Functional Structure/Activity Relationships

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Identification of Racemic and Chiral Carbazole Derivatives Containing an Isopropanolamine Linker as Prospective Surrogates Against Plant Pathogenic Bacteria: In Vitro and In Vivo Assays, and Quantitative Proteomics

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Abstract

Recent observations on the emergence of drug-resistant plant pathogenic bacteria have highlighted and elicited an acute campaign to develop novel, highly efficient antibiotic surrogates for managing bacterial diseases in agriculture. Thus, a type of racemic and chiral carbazole derivative containing an isopropanolamine pattern was systematically synthesized to discover low-cost and efficient antibacterial candidates. Screening results showed that compounds 2f, 6c, and 2j could significantly suppress the growth of tested plant pathogens, namely Xanthomonas oryzae pv. oryzae, X. axonopodis pv. citri, and Pseudomonas syringae pv. actinidiae, and provided the corresponding EC_{50} values of 1.27, 0.993, and 0.603 µg/mL, which were extremely better than those of existing commercial drugs. In vivo study confirmed their prospective applications for controlling plant bacterial diseases. Label-free quantitative proteomics analysis indicated that compound 2f could dramatically induce the up- and down-regulation of a total of 247 differentially expressed proteins, which was further validated by the parallel reaction monitoring technique. Moreover, fluorescence spectra and SEM images were performed to further explore the antibacterial mechanism.

Keywords

carbazole, isopropanolamine, antibacterial, proteomics, action mechanism

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1. Introduction

Despite the persistent threat posed by plant bacterial diseases and the identification of a variety of widespread, highly phytopathogenic bacteria, the shortage of effective antibacterial agents in agriculture causes considerable concern.\textsuperscript{1-3} Currently, a limited number of marketed bactericides, such as bismuthiazol (\textbf{BT}), thiodiazole copper (\textbf{TC}), streptomycin (has been disabled for the risk potentials), triazoles, kocide, and amobam, were exploited to manage these destructive bacterial diseases.\textsuperscript{4,5} However, these agents have performed reduced field control efficiency, not only due to their long-term usage and abuse, but also ascribing to the single mode of action performed.\textsuperscript{6,7} Particularly, some of these pathogenic bacterial strains have progressively experienced an elusive and prompt variation to acquire the flexible defense mechanisms. Such complicated resistant pathogens can provide a quick and adequate response against various existing external stimuli, putting agriculture in considerable difficulty. For example, the application of marketed bactericide \textbf{BT} has already resulted in the emergence of \textbf{BT}-resistant strains of \textit{Xanthomonas oryzae} pv. \textit{oryzae} (\textit{Xoo}) in Anhui Province, China.\textsuperscript{2,4} Therefore, exploring and developing new chemotherapeutic agents normally bearing different modes of action to combat wild-type and drug-resistant pathogens is urgently required.

Elaborate observation found that agrochemicals, which can be commercialized preferentially, possess the following features: simple structural architecture, short synthetic procedures, facile operations for drug production, low manufacturing cost, and superior biological activity. Concurrently, modern agricultural science has
proposed strict or even higher requirements, such as good biocompatibility and degradability, low residues, negligible phytotoxicity, permissible environmental friendliness, high selectivity for the target species, and low cytotoxicity toward non-target organisms, for the development of pesticide products. Therefore, the reasonable capture of a building block as the original template for exploring highly active antibacterial leads has elicited considerable concern. One of the effective sources for the selection is from the natural products in which the biological effects of various key skeletons have been carefully explored and highlighted. Carbazole skeleton with outstanding electronic and charge-transport properties has been frequently discovered and reported from many naturally occurring products.\textsuperscript{8,9} Moreover, this privileged building block can be easily decorated with various functional groups, which endows carbazole-based derivatives and analogs with extensive and distinct biopharmaceutical activities, including antimicrobial,\textsuperscript{10-14} anti-cancer,\textsuperscript{15-17} insecticidal,\textsuperscript{18,19} anti-viral,\textsuperscript{20} anti-α-glucosidase,\textsuperscript{21,22} anti-inflammatory,\textsuperscript{23,24} anti-malarial,\textsuperscript{25,26} anti-diabetic,\textsuperscript{27,28} neuroprotective,\textsuperscript{29,30} and anti-Alzheimer activities.\textsuperscript{31-33} Given that the carbazole motif plays a crucial and versatile role in determining the final biological action of a target molecule, a large batch of natural and non-natural carbazole-tailored substrates acquiring prospective and practical applications in many fields, especially in the antimicrobial aspect, has also been presented.\textsuperscript{8,34} As shown in Figure 1a, these kinds of typical natural carbazole derivatives were discovered to possess good antimicrobial effects against a wide range of medicinal bacteria and fungi including \textit{Escherichia coli}, \textit{Salmonella}
typhi, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Proteus vulgaris, Candida albicans, and Trichophyton rubrum.\textsuperscript{35,36}

Considering the powerfully broad-spectrum bioactivity of carbazole-based compounds, the relevant action mechanisms for their pharmaceutical effects have also been extensively studied. Consequently, a variety of target species, such as diverse enzymes or kinases (Pim-1 kinase, anaplastic lymphoma kinase, topoisomerase I, and topoisomerase II), DNA, and G-quadruplexes (G-tetrads or G4-DNA), were reported.\textsuperscript{37-39} Inspired by the aforementioned studies, the rational design of title compounds based on natural carbazole skeleton may probably lead to the discovery of highly active antibacterial surrogates intended for the future battle against plant bacterial diseases. Moreover, this kind of carbazole-tailored molecules may be endowed with flexibly privileged functions for interacting with multiple targets of pathogenic microorganisms, which probably reduces the risk of developing bacteria-resistance. Few studies were performed to describe the general antibacterial effects of carbazole-tailored compounds toward plant pathogens. Herein, a type of simple racemic and chiral $N$-substituted carbazole derivative bridging by isopropanolamine patterns (Figure 1b) was systematically synthesized and their antibacterial effects were screened. In this study, three seriously invasive phytopathogenic bacterial strains, including \textit{Xoo}, \textit{Xanthomonas axonopodis pv. citri} (\textit{Xac}), and \textit{Pseudomonas syringae pv. actinidiae} (\textit{Psa}), will be evaluated \textit{in vitro}. These pathogenic strains are known to cause destructive and widespread bacterial leaf
blight, citrus bacterial canker, and kiwifruit bacterial canker in agriculture, which makes a mass of economic losses every year.\cite{3,40,41} Subsequently, \textit{in vivo} study will be performed to verify the practical application for controlling bacterial diseases. In addition, label-free quantitative proteomics and parallel reaction monitoring (PRM) techniques, fluorescence spectra, and scanning electron microscopy (SEM) will be utilized to study the possible antibacterial mechanism.

2. Materials and methods

2.1 Instruments and Chemicals

NMR spectra were obtained by using a Bruker Biospin AG-400 apparatus. Chemical shifts were reported in parts per million (ppm) down field from TMS with the solvent resonance as the internal standard. Coupling constants (\(J\)) were reported in Hz and referred to apparent peak multiplications. SEM images were visualized and obtained using Nova Nano SEM 450. Fluorescence spectra measurements were performed on a Fluoromax-4 spectrofluorimeter (Horiba Trading CO., LTD).

2.2 \textit{In vitro} antibacterial bioassay (turbidimeter test)

In our study, all the synthesized target compounds were evaluated for their antibacterial activities against \textit{Xoo}, \textit{Xac}, and \textit{Psa} by the turbidimeter test \textit{in vitro}.\cite{6} Dimethylsulfoxide in sterile distilled water served as a blank control, Bismertonazol and Thiodiazole Copper served as positive controls. Approximately 40 \(\mu\)L of solvent NB (1.5 g beef extract, 2.5 g peptone, 0.5 g yeast powder, 5.0 g glucose, and 500 mL distilled water; pH = 7.0-7.2) containing \textit{Xoo} (or \textit{Xac}, or \textit{Psa}), incubated on the phase of logarithmic growth, was added to 5 mL of solvent NB containing different
concentrations of the test compounds and positive control, such as 20, 10, 5, 2.5, 1.25 μg/mL or 10, 5, 2.5, 1.25, 0.625 μg/mL (depending on the bioactivity of different compounds, the concentrations were chosen in two times decline trend to make sure the EC$_{50}$ values are inside the concentration ranges tested). The inoculated test tubes were incubated at 28 ± 1 °C and continuously shaken at 180 rpm for 24-48 h until the bacteria were incubated on the logarithmic growth phase. The growth of the cultures was monitored on a microplate reader by measuring the optical density at 595 nm (OD$_{595}$) given by turbidity corrected values = OD$_{bacterial\ wilt}$ − OD$_{no\ bacterial\ wilt}$, and the inhibition rate $I$ was calculated by $I = \frac{(C - T)}{C} \times 100\%$. C is the corrected turbidity values of bacterial growth on untreated NB (blank control), and T is the corrected turbidity values of bacterial growth on treated NB. By using the SPSS 17.0 software and the obtained inhibition rates at different concentrations, a regression equation was provided. The results of antibacterial activities (expressed by EC$_{50}$) against Xoo, Xac, and Psa were calculated from the equation and the value was within the concentration ranges. The experiment was repeated three times.

2.3 In vivo bioassay against rice bacterial leaf blight

The curative and protection activities of compound 2f against rice bacterial leaf blight were determined by Schaad’s method with some slight modifications.$^{42}$ Bismerthiazol (20% wettable powder) and thiodiazole copper (20% suspending agent), the bactericides registered for rice bacterial leaf blight and purchased from the market, served as the positive controls. The curative activity in potted plants for reducing rice bacterial leaf blight of compound 2f was determined under controlled conditions in a
growth chamber. After sowing the rice seeds of variety “Fengyouxiangzhan” approximately 8 weeks, rice leaves were inoculated with Xoo, which was incubated at logarithmic growth using sterilized scissors. One day after inoculation, 2f solution at 200 μg/mL was uniformly sprayed onto the rice leaves until dripping down, whereas distilled water was uniformly sprayed onto the negative control plants. Then, all inoculated rice plants were placed in a plant growth chamber (28 °C and 90% RH). At 14 days after spraying, the disease index of the inoculated rice leaves was measured. Similarly, the protection activity for reducing rice bacterial leaf blight of compound 2f was also conducted under controlled conditions. After sowing the rice seeds of variety “Fengyouxiangzhan” approximately 8 weeks, compound 2f solution at 200 μg/mL was uniformly sprayed onto the rice leaves until dripping down, whereas distilled water was uniformly sprayed onto the negative control plants. One day after spraying, Xoo, which was incubated at logarithmic growth, was inoculated on the rice leaves using sterilized scissors. All inoculated rice plants were placed in a growth chamber (28 °C and 90% RH). At 14 days after inoculation, the disease index (C or T) of the inoculated rice leaves was measured.6 Firstly, the spot area of each leaf and the whole leaf area were measured, and then the percentage of the spot area in the whole leaf area was calculated. Secondly, these leaves were classed according to the following grading standards. Grade 1: the area of disease spot accounts for less than 5% of the whole leaf area; Grade 3: the area of disease spot accounts for 6-10% of the whole leaf area; Grade 5: the area of disease spot accounts for 11-20% of the whole leaf area; Grade 7: the area of disease spot accounts for 21-50% of the whole leaf area.
area; Grade 9: the area of disease spot accounts for more than 50% of the whole leaf area. Finally, the disease index (C or T) was calculated using the following formula:

\[
\text{Disease index (C or T)} = \frac{\sum (\text{the number of leaves at each Grade} \times \text{the corresponding Grade})}{(\text{the total number of leaves} \times \text{the superlative Grade})}.
\]

The control efficiencies \(I\) for the curative and protection activities are calculated by the following equation: Control efficiency \(I\) (%) = \(\frac{(C - T)}{C} \times 100\). In the equation, \(C\) is the disease index of the negative control and \(T\) is the disease index of the treatment group.

### 2.4 Label-free quantitative proteomics analysis

#### 2.4.1 Bacterial strains and growth conditions

The bacterial strain used in this study was \(Xoo\), which is the causative pathogen of rice bacterial leaf blight. The \(Xoo\) was cultivated in liquid nutrient broth medium (1.5 g beef extract, 2.5 g peptone, 0.5 g yeast powder, 7.5 g sucrose, added into 500 mL distilled water, pH 7.0–7.2) at 28–30 °C for 12 h. Cells were harvested when \(\text{OD}_{600}\) (optical density at 600 nm) reached approximately 0.1. Then compound 2f with the concentration of 6.35 μg/mL (5×EC\(_{50}\)) was added into the mixture and cultured at 28-30 °C. When the \(\text{OD}_{600}\) of the control group reached to 0.6-0.8, all the samples were collected and centrifuged at 8000 rpm at 4 °C, respectively. Three biological replicates were prepared for each condition.

#### 2.4.2 Protein extraction and trypsin digest

The extraction of all \(Xoo\) proteins were performed based on the methods with a slight modification.\(^{43}\) Briefly, the sample was sonicated three times on ice using a high
intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail). The remaining debris was removed by centrifugation at 12,000 g at 4 °C for 10 min. Finally, the supernatant was collected and the protein concentration was determined with BCA kit according to the manufacturer’s instructions. For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted to a urea concentration (less than 2 M). Finally, the trypsin was added into the protein sample by 1:50 trypsin-to-protein mass ratio for the first digestion overnight, and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion.

2.4.3 LC-MS/MS analysis

The tryptic peptides were dissolved in 0.1% formic acid, directly loaded onto a home-made reversed-phase analytical column (15 cm length, 75 μm i.d.). The gradient was comprised of an increase from 6% to 23% (0.1% formic acid in 98% acetonitrile) over 26 min, 23% to 35% in 8 min and climbing to 80% in 3 min, then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments
were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set at 50000. Fixed first mass was set as 100 m/z.

2.4.4 Protein identification

The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against \textit{Xoo} database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 2 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in First search and 5 ppm in Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and oxidation on Met was specified as variable modifications. The false discovery rate (FDR) for peptide and protein identifications was set to 0.01 and minimum score for peptides was set >40. It should be combined and reported as one protein group to identify peptides which all appear between two proteins. We used label-free quantification with a minimum of two ratio counts to determine the normalized protein intensity to compare between samples. We used the iBAQ algorithm to rank the absolute abundance of different proteins within a single sample. Protein tables were filtered to eliminate the interference from common contaminants and reverse database. A two-sample unpaired t-test was used to identify the differentially accumulated proteins between control and treatment groups. The iBAQ data were used for the t-test, and proteins with p value $\leq 0.05$ were considered differentially expressed.
2.4.5 Bioinformatics analysis

Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). Firstly, Converting identified protein ID to UniProt ID and then mapping to GO IDs by protein ID. If some identified proteins were not annotated by UniProt-GOA database, the InterProScan soft would be used to annotated protein’s GO functional based on protein sequence alignment method. GO items can be divided into three categories, namely, biological process (BP), cellular components (CC), and molecular functions (MF). In this study, we mapped the differentially displayed proteins (fold changes >1.5, P<0.05) into the GO database (http://www.geneontology.org/). It was computable for the amount of proteins at each GO term, and the target list used for result which came from label-free. The list was constructed by downloading the data on the GO database.

Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate protein pathway. Firstly, using KEGG online service tools KAAS to annotated protein’s KEGG database description. Then mapping the annotation result on the KEGG pathway database used KEGG online service tools KEGG mapper.

2.5 Fluorescence analysis

The total DNA was isolated from Xoo by TIANamp bacterial DNA kit. The concentration of DNA was determined by UV absorption at 260 nm using a molar absorption coefficient $\varepsilon_{260} = 6600$ L mol$^{-1}$ cm$^{-1}$ (expressed as molarity of phosphate groups) by Bouguer-Lambert-Beer law. The purity of DNA was checked by monitoring the ratio of the absorbance at 260 nm to that at 280 nm. The solution gave
a ratio of >1.8 for A\textsubscript{260}/A\textsubscript{280}, which indicated that DNA was sufficiently free from protein. Then compound 2f was added into a TRIS-HCl buffer solution (50 mM, pH = 7.4) containing different amounts of DNA. The final volume was fixed to 10 mL and the final concentration of compound 2f was 1×10\(^{-8}\) mol L\(^{-1}\). After incubating the solution for 20 min at room temperature, fluorescence spectra were recorded on F-7000 Spectrofluorimeter (Hitachi, Tokyo, Japan) equipped with 1.0 cm quartz cells, the widths of both the excitation and emission slit were fixed as 5 nm, and the excitation wavelength was 363 nm.

2.6 Scanning electron microscopy (SEM)

In this assay, 1.5 mL Xoo (or Xac or Psa) cells incubated at the logarithmic phase were centrifuged and washed with PBS (pH = 7.2), and re-suspended in 1.5 mL of PBS buffer (pH = 7.2). After that, bacteria Xoo (or Xac or Psa) were incubated with compound 2f at concentrations of 12.5 μg/mL, 25.0 μg/mL, and an equivalent volume of DMSO (solvent control) for 6 h at room temperature. After incubation, these samples were washed 3 times with PBS (pH = 7.2). Subsequently, the bacterial cells were fixed for 8 h at 4 °C with 2.5% glutaraldehyde, and then dehydrated with graded ethanol series and pure tert-butanol (2 times with 10 min/time). Following dehydration, samples were freezing dried and coated with gold, and visualized using Nova Nano SEM 450.

3. Results and Discussion

An isopropanolamine linker was elaborately investigated and will be integrated into the title compounds to efficiently fabricate a type of simple carbazole derivative,
which probably possesses adequate competence to inhibit the growth of plant pathogens; this linker has been extensively exploited as a versatile tool or bridge to construct pharmacological substrates, which displayed impressive biological activities in many fields.\textsuperscript{14,34} Therefore, a series of carbazole compounds bridging by this isopropanolamine linker was synthesized following Figure 2. Briefly, carbazole was treated with racemic epichlorohydrin under strong alkali conditions of KOH to give the key intermediate 1 bearing an epoxy tail, which was subsequently ring-opened by various substituted benzylamines to afford title molecules 2a–2k. All these racemic molecular structures were confirmed by \textsuperscript{1}H NMR, \textsuperscript{13}C NMR, and HRMS. For their antibacterial evaluation, the typical turbidimeter test was conducted, while the mainly used agricultural agents BT and TC were co-assayed as reference drugs. As indicated in Table 1, most of the designed compounds displayed significant antibacterial powers against the tested three bacterial strains. For anti-Xoo and anti-Xac activity, numerous compounds provided the corresponding EC_{50} values ranging from 1.39 to 4.30 µg/mL and 1.80 to 2.54 µg/mL except compounds 2d and 2g, which afforded relatively high EC_{50} values of 10.2, 35.4 µg/mL and 8.01, 30.5 µg/mL, respectively. For anti-Psa assays, these compounds also showed outstanding performances in suppressing the pathogen growth with EC_{50} values within 0.603 and 29.7 µg/mL apart from compounds 2d and 2i, which presented the negligible antibacterial ability with EC_{50} values exceeding 100 µg/mL. The above findings indicated that the design of carbazole-based compound bridging by the isopropanolamine linker could promote the discovery of highly efficient molecules probably applied in agriculture. The
structure–activity relationship (SAR) was elucidated according to the obtained EC\textsubscript{50} values and the effect of different substituents on the benzene ring. The electronic property, the type of halogens, the position, and steric hindrance of substituents clearly have a significant influence toward the bioactivity. Compared with unsubstituted compound \textit{2c} (EC\textsubscript{50} values of 2.76, 2.46, 3.94 µg/mL against \textit{Xoo}, \textit{Xac}, and \textit{Psa}, respectively), introducing a weak electron-donating group 4–CH\textsubscript{3} (\textit{2b}, 1.51, 1.80, and 3.73 µg/mL) or weak electron-withdrawing groups 4-Cl (\textit{2f}, 1.27, 1.96, and 3.83 µg/mL) or 4-F (\textit{2j}, 2.04, 2.42, and 0.603 µg/mL) would improve the bioactivity. Notably, the antibacterial effects on \textit{Psa} were increased by approximately two folds after introducing a strong electron-donating group (4-OCH\textsubscript{3}, \textit{2a}, 1.79 µg/mL) and were reduced by approximately two folds after placing a strong electron-withdrawing group (4-CF\textsubscript{3}, \textit{2k}, 9.24 µg/mL) on the benzene ring. Appreciably, the position of the substituents provided different tendencies toward the three strains. For anti-\textit{Xoo} activity, the order of activities followed para (R = 4-Cl or 4-F, \textit{2f} and \textit{2j}, 1.27 and 2.04 µg/mL) > ortho (R = 2-Cl or 2-F, \textit{2d} and \textit{2h}, 10.2 and 4.30 µg/mL). By contrast, different patterns were observed for the anti-\textit{Xac} activity, providing the results of para (R = 4-Cl, \textit{2f}, 1.96 µg/mL) > ortho (R = 2-Cl, \textit{2d}, 8.01 µg/mL). Additionally, the substituent located at the para-position afforded the most powerful potency against \textit{Psa} with the EC\textsubscript{50} value of 0.603 µg/mL (4-F, \textit{2j}). In another aspect, a remarkable observation indicated that the antibiotic action was dramatically decreased after introducing an additional steric group on the benzene ring, which was illustrated by comparing the bioactivity of compounds \textit{2f} (4-Cl, 1.27, 1.96, 3.83 µg/mL) and \textit{2g}}
(2,4-diCl, 35.4, 30.5, 29.7 µg/mL). From the above results, admirable antibacterial effects with the minimal EC₅₀ values of 1.27, 1.80, and 0.603 µg/mL against the corresponding strains of Xoo, Xac, and Psa were achieved.

Further modification from various aspects was conducted based on the preceding study to explore even superior bioactive molecules. First, influence of replacing the benzene ring into the heterocyclic scaffolds on the antibacterial effect was examined, and compounds 2l, 2m, and 2n bearing furan, thiofuran, or pyridine moieties were prepared. Although these compounds still performed strong inhibition powers against the three pathogens with EC₅₀ values ranging from 2.06 to 7.23 µg/mL, except for compound 2m against Psa (EC₅₀ value > 100 µg/mL), they could not afford a better antibacterial action. Second, substituted arylamines were employed to ring-open the epoxy motif instead of substituted benzylamines and resulted in the fabrication of title compounds 2o–2r. Even though compounds 2p and 2r provided a tolerable inhibition action toward Psa with the corresponding EC₅₀ values of 12.2 and 32.0 µg/mL, the antibacterial effects of these compounds were significantly blocked after removing the methylene group. This finding suggested that ring-opening the epoxy motif with a relatively flexible and relaxed amine would be beneficial to the bioactivity. A series of title compounds (2s–2x) bearing the saturated alkyl amine motifs were synthesized to verify this hypothesis and their bioactivities were tested. Notably, the EC₅₀ values of these compounds experienced three steps of variation tendencies: first, increasing to extend the alkyl chain length, suggesting that slightly raising the hydrophobicity of title compounds was disadvantageous to the activity; then being decreased after
introducing a long alkyl chain (for example, n-butyl, 2w, providing the minimal EC$_{50}$ values of 2.09, 1.90, and 4.84 µg/mL against $Xoo$, $Xac$, and $Psa$, respectively), indicating that the title molecule bearing a providential and adjustable lipophilic tail would elevate the antibacterial potency; finally, elevation along with the introduction of a sterically hindered group (tert-butyl, 2x, providing EC$_{50}$ values of 7.34, 12.5, and 5.55 µg/mL against $Xoo$, $Xac$, and $Psa$, respectively), indicating that a bulk group located at the molecular tail would block the additional interactions with target species. Based on the preceding modification and obtained bioassay results, even slight changes in the molecular patterns would significantly affect the final bioactivity. Therefore, molecular framework maps must be carefully and systematically optimized.

Given that compounds 2f, 2b, and 2j bearing the corresponding 4-chlorobenzyl, 4-methylbenzyl, and 4-fluorobenzyl groups exerted the best antibacterial potency against $Xoo$ (EC$_{50}$ = 1.27 µg/mL), $Xac$ (EC$_{50}$ = 1.80 µg/mL), and $Psa$ (EC$_{50}$ = 0.603 µg/mL), respectively, the substituents on the carbazole ring toward bioactivity were investigated. Therefore, compounds 4a–4h bearing 3,6-diBr, 3,6-ditert-butyl, or 3,6-diacetyl motifs were prepared (Figure 3) and biologically evaluated. As shown in Table 2, the anti-$Psa$ activity was significantly decreased after introducing the substituents on the carbazole ring and resulted in the EC$_{50}$ values exceeding 100 µg/mL, suggesting that the additional substituted groups could seriously block the interactions with $Psa$ target species. For anti-$Xoo$ and anti-$Xac$ activity, an increased pharmacological effect was observed via the introduction of the 3,6-diBr moiety (4c)
and provided the corresponding EC$_{50}$ values of 1.10 and 1.62 μg/mL. By contrast, an opposite pattern was observed from EC$_{50}$ values of compounds 4a (17.9 and $>$100 μg/mL) and 4b (17.2 and $>$100 μg/mL), indicating that an electron-donating group (-CH$_3$, 4c) on the benzene ring was beneficial to bioactivity in comparison to those weak electron withdrawing groups (4-Cl, 4a; 4-F, 4b). Notably, introducing a hydrophobic and bulk group (3,6-ditert-butyl, 4d-4f) led to a comparative activity toward Xoo and slightly improved efficiency toward Xac, respectively. By contrast, the introduction of 3,6-diacetyl motif (4g and 4h) significantly reduced the antibacterial potency, demonstrating that an electron-withdrawing group was unfavorable to the bioactivity. Given the preceding results, the antibacterial competence could be affected by various substituents on the carbazole ring, which provides a new template and guidance for the careful optimization of the molecular framework.

After evaluating the antibacterial effect of substituents on the carbazole ring, the absolutely chiral carbazole derivatives toward activity must be clarified. Therefore, 6a (R), 6b (S), 6c (R), and 6d (S) were synthesized by replacing the racemic epichlorohydrin into the corresponding chiral material (Figure 4). Bioassay results indicated that the fixed configuration had certain effects toward the antibacterial efficiency (Table 3). For the anti-Xoo activity, their EC$_{50}$ values presented the following order, $R$-forms (1.38 μg/mL, 6a; 0.902 μg/mL, 6c) $< R/S$-forms (1.47 μg/mL, 4d; 1.32 μg/mL, 4f) $< S$-forms (1.51 μg/mL, 6b; 1.43 μg/mL, 6d), suggesting that the $R$-forms of target compounds were beneficial to bioactivity. For anti-Xac
bioassay, a distinct pattern was provided as $R_1 = 4$-chlorobenzyl, and their $EC_{50}$ values followed the order of $R/S$-form (2.42 µg/mL, 4d) < $S$-form (3.11 µg/mL, 6b) < $R$-form (5.49 µg/mL, 6a). This finding indicates that the combination of $S$- and $R$-form can improve the antibacterial power. Based on the preceding investigation, the final bioactivity could be affected by the absolute configuration of the chiral center in the designed molecular structure.

In vivo studies were conducted to fight against rice bacterial leaf blight and estimate the prospective applications of this kind of compounds against plant bacterial diseases. In this study, compound 2f was selected due to its superior anti-$Xoo$ activity (1.27 µg/mL) and accessibility for synthesis. As shown in Table 4 and Figure 5, compound 2f exhibited good in vivo curative and protection activities toward bacterial leaf blight diseases with the corresponding control efficiency of 50.77% and 48.71% at 200 µg/mL, respectively, which were superior to those of BT (43.47% and 40.91%) and TC (42.60% and 39.17%). This outcome suggests that this type of molecular structure can be considered as a template for the future exploration and development of highly efficient alternatives.

A label-free quantitative proteomic analysis was conducted in response to the compound 2f stimulation to examine the underlying antibacterial mechanism of this type of designed compound toward $Xoo$. In this study, three biological replicates were conducted for each group (2f and CK) to ensure the reliability of the results (detected by relative standard deviation (RSD) analysis, Figure 6a) and the total proteins of samples were identified through label-free LC-MS/MS. The result showed that a total
of 2337 proteins were preliminary monitored for CK and 2f samples, among which 2095 proteins with quantitative information were identified (Table S1). Comparing the treatment (2f) and control (CK) groups, 1848 proteins (88.2%) presented common expression levels, while 247 proteins (100 up-regulated, 147 down-regulated; fold changes > 1.5, p < 0.05) were confirmed differentially expressed (Table S2, Figure 6b). A volcano plot map was also utilized to filter the significant differentially expressed proteins (Figure 6c). This outcome indicates that compound 2f could significantly provoke the pathogen to provide a physiological response against the external stimulus, and consequently produced a series of differentially expressed proteins.

These differentially expressed proteins were analyzed for GO categories in biological process (BP), molecular function (MF), and cellular component (CC) to preliminarily clarify their biological functions. According to their BP (Figure 7A), a large proportion of these proteins were clearly involved in the metabolic process, localization, cellular process, and single-organism process. Figure 7B showed that a large quantity of these differentially expressed proteins was involved in catalytic activity, binding transporter activity on the basis of their MF. Meanwhile, CC analysis showed that most of these proteins were found in membrane and cell (Figure 7C). Moreover, the subcellular localization chart revealed that most of these differentially expressed proteins were mainly found in the cytoplasmic (Figure Sa). Clusters of Orthologous Groups (COG) chart was exploited to further expound the functional classification of these unique proteins. As indicated in Figure Sb, 21 representative
functions, such as “Energy production and conversion,” “Amino acid transport and metabolism,” “Carbohydrate transport and metabolism,” “Lipid transport and metabolism,” “Cell wall/membrane/envelope biogenesis,” and “General function prediction only”, were predicted. This observation suggests that a variety of physiological processes could be dramatically influenced and triggered by the stimulation from compound 2f.

The KEGG pathway enrichment bubble plot chart (Figure 5c) was presented to determine the receivable action pathways containing multiple differentially expressed proteins induced by compound 2f. Two main metabolic pathways containing biotin metabolism pathway and starch and sucrose metabolism pathway involving additional differentially expressed proteins were locked. The two pathways are known to play one of the key approaches in the biosynthesis of biotin and D-glucose, which are normally participants in a variety of physiological processes including more than the metabolisms of fat, protein, and energy. The disturbance or blockage of the access will result in serious problems, such as delayed growth, energy deficiency, and biochemical disorder. In the biotin metabolism pathway (Figure 8), the harmonious cooperation of these proteins is essential for the normal operation. However, an array of down-regulated proteins was observed in this pathway. For example, beta-ketoacyl-synthase I (Fab B) and 3-oxoacyl-(Acyl-carrier-protein) reductase (Fab G) are the main enzymes performing significant roles in the biosynthesis of fatty acid; by contrast, the expression quantity of biotin synthase (2.8.1.6), ATP-dependent dethiobiotin synthetase BioD (6.3.3.3), and
8-amino-7-oxononanoate synthase (2.3.1.47) would further influence the synthesis of biotin. Simultaneously, in the starch and sucrose metabolism pathway, the evidently decreased expression of beta-glucosidase (3.2.1.21) might directly lead to the reduced conversion of GDP-glucose into D-glucose, which would disrupt the normal energy metabolism of bacteria and consequently resulted in the failure of normal life activities of bacteria. By contrast, the up-regulated proteins, including endoglucanase (3.2.1.4), trehalose-6-phosphate synthase (2.4.1.15), and trehalose 6-phosphate phosphatase (3.1.3.12), would cause the imbalance of these key metabolic intermediates, further breaking the normal pathway metabolism. This outcome suggested that compound 2f might have the privileged competence to induce the expression of differential proteins affecting the crucial metabolic pathways, and consequently resulted in the disorder of normal physiological processes and final bacteria death.

Protein–protein interaction networks were presented using STRING database version 10.5 to further study and assign the function and association of these differentially expressed proteins. As indicated in Figure 9, 12 functions, including “Fatty acid biosynthesis,” “Translation ribosomal structure and biogenesis,” “Lipid transport and metabolism,” “RNA processing and modification,” “Ion transport and metabolism,” “Amino acid transport and metabolism,” “Phosphotransferase system,” “Nucleotide transport and metabolism,” “Cytochrome oxidoreductase,” “Oxidoreductase,” “Biotin metabolism,” and “Starch and sucrose metabolism” were enriched. This finding further suggested that various physiological processes could be
significantly affected by compound 2f stimulation and consequently led to the disturbance and final bacteria death.

PRM technique was performed to further validate the label-free quantitative proteomics result, because it is another widely used and efficient method which can precisely quantify and verify an array of target proteins of interest.\textsuperscript{50-52} In this study, twenty significant differentially produced proteins involved in these GO terms and pathways were selected for PRM analysis (Table S3). Moreover, two unique peptides with anticipated chemical stability were chosen for each protein, and the relative protein abundance was expressed as the average of the two normalized peptide peak areas (Table S4). The PRM result was shown in Figure 10 and afforded the same trends with the label-free quantitative proteomics data.

The natural carbazole scaffold is known to normally possess the privileged ability to interact with DNA, which results in changes in fluorescence intensity.\textsuperscript{13,34} Fluorescence spectrometry was used to monitor the interactions between the total DNA extracted from pathogen Xoo and compound 2f. Primarily, time dependent fluorescent intensity of compound 2f in the TRIS-buffer revealed that the fluorescence was constant after 20 min (Figure 11a). After the addition of an equivalent DNA, the fluorescent intensity was first decreased, and then became constant for 20 min incubation (Figure 11b). This outcome might be attributed to the encapsulation of the carbazole moiety into the grooves of DNA helical structures, and consequently reduced the total amount of carbazoles to produce fluorescence. A gradually decreased fluorescent intensity for compound 2f with different dosages of DNA.
further confirmed that the designed compounds bearing carbazole patterns had strong interactions with the DNA of pathogens (Figure 11c), which was in accordance with the observation of differentially expressed proteins.

The SEM images of Xoo, Xac, and Psa were presented to study the morphology changes after being subjected to different dosages of compound 2f. As indicated in Figure 12, the shapes of these pathogens were changed from well-proportioned (Figures 12a, 12d, and 12g) to partially corrugated or broken (Figures 12b, 12e, and 12h) after treatment with compound 2f at the dosage of 12.5 µg/mL. Furthermore, nearly all these pathogens were destroyed when the drug concentration was elevated to 25 µg/mL and consequently afforded the observation of various debris and large leakage holes on the bacteria surfaces (Figures 12c, 12f, and 12i). This result further confirmed that the designed compounds performed strong interactions with the target species of bacteria, which subsequently led to a series of physiological process changes and final bacteria death.

In summary, a type of simple racemic and chiral carbazole derivative possessing an isopropanolamine pattern was systematically synthesized and evaluated their antibacterial activities. Bioassay results showed that compounds 2f, 6c, and 2j could evidently inhibit the growth of plant pathogens Xoo, Xac and Psa with the corresponding EC$_{50}$ values of 1.27, 0.993, and 0.603 µg/mL, which were extremely superior to those of BT (92.6 µg/mL) and TC (121.8, 77.0, and 87.0 µg/mL). Moreover, the SAR analysis indicated that the electronic property, the type of halogens, the position, steric hindrance of substituents, and molecular absolute
configuration had a dramatic influence toward the bioactivity. *In vivo* study confirmed the prospective application for developing these compounds as agricultural agents against plant bacterial diseases. Quantitative proteomics analysis indicated that compound 2f could induce a total of 247 proteins (100 up-regulated, 147 down-regulated) differentially expressed comparing to the negative control, which was further validated by the PRM result. This occurrence significantly affected various physiological processes of pathogens and eventually resulted in their morphological variations from SEM images. Moreover, fluorescence spectra suggested that this kind of compounds had strong interactions with the DNA of pathogens. Considering their privileged features and multi-interactions with the target species of tested pathogens, these versatile carbazole derivatives could be considered as a suitable template for exploring highly efficient antibacterial surrogates against plant bacterial infections and disabling the bacterial resistance.

**Acknowledgment**

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**Supporting Information**

Supplementary data including experimental procedures for PRM, partial racemic or chiral intermediates and target compounds were analyzed by HPLC (Figures S1-S10), Figures Sa-Sc, Tables S1-S5, synthetic procedures for the intermediates and target
compounds and $^1$H NMR, $^{13}$C NMR, and HRMS spectra of target compounds associated with this article can be found, in the online version, at https://pubs.acs.org/journal/jafcau.

**Conflict of interest**

The authors declare no competing financial interest.


(37) Surineni, G.; Yogeeswari, P.; Sriram, D.; Kantevari, S. Design and synthesis of novel carbazole tethered pyrrole derivatives as potent inhibitors of


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Figure captions

**Figure 1.** a) Some natural antimicrobial structures containing carbazole moieties; b) Design strategy for the target molecules.

**Figure 2.** Synthetic route for the target molecules 2a–2x.

**Figure 3.** Synthetic route for the target molecules 4a–4h.

**Figure 4.** Synthetic route for the target molecules 6a–6d.

**Figure 5.** Curative and protection activities of compound 2f against rice bacterial leaf blight under greenhouse conditions at 200 μg/mL; BT and TC were the positive controls at the same conditions.

**Figure 6.** a) Box plot of RSD distribution of repeated samples (three repetitions) using quantified proteins; b) Venn diagrams for proteome comparison on control and treatment groups. The blue part is differentially expressed down-regulated proteins, the brown part is up-regulated proteins, and the center is unchanged; c) Volcano plot of the relative protein abundance changes between control and treatment groups. The red and green points are significantly up- and down-regulated proteins, respectively.

**Figure 7.** Differential expression proteins between control and treatment groups were classified based on known biological process (A), molecular functions (B), and cellular components (C).

**Figure 8.** Starch and sucrose metabolism and biotin metabolism signaling pathways in response to compound 2f. Red color represents up-accumulated genes or proteins in this pathway, while the blue color represents down accumulation.

**Figure 9.** Protein-protein interaction networks for differentially expressed proteins.
The circles and different colors in the figure represent the differentially expressed proteins (blue and red respectively represent the down- and the up-regulated proteins).

**Figure 10.** Confirmation of 20 selected differentially produced proteins detected by PRM technique.

**Figure 11.** Fluorescence spectra for a) time-dependent fluorescent intensity of compound 2f (1 × 10⁻⁸ M) in TRIS-buffer (50 nM, pH = 7.4); b) time-dependent fluorescent intensity of compound 2f after adding a 1eq. DNA in TRIS-buffer; c) concentration-dependent fluorescent intensity of compound 2f with different dosages of DNA.

**Figure 12.** SEM images for Xoo, Xac and Psa after being incubated with different concentrations of compound 2f, Xoo images for (a) 0 µg/mL, (b) 12.5 µg/mL, and (c) 25.0 µg/mL; Xac images for (d) 0 µg/mL, (e) 12.5 µg/mL, and (f) 25.0 µg/mL; Psa images for (g) 0 µg/mL, (h) 12.5 µg/mL, and (i) 25 µg/mL. Scale bars are 1 µm.
Table 1. Antibacterial activities of target compounds 2a-2x against plant pathogens

**Xoo, Xac, and Psa in vitro.**

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Regression equation</th>
<th>EC$_{50}$ (µg/mL)</th>
<th>Regression equation</th>
<th>EC$_{50}$ (µg/mL)</th>
<th>Regression equation</th>
<th>EC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>y = 3.650x + 3.357</td>
<td>2.82 ± 0.09</td>
<td>y = 2.883x + 4.136</td>
<td>1.99 ± 0.03</td>
<td>y = 1.019x + 4.742</td>
<td>1.79 ± 0.35</td>
</tr>
<tr>
<td>2b</td>
<td>y = 5.573x + 4.000</td>
<td>1.51 ± 0.03</td>
<td>y = 4.085x + 3.958</td>
<td>1.80 ± 0.04</td>
<td>y = 3.919x + 2.759</td>
<td>3.73 ± 0.09</td>
</tr>
<tr>
<td>2c</td>
<td>y = 8.939x + 1.059</td>
<td>2.76 ± 0.02</td>
<td>y = 2.214x + 4.133</td>
<td>2.46 ± 0.09</td>
<td>y = 1.804x + 3.925</td>
<td>3.94 ± 0.07</td>
</tr>
<tr>
<td>2d</td>
<td>y = 1.482x + 3.506</td>
<td>10.2 ± 0.5</td>
<td>y = 0.720x + 4.349</td>
<td>8.01 ± 0.63</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2e</td>
<td>y = 8.837x + 3.747</td>
<td>1.39 ± 0.01</td>
<td>y = 2.353x + 4.382</td>
<td>1.83 ± 0.05</td>
<td>y = 4.619x + 1.129</td>
<td>6.88 ± 0.04</td>
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<tr>
<td>2f</td>
<td>y = 14.74x + 3.46</td>
<td>1.27 ± 0.01</td>
<td>y = 4.418x + 3.713</td>
<td>1.96 ± 0.03</td>
<td>y = 4.461x + 2.400</td>
<td>3.83 ± 0.05</td>
</tr>
<tr>
<td>2g</td>
<td>y = 4.149x - 1.428</td>
<td>35.4 ± 0.5</td>
<td>y = 4.581x - 1.801</td>
<td>30.5 ± 1.2</td>
<td>y = 2.065x + 1.959</td>
<td>29.7 ± 1.7</td>
</tr>
<tr>
<td>2h</td>
<td>y = 5.985x + 1.209</td>
<td>4.30 ± 0.02</td>
<td>y = 1.450x + 4.610</td>
<td>1.86 ± 0.05</td>
<td>y = 1.229x + 3.961</td>
<td>7.01 ± 0.38</td>
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<tr>
<td>2i</td>
<td>y = 11.73x + 1.346</td>
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<td>y = 1.343x + 4.652</td>
<td>1.81 ± 0.15</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2j</td>
<td>y = 3.763x + 3.834</td>
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<td>y = 2.006x + 4.229</td>
<td>2.42 ± 0.05</td>
<td>y = 0.388x + 5.085</td>
<td>0.603 ± 0.292</td>
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<td>2k</td>
<td>y = 10.16x + 1.481</td>
<td>2.22 ± 0.04</td>
<td>y = 2.311x + 4.063</td>
<td>2.54 ± 0.18</td>
<td>y = 2.479x + 2.607</td>
<td>9.24 ± 0.06</td>
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<td>2l</td>
<td>y = 12.80x - 5.574</td>
<td>6.70 ± 0.04</td>
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<td>2.08 ± 0.12</td>
<td>y = 1.129x + 4.359</td>
<td>3.70 ± 0.21</td>
</tr>
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<td>2m</td>
<td>y = 3.544x + 2.492</td>
<td>5.10 ± 0.12</td>
<td>y = 0.850x + 4.475</td>
<td>4.15 ± 0.18</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2n</td>
<td>y = 4.058x + 3.727</td>
<td>2.06 ± 0.08</td>
<td>y = 2.326x + 3.224</td>
<td>5.80 ± 0.14</td>
<td>y = 0.492x + 4.577</td>
<td>7.23 ± 0.78</td>
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<tr>
<td>2o</td>
<td>/</td>
<td>&gt;100</td>
<td>/</td>
<td>&gt;100</td>
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<td>&gt;100</td>
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<tr>
<td>2p</td>
<td>/</td>
<td>&gt;100</td>
<td>/</td>
<td>&gt;100</td>
<td>y = 1.493x + 3.378</td>
<td>12.2 ± 0.1</td>
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<tr>
<td>2q</td>
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<td>&gt;100</td>
<td>/</td>
<td>&gt;100</td>
<td>/</td>
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<tr>
<td>2r</td>
<td>/</td>
<td>&gt;100</td>
<td>/</td>
<td>&gt;100</td>
<td>y = 0.660x + 4.007</td>
<td>32.0 ± 1.3</td>
</tr>
<tr>
<td>2s</td>
<td>y = 3.345x + 1.494</td>
<td>11.2 ± 0.4</td>
<td>y = 1.609x + 3.458</td>
<td>9.08 ± 0.38</td>
<td>y = 1.211x + 4.094</td>
<td>5.60 ± 0.20</td>
</tr>
<tr>
<td>2t</td>
<td>y = 7.091x - 4.347</td>
<td>20.8 ± 0.5</td>
<td>y = 1.661x + 3.828</td>
<td>5.07 ± 0.33</td>
<td>y = 2.242x + 2.058</td>
<td>20.5 ± 1.0</td>
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<td>2u</td>
<td>y = 10.83x - 9.844</td>
<td>23.5 ± 0.4</td>
<td>y = 2.311x + 2.672</td>
<td>10.2 ± 0.8</td>
<td>y = 1.265x + 3.340</td>
<td>20.5 ± 2.6</td>
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<td>2v</td>
<td>y = 5.837x + 1.748</td>
<td>14.3 ± 0.1</td>
<td>y = 1.117x + 4.178</td>
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<td>5.14 ± 0.02</td>
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<td>2w</td>
<td>y = 3.345x + 3.931</td>
<td>2.09 ± 0.04</td>
<td>y = 2.220x + 4.382</td>
<td>1.90 ± 0.06</td>
<td>y = 0.696x + 4.523</td>
<td>4.84 ± 0.22</td>
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<tr>
<td>2x</td>
<td>y = 9.773x + 3.458</td>
<td>7.34 ± 0.13</td>
<td>y = 0.763x + 4.163</td>
<td>12.5 ± 2.04</td>
<td>y = 0.867x + 4.355</td>
<td>5.55 ± 0.53</td>
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<tr>
<td>BT</td>
<td>y = 1.499x + 2.052</td>
<td>92.6 ± 2.1</td>
<td>/</td>
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<td>y = 4.913x - 4.246</td>
<td>111.2 ± 4.9</td>
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<tr>
<td>TC</td>
<td>y = 1.540x + 1.788</td>
<td>121.8 ± 3.6</td>
<td>y = 2.153x + 0.938</td>
<td>77.0 ± 2.0</td>
<td>y = 5.669x - 5.994</td>
<td>87.0 ± 2.1</td>
</tr>
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</table>
Table 2. Antibacterial activities of target compounds 4a–4h against plant pathogens *Xoo, Xac, and Psa in vitro.*

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Regression equation</th>
<th>EC₅₀ (µg/mL)</th>
<th>Regression equation</th>
<th>EC₅₀ (µg/mL)</th>
<th>Regression equation</th>
<th>EC₅₀ (µg/mL)</th>
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</thead>
<tbody>
<tr>
<td>4a</td>
<td>y = 1.186x + 3.513</td>
<td>17.9 ± 0.3</td>
<td>/</td>
<td>&gt;100</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4b</td>
<td>y = 3.208x + 0.990</td>
<td>17.2 ± 0.4</td>
<td>/</td>
<td>&gt;100</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4c</td>
<td>y = 9.455x + 4.627</td>
<td>1.10 ± 0.01</td>
<td>y = 4.378x + 4.085</td>
<td>1.62 ± 0.06</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4d</td>
<td>y = 9.696x + 3.354</td>
<td>1.47 ± 0.02</td>
<td>y = 5.977x + 2.709</td>
<td>2.42 ± 0.01</td>
<td>/</td>
<td>&gt;100</td>
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<tr>
<td>4e</td>
<td>y = 15.14x + 0.579</td>
<td>1.96 ± 0.01</td>
<td>y = 3.776x + 4.482</td>
<td>1.37 ± 0.05</td>
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<tr>
<td>4f</td>
<td>y = 10.13x + 3.772</td>
<td>1.32 ± 0.02</td>
<td>y = 5.150x + 4.115</td>
<td>1.49 ± 0.01</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4g</td>
<td>y = 3.340x - 0.634</td>
<td>20.3 ± 0.4</td>
<td>y = 3.746x + 0.188</td>
<td>24.3 ± 0.8</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4h</td>
<td>y = 4.202x - 0.242</td>
<td>17.7 ± 0.4</td>
<td>y = 5.877x + 1.018</td>
<td>10.6 ± 0.3</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>BT</td>
<td>y = 1.499x + 2.052</td>
<td>92.6 ± 2.1</td>
<td>/</td>
<td>/</td>
<td>y = 4.913x - 4.246</td>
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<tr>
<td>TC</td>
<td>y = 1.540x + 1.788</td>
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<td>77.0 ± 2.0</td>
<td>y = 5.669x - 5.994</td>
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</tbody>
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Table 3. Antibacterial activities of target compounds 6a–6d against plant pathogens *Xoo*, *Xac*, and *Psa* in vitro.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Regression equation</th>
<th>EC$_{50}$ (µg/mL)</th>
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<th>Regression equation</th>
<th>EC$_{50}$ (µg/mL)</th>
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</thead>
<tbody>
<tr>
<td>6a (R)</td>
<td>$y = 17.59x + 2.52$</td>
<td>1.38 ± 0.03</td>
<td>$y = 1.775x + 3.687$</td>
<td>5.49 ± 0.08</td>
<td>/</td>
<td>&gt;100</td>
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<tr>
<td>6b (S)</td>
<td>$y = 8.457x + 3.475$</td>
<td>1.51 ± 0.02</td>
<td>$y = 2.939x + 3.552$</td>
<td>3.11 ± 0.11</td>
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<tr>
<td>4d (R/S)</td>
<td>$y = 9.696x + 3.354$</td>
<td>1.47 ± 0.02</td>
<td>$y = 5.977x + 2.709$</td>
<td>2.42 ± 0.01</td>
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<td>&gt;100</td>
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<tr>
<td>6c (R)</td>
<td>$y = 10.81x + 5.49$</td>
<td>0.902±0.021</td>
<td>$y = 6.405x + 5.040$</td>
<td>0.993±0.012</td>
<td>/</td>
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<td>6d (S)</td>
<td>$y = 4.133x + 4.358$</td>
<td>1.43 ± 0.04</td>
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<td>1.16 ± 0.02</td>
<td>/</td>
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<td>4f (R/S)</td>
<td>$y = 10.13x + 3.77$</td>
<td>1.32 ± 0.02</td>
<td>$y = 5.150x + 4.115$</td>
<td>1.49 ± 0.01</td>
<td>/</td>
<td>&gt;100</td>
</tr>
<tr>
<td>BT</td>
<td>$y = 1.499x + 2.052$</td>
<td>92.6 ± 2.1</td>
<td>/</td>
<td>/</td>
<td>y = 4.913x-4.246</td>
<td>111.2 ± 4.9</td>
</tr>
<tr>
<td>TC</td>
<td>$y = 1.540x + 1.788$</td>
<td>121.8 ± 3.6</td>
<td>y = 2.153x + 0.938</td>
<td>77.0 ± 2.0</td>
<td>y = 5.669x-5.994</td>
<td>87.0 ± 2.1</td>
</tr>
</tbody>
</table>
Table 4. Curative and protection activities of compound 2f against rice bacterial leaf blight under greenhouse conditions at 200 μg/mL *in vitro*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Curative activity (14 days after spraying)</th>
<th>Protection activity (14 days after spraying)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morbidity (%)</td>
<td>Disease index (%)</td>
</tr>
<tr>
<td>2f</td>
<td>100</td>
<td>45.32</td>
</tr>
<tr>
<td>BT</td>
<td>100</td>
<td>52.04</td>
</tr>
<tr>
<td>TC</td>
<td>100</td>
<td>51.02</td>
</tr>
<tr>
<td>CK*</td>
<td>100</td>
<td>92.06</td>
</tr>
</tbody>
</table>

*Negative control. * Statistical analysis was conducted by ANOVA method under the condition of equal variances assumed (P > 0.05) and equal variances not assumed (P < 0.05). Different uppercase letters indicate the values of protection activity with significant difference among different treatment groups at P < 0.05.
Figures

Figure 1

(a) Bioactive scaffolds

(b) Bridging by isopropanolamine linker

Investigate substituent groups

Investigate chiral configuration
Figure 2

\[
\begin{align*}
\text{KOH, DMF, 0°C} & \quad \text{Isopropanol, K}_2\text{CO}_3, 60°C \\
\end{align*}
\]

\[R_1 = \begin{array}{c}
\text{H}_3\text{C} \\
\text{H}_3\text{C} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\text{F} \\
\text{F} \\
\text{F} \\
\text{F} \\
\text{Cl} \\
\text{Cl} \\
\text{OCH}_3 \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
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\text{Cl} \\
\text{Cl} \\
\end{array}
\]
Figure 3

4a: $R_1 = 4$-chlorobenzyl, $R_2 = 3,6$-diBr
4b: $R_1 = 4$-fluorobenzyl, $R_2 = 3,6$-diBr
4c: $R_1 = 4$-methylbenzyl, $R_2 = 3,6$-diBr
4d: $R_1 = 4$-chlorobenzyl, $R_2 = 3,6$-diter-butyl
4e: $R_1 = 4$-fluorobenzyl, $R_2 = 3,6$-diter-butyl
4f: $R_1 = 4$-methylbenzyl, $R_2 = 3,6$-diter-butyl
4g: $R_1 = 4$-chlorobenzyl, $R_2 = 3,6$-diacetyl
4h: $R_1 = 4$-methylbenzyl, $R_2 = 3,6$-diacetyl
Figure 4

6a: $R_1 = 4$-chlorobenzyl, $R_2 = 3,6$-diter-butyl
6b: $R_1 = 4$-chlorobenzyl, $R_2 = 3,6$-diter-butyl
6c: $R_1 = 4$-methylbenzyl, $R_2 = 3,6$-diter-butyl
6d: $R_1 = 4$-methylbenzyl, $R_2 = 3,6$-diter-butyl
Figure 5
Figure 6
Figure 7

A Biological Process
- other signal response to stimulus cellular component organization or biogenesis biological regulation multi-organism process single-organism process cellular process localization metabolic process

B Molecular Function
- other signal transducer activity molecular transducer activity transporter activity binding catalytic activity

C Cellular Component
- organelle macromolecular complex cell membrane
Figure 8

Starch and sucrose metabolism

Cytoplasm

UDP-glucose
Cellulose
p-D-glucoside
Cellodextrin
Cellulobiose
Trehalose
D-Glucose

Amino acid ion

ATP

TCA

Biotin metabolism

NAD^+
NADPH+H^+

FabB
FabG
FabA
FabZ

H_2O

NADP^+

Cell death

Biotin
Dethiobiotin

7,8-Diaminocinnamate

Pimeloyl-ACP
Pimeloyl-CoA
Pimelate

ACS Paragon Plus Environment
Figure 9

- Up-regulated proteins
- Down-regulated proteins

Categories:
- Fatty acid biosynthesis
- Biotin metabolism
- Lipid transport and metabolism
- Ion transport and metabolism
- RNA processing and modification
- Starch and sucrose metabolism
- Amino acid transport and metabolism
- Phosphotransferase system
- Nucleotide transport and metabolism
- Cytochrome oxidoreductase
- Translation ribosomal structure and biogenesis

ACS Paragon Plus Environment
Figure 10

Fold Change of Protein Level

Label free vs PRM
Figure 11
Figure 12