral pesticides is the herbicide metolachlor (Figure 1b).[27] The chiral C−N axis presented in metolachlor was found to have little influence on the efficacy[41,42] but different toxicity on aquatic organisms.[25,43] Other studies of axially chiral molecules as potential pesticides mainly involve isolated natural products bearing a chiral axis, such as laurokamurol and ustilaginoidin.[44,45] Overall, very few studies or little background knowledge are available in the current literature concerning axially chiral pesticides. There are very few deliberate designs of axially chiral molecules for agricultural uses. Part of the reason, to the best of our understanding, is due to the costs/challenges for access to such axially chiral molecules. On the other hand, in the recent 1−2 decades, with explosive development on asymmetric catalysis and chemical synthesis, the synthesis of axially chiral molecules with low cost becomes feasible. We therefore believe that it is now a critical time to start serious evaluation of axially chiral molecules for plant diseases, especially those such as *Xoo* infection, which currently do not have an effective chemical solution.

Our strategy and key findings are briefed in Figure 1c. We started with two building blocks (**1a** and **2a−m**) that are readily available and contain typical functional moieties found in pesticides and other bioactive molecules. The first building block (**1a**) is an ynal that after reactions becomes an *α,β*-unsaturated amide moiety (as in **3a−m**). Similar unsaturated amide groups are found in pesticides such as maleic hydrazide and nicobifen.[46−48] The second building block (**2a−m**) is an acylated thiourea that after reaction can form a heterocycle (a nitrogen and sulfur atom-containing thiazine derivative) connecting to multiple heteroatoms. Heterocycles of this type are common moieties in bactericides such as cefradine and omonasteine. The substituents (R, R′, and R″) on both building blocks can be easily modified to offer tunable structures. The two building blocks can react with each other, enabled by N-heterocyclic carbene organocatalysts studied in our labs and others.[49,50] Importantly, this organocatalytic reaction (eq 1) creates a new carbon−nitrogen (C−N) bond as a chiral axis, leading to a novel type of axially chiral molecules (**3a−m**). The axial chirality of most of these molecules (*R*- or *S*-atropisomer) shows a clear impact on the in vitro inhibitory activities of these molecules against *Xoo*. For example, for a best-performing molecule [(*S*)-**3a**] with the *Xoo* inhibition rate at about 92%, the corresponding enantiomer [(*R*)-**3a**] exhibits a much lower inhibition rate at around 52% under otherwise identical experimental conditions (Figure 1c). The EC_{50} of (*S*)-**3a** (4.18 μg/mL) of the in vitro studies is better than those of commercial pesticides [thiodiazole-copper (TC) and BT]. This lead compound (*S*)-**3a** also shows promising in vivo curative and protective effects on rice plants, compared to blank controls and commercial pesticides. These promising results clearly show the effects of axial chirality on antimicrobial activities against *Xoo* for rice protection. This information obtained here with this new class of axially chiral molecules is unprecedented and has potential for probing new mechanisms of actions. It provides conceptual insights for further explorations of axially chiral molecules for agricultural applications.

MATERIALS AND METHODS

Instruments and Chemicals. Commercially available materials were purchased from J&K Scientific and Energy Chemical. NMR spectra were obtained using a JEOL-ECX-500 (500 MHz) or Bruker ASCEND 400 (400 MHz) spectrometer. Scanning electron microscopy (SEM) images were observed using a Nova Nano SEM 450.

Synthesis of Axially Chiral Compounds (3a−m). The synthetic scheme (eq 2) and structures of compounds **3a−m** are illustrated in Figure 2. The synthetic procedures and product characterizations follow a recent report from our own laboratories.[51] A typical procedure is as follows: chiral NHC pre-catalyst (+)-**A** (0.02 mmol, 0.2 equiv, 8.4 mg) was added into a 4.0 mL vial equipped with a magnetic stir bar; then, DMAP (0.1 mmol, 1.0 equiv, 12.2 mg), $Sc(OTf)_3$ (0.02 mmol, 0.2 equiv, 9.8 mg), 5 Å molecular sieves (150.0

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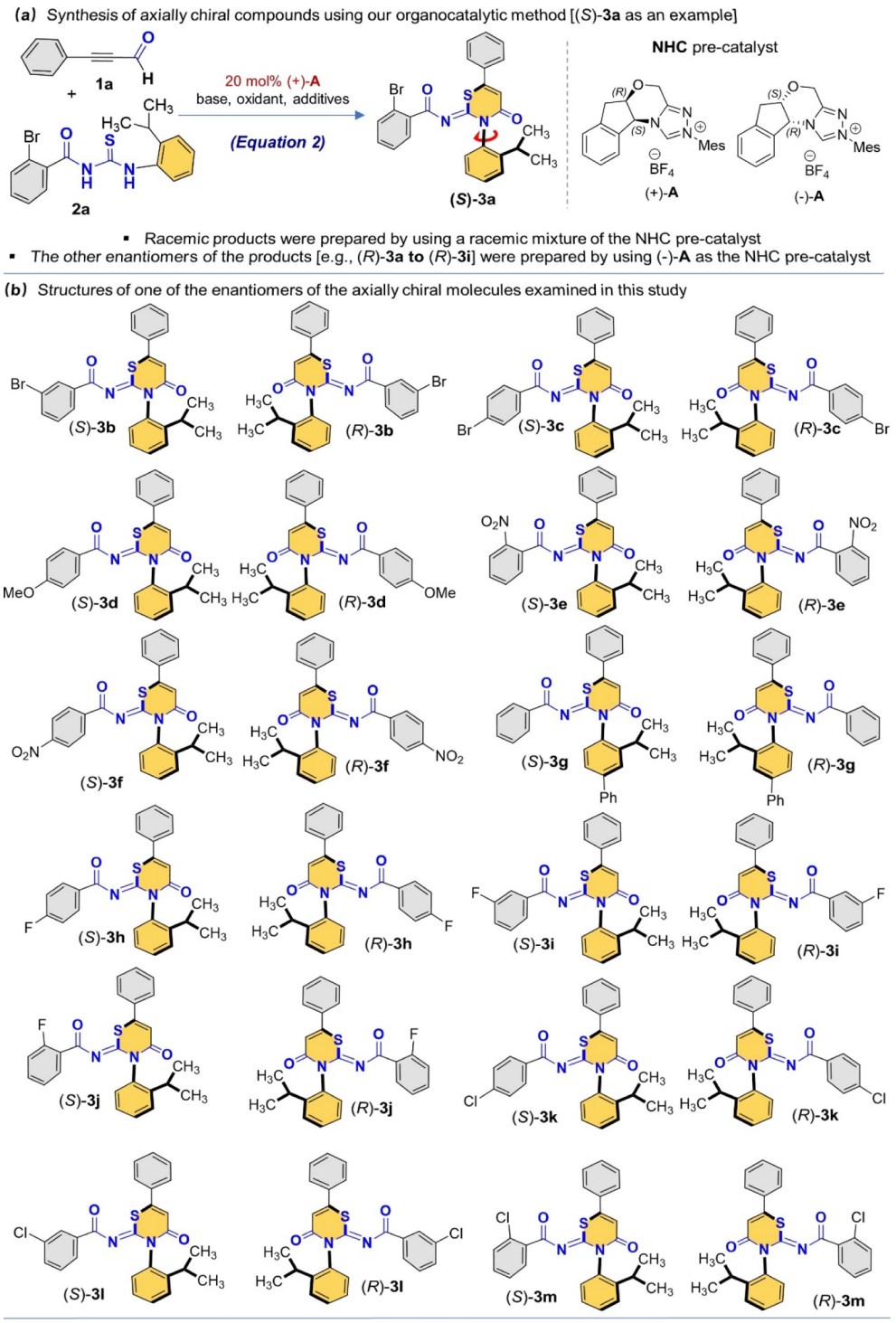

Figure 2. Synthesis and structures of the axially chiral molecules.

mg), 3,3',5,5'-tetra-tert-butyldiphenoquinone (0.3 mmol, 3.0 equiv, 122.4 mg), and substituted thiourea **2a** (0.1 mmol) were added. After that, furan (2.0 mL) and ynal **1a** (0.3 mmol) was added, and the reaction mixture was allowed to stir for 12 h at 30 °C. After consumption of thiourea substrate **2**, the reaction mixture was directly subjected to column chromatography on silica gel (20:1 hexanes/EtOAc) to afford the desired product (*S*)-**3a**. The other enantiomer (*R*)-**3a** was prepared by using the NHC catalyst with the opposite chirality [(−)-**A**] as the catalyst under an otherwise identical condition. The racemic form of the compound [(±)-**3a**] was prepared by using a racemic mixture of the NHC catalyst. Each of the compounds was characterized by ^{1}H, ^{13}C, and ^{19}F NMR, as documented in the earlier report.[51]

Methods for Antibacterial Activities against *Xoo*. The in vitro and in vivo experiments against *Xoo*, SEM analysis, and label-free quantitative proteomics analysis were carried out,[52−57] and more details could be seen in the Supporting Information.

RESULTS AND DISCUSSIONS

Chemistry. The model axially chiral compounds for antimicrobial activities (**3a−3m**) were readily synthesized from acylthioureas and ynals as the starting materials (Figure 2,

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Table 1. In Vitro Antibacterial Activities of Title Compounds against the Pathogen Xoo; (a) Preliminary Anibacterial Activities of Title Compounds against the Pathogen *Xoo* In Vitro; (b) EC_{50} Values of the Target Compounds (*S*)-3a, (*S*)-3d, and (*S*)-3e against *Xoo*

(a)

	Xoo inhibition rate [%]			*Xoo* inhibition rate [%]	
compounds	100 μg/mL	50 μg/mL	compounds	100 μg/mL	50 μg/mL
(*R*)-**3a**	52.25 ± 5.57	28.87 ± 8.01	(*R*)-**3h**	9.04 ± 9.09	14.04 ± 5.51
(*S*)-**3a**	92.16 ± 9.38	58.91 ± 3.61	(*S*)-**3h**	35.02 ± 7.57	18.41 ± 3.12
(*rac*)-**3a**	70.18 ± 7.67	31.52 ± 5.45	(*rac*)-**3h**	20.18 ± 7.68	11.64 ± 3.18
(*R*)-**3b**	37.96 ± 2.86	32.67 ± 8.21	(*R*)-**3i**	44.26 ± 7.51	7.65 ± 3.59
(*S*)-**3b**	18.28 ± 9.15	2.95 ± 9.73	(*S*)-**3i**	47.16 ± 8.84	31.43 ± 2.37
(*rac*)-**3b**	77.33 ± 1.62	46.06 ± 3.69	(*rac*)-**3i**	19.28 ± 5.30	36.27 ± 3.64
(*R*)-**3c**	25.83 ± 3.51	4.40 ± 2.47	(*R*)-**3j**	47.85 ± 1.55	21.11 ± 2.21
(*S*)-**3c**	34.07 ± 3.58	15.84 ± 7.24	(*S*)-**3j**	68.28 ± 8.15	32.95 ± 4.73
(*rac*)-**3c**	31.57 ± 9.16	26.77 ± 6.18	(*rac*)-**3j**	51.33 ± 2.62	16.06 ± 3.69
(*R*)-**3d**	74.28 ± 5.44	23.88 ± 4.76	(*R*)-**3k**	46.47 ± 9.54	5.65 ± 4.84
(*S*)-**3d**	86.76 ± 2.62	47.00 ± 1.90	(*S*)-**3k**	39.73 ± 8.59	9.95 ± 3.96
(*rac*)-**3d**	42.01 ± 3.92	34.47 ± 2.67	(*rac*)-**3k**	13.63 ± 6.98	4.17 ± 2.08
(*R*)-**3e**	54.75 ± 4.71	23.78 ± 4.39	(*R*)-**3l**	37.39 ± 0.68	15.83 ± 9.19
(*S*)-**3e**	68.23 ± 5.38	53.90 ± 2.29	(*S*)-**3l**	45.56 ± 4.47	15.97 ± 7.46
(*rac*)-**3e**	65.48 ± 2.78	62.19 ± 6.42	(*rac*)-**3l**	18.32 ± 6.52	27.25 ± 3.15
(*R*)-**3f**	44.96 ± 7.32	25.42 ± 1.65	(*R*)-**3m**	32.47 ± 9.57	23.08 ± 6.54
(*S*)-**3f**	37.96 ± 1.95	37.11 ± 4.14	(*S*)-**3m**	38.11 ± 6.59	34.42 ± 3.20
(*rac*)-**3f**	40.56 ± 9.94	36.11 ± 1.65	(*rac*)-**3m**	22.58 ± 9.02	11.54 ± 2.33
(*R*)-**3g**	28.72 ± 2.34	17.78 ± 1.59	TC	87.79 ± 4.69	53.45 ± 2.11
(*S*)-**3g**	17.48 ± 3.70	19.88 ± 8.26	BT	98.30 ± 2.19	79.53 ± 1.11
(*rac*)-**3g**	21.88 ± 1.23	18.11 ± 5.11			

(b)

	Xoo inhibition rate [%]		
compounds	regression equation	EC_{50} (μg/mL)	R^2
(*S*)-**3a**	$y = 0.5285x + 4.6729$	4.18	0.9257
(*S*)-**3d**	$y = 0.4730x + 4.1628$	58.88	0.9165
(*S*)-**3e**	$y = 0.9932x + 3.7594$	17.75	0.9677
BT	$y = 2.5275x + 1.9419$	16.22	0.9514
TC	$y = 0.4267x + 4.3291$	37.35	0.9758

eq 2). The synthesis involves a catalytic one-pot operation developed in our laboratories earlier.[51] Unlike our previous report[51] that concerns new catalysis concepts and reaction designs, here in the present study, we focused on how the axial chirality and the functional groups presented in the molecules influence the bioactivities. In particular, we introduced different substituents and substitution patterns into the structure of compound **3** to investigate their antibacterial activities against *Xoo* (Figure 2). Both enantiomers of the target molecules were prepared and evaluated. Substituents commonly found to improve bioactivities in pesticides (such as F, Cl, Br) were found in our study to give high antimicrobial activities against *Xoo* (compound **3a**−**3c**, **3h**, **3i**). Meanwhile, the electron-withdrawing group (NO_2) or electron-donating group (CH_3O) was also installed on the phenyl group to explore the influence of the electronic effect on target compounds (**3d**−**3f**). Although a comprehensive structure−activity relationship cannot be established at this point, our chemistry designs do provide sufficient insights for further development. For the first time, the chemical entities and their unique spatial arrangements induced by the axial chirality were found to have a drastic influence on the antimicrobial activities against *Xoo*, which infects rice plants.

In Vitro Antibacterial Bioassays. A series of axially chiral compounds (*R*- or *S*-atropisomer) were prepared to investigate the in vitro inhibitory activities against *Xoo*. The agricultural antibiotic agents TC and BT were used as the positive controls. Among the compounds evaluated (Table 1), (*S*)-**3a** was found to exhibit the best antibacterial activity against *Xoo* at the concentrations of 50 and 100 μg/mL. The stereoconfigurations (*R*, *S*, *rac*) of the compounds were found to significantly affect their antimicrobial activities. For example, the inhibition rate of (*S*)-**3a** was much better than that of its enantiomer (*R*)-**3a** and the corresponding racemic mixture [(*rac*)-**3a**]. Additional bioactivity studies with (*S*)-**3a** revealed an EC_{50} value of 4.18 μg/mL, which was superior to those of BT (16.22 μg/mL) and TC (37.35 μg/mL) under the same conditions (Table 1b). Different substituents or substitution patterns also have a significant influence on the antibacterial activities of the compounds. For instance, when the Br group of **3a** was moved from the *ortho*-position to the *meta*- (**3b**) or *para*-position (**3c**) on the benzene ring of the benzoyl group, a clear decrease on activities was observed. Replacing the *o*-Br group of (*S*)-**3a** with an electron-donating methoxy unit (to get compound **3d**) led to a moderate activity decrease. Multiple other substituents and substitution patterns commonly evaluated for drug and pesticide developments were also studied, as exemplified by compounds **3e**−**m**. Notably, compound (*S*)-**3e** was found to have an inhibition rate of 53.90% at a concentration of 50 μg/mL and a EC_{50} value of 17.75 μg/mL, which is comparable to

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Compounds	Morbidity(%)	Curative activity (14 days after spraying)		Protective activity (14 days after spraying)	
		disease index	control efficiency(%)[b]	disease index	control efficiency(%)[b]
(*S*)-**3a**	100	45.80	45.90	39.20	53.70
TC	100	53.17	37.22	67.74	20.02
BT	100	36.67	56.71	44.67	47.26
CK[a]	100	84.70	/	84.70	/

[a] Negative control. [b] Statistical analysis was conducted by the ANOVA method under the condition of equal variances assumed ($P > 0.05$) and equal variances not assumed ($P < 0.05$).

Figure 3. Curative and protective activities of (*S*)-**3a** against rice bacterial blight at 200 μg/mL. BT and TC were the positive controls.

Figure 4. SEM images for *Xoo* after incubation in various dosages of (*S*)-**3a** or TC. (*S*)-**3a**: (a) 0 μg/mL, (b) 10 μg/mL, and (c) 5 μg/mL; TC: (d) 10 μg/mL and (e) 5 μg/mL. Scale bars for (a–e) are 2000 nm.

those of the positive controls using commercial pesticides (Table 1). As a technical note, as the solubility of these compounds is relatively low in the experimental medium, the observed inhibition rates at the concentrations of 50 and 100 μg/mL are likely underestimated (Table 1). The EC_{50} values (Table 1b) of the compounds in comparison with the positive controls shall be relatively accurate.

In Vivo Bioassay against Rice Bacterial Leaf Blight. To further evaluate the potential applications of (*S*)-**3a** against rice bacterial leaf blight, in vivo experiments were carried out. As shown in Figure 3, (*S*)-**3a** displayed a promising in vivo curative activity against this disease with a control efficiency of 45.90% at 200 μg/mL, which was better than that of TC (37.22%). For the protective activity, compound (*S*)-**3a** exerted a control efficiency of 53.70%, which was superior to those of BT (20.02%) and TC (47.26%). The results from both in vitro and in vivo studies suggest that these types of axially chiral thiazine derivatives are a class of new scaffolds worth of further explorations as pesticides to fight against bacterial infections and other plant diseases.

Scanning Electron Microscopy. SEM images were used to detect the morphological variations of pathogens after treatments with compounds (*S*)-**3a** and TC (10, 5 μg/mL) (Figure 4) that expressed in a concentration-dependent manner. The morphology of the pathogen *Xoo* was changed from intact to partially damaged or distorted after treatment with (*S*)-**3a** (10, 5 μg/mL). Compared with the positive control (of TC), the morphology of bacteria showed more deformity, corrugations, and cracking with the increase of the drug dosages of (*S*)-**3a**. Observations of the SEM analysis suggested that compound (*S*)-**3a** could cause deformation of the cell membranes for *Xoo*. This disruption of cell membranes likely contributes to (part of) the activities observed from the in vitro and in vivo studies.

Label-Free Quantitative Proteomics Analysis. We obtained a label-free quantitative proteomics profile[58] by treating a rice plant with *Xoo* and compound (*S*)-**3a** successively (Figure 5), which can derive a preliminary understanding of the adjusting effect and antibacterial mechanism caused by our axially chiral thiazine derivatives. From the rice plant treated with *Xoo* and the blank control, approximately 5797 proteins (5620 + 139; Figure 5a) were initially excavated. From the plant treated with *Xoo* and compound (*S*)-**3a**, the same analysis revealed 5658 (5620 + 38) proteins. Among these proteins, 5620 (96.9%) of them were commonly observed from plants treated with the blank control and our compound. From comparative proteomics analysis [(*S*)-**3a**/CK], 366 proteins (97 + 269; Figure 5b) were revealed to be differently expressed when triggered by compound (*S*)-**3a**. Under the screening conditions of multiple change >1.5 and $P < 0.05$, 97 proteins were up-regulated and 269 proteins were down-regulated.

The biological functions of the different expressed proteins were detected by three main GO categories (Figure 5d). The

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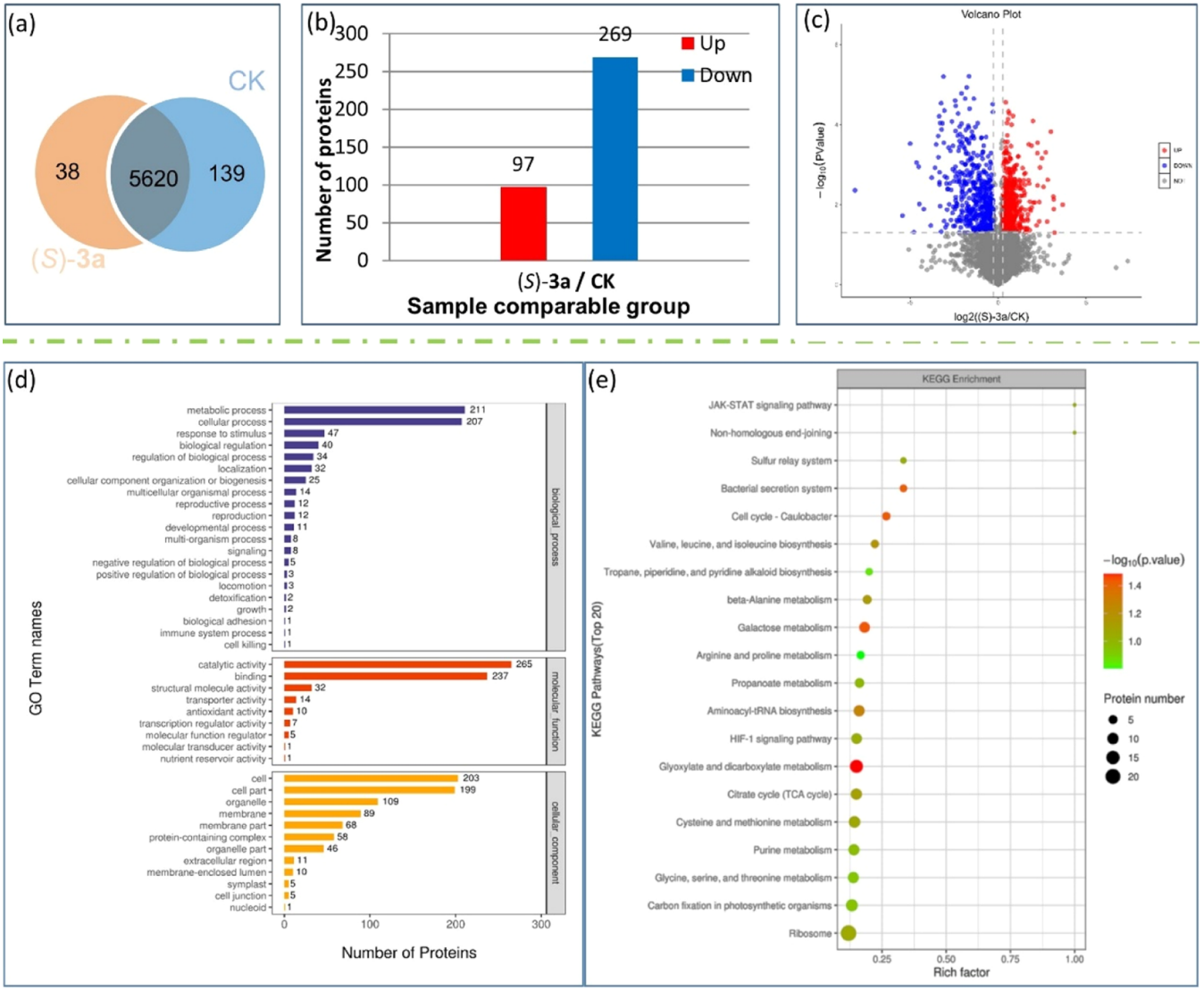

Figure 5. (a) Venn diagrams for proteome comparison on control and treatment groups; (b) histogram of the number distribution of differentially expressed proteins in different comparison groups [(*S*)-**3a**/CK]; (c) volcano plot of differentially expressed proteins [(*S*)-**3a**/CK]; (d) differentially expressed proteins in control and treatment groups; (e) KEGG pathway enrichment bubble plot of differentially expressed proteins.

results of biological process analysis showed that these proteins were mainly related to the metabolic process, the cellular process, biological regulation, localization, and response to stimuli. For the molecular function, these proteins were involved in multiple biological actions such as catalytic activity, binding, and transporter activity. Meanwhile, cellular component analysis presented that most of the differentially expressed proteins were concentrated in the cell, the cell part, the organelle, and the membrane.

Compound (*S*)-**3a** induced different expressions of the multiple proteins, which reveals the reasonable action pathways though the KEGG pathway enrichment bubble plot chart (Figure 5e). The galactose metabolism is one of the main metabolic pathways. It is well known that the galactose metabolism plays significant roles in the bioactivities of malate dehydrogenase and isocitrate lyase, which is crucial in multifarious physiological processes incorporating the metabolisms of fat, glucose, and energy. It is also observed that the citrate, malic enzyme, and glyoxylic acid in the glyoxylate and diacid metabolism pathways are down-regulated, which might result in the indirect up-regulation of cytochrome P450, which plays significant roles in the nitrogen metabolism pathway. The up-regulation of cytochrome P450 can cause the death of the bacteria via improvement of the self-protective ability of the plants. Moreover, the amount of the peroxidase is significantly increased and can strengthen the plant entities to help resist various plant pathogens.

In summary, we have developed a new class of axially chiral thiazine derivatives with antibacterial activities against the plant pathogen *Xoo*. Among these molecules, compound (*S*)-**3a** displayed potent inhibition effects with an EC_{50} value of 4.18 μg/mL, which are better than those of commercial antibacterial agents (BT and TC). In vivo studies revealed promising protective activities of (*S*)-**3a** against rice bacterial blight with a control efficiency of 53.70%, which is better than those for BT and TC. The chiralities of these molecules have profound effects on the corresponding antibacterial activities. Preliminary mechanistic studies via SEM images showed that the hit compound (*S*)-**3a** could clearly destroy the bacterial integrity of *Xoo*. Label-free quantitative proteomics analysis indicated that a total of 366 proteins were differentially expressed in plant cells as induced by compound (*S*)-**3a**. Our

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study opens a previously unexplored window in developing axially chiral molecules as effective cures of *Xoo* infections on rice plants. Insights generated from present and future studies in the direction of bioactive axially chiral scaffolds shall likely benefit the area of plant protection beyond *Xoo* infections. Ongoing studies in our laboratories include further structural optimizations and mechanistic evaluations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c01407.

General information, biological assay methods, SEM characterization, and label-free quantitative proteomics analysis (PDF)

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#Y.Chen and T.L contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Xoo, *Xanthomonas oryzae* pv. oryzae; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EC_{50}, half-maximal effective concentration; TC, thiodiazole-copper; BT, bismerthiazol; DMAP, 4-dimethylaminopyridine; SEM, scanning electron microscopy

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