JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Agricultural and Environmental Chemistry

Sulfone-based probes unraveled dihydrolipoamide S-succinyltransferase as an unprecedented target in phytopathogens

Biao Chen, Qingsu Long, Yong-Liang Zhao, Yuanyuan Wu, Shasha Ge, Peiyi Wang, Cai-Guang Yang, Yonggui Robin Chi, Baoan Song, and Song Yang

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.9b02059 • Publication Date (Web): 31 May 2019

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Sulfone-based probes unraveled dihydrolipoamide S-succinyltransferase as an unprecedented target in phytopathogens

Biao Chen,[†] Qingsu Long,[†] Yongliang Zhao,[†] Yuanyuan Wu,[†] Shasha Ge,[†] Peiyi Wang,[†] Cai-Guang Yang,[§] Yonggui Chi,[†] Baoan Song,[†,*] and Song Yang[†,‡,*]

[†]Laboratory Breeding Base of Green Pesticide and Agricultural Bioengineering, Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University, Huaxi District, Guiyang 550025, China.

[§]Laboratory of Chemical Biology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China.

[‡]College of Pharmacy, East China University of Science & Technology, Shanghai 200237, China.

[*]Corresponding author

*E-mail: jhzx.msm@gmail.com (S.Y.); basong@gzu.edu.cn (B.S.).

Abstract

Target validation of current drugs remains the major challenge for target-based drug discovery, especially for the agrochemical discovery. The bactericide **0** represents a novel lead structure and has showed potent efficacy against those diseases that are extremely difficult to control, such as rice bacterial leaf blight. However, no detailed target analysis of this bactericide has been reported. Here we developed a panel of **0**-derived probes **1**–**6**, in which a conservative modification (alkyne tag) was introduced to keep the antibacterial activity of **0** and provide functionality for target identification via click chemistry. With these cell-permeable probes, we were able to discover dihydrolipoamide S-succinyltransferase (DLST) as an unprecedented target in living cells. The probes showed a good preference for DLST, especially probe **1**, which demonstrated distinct selectivity and reactivity. Also, we reported **0** as the first covalent DLST inhibitor, which has been used to confirm the involvement of DLST in the regulation of energy production.

Keywords

Bactericide **0**; Target; DLST; Covalent inhibitor; Energy production

1. Introduction

Since the late 19th century, crop protection chemistry has become a high-tech science in supporting the sustainable production of food from the available farmland.[1] Advancements in the discovery of highly effective agrochemicals will be required to tackle the challenge of increasing food production. Generally, the discovery of a commercialized agrochemical will take about 10 years and a cost of approximately U.S. $286 million and a screening of above 150,000 compounds.[2] An important step that accelerates the agrochemical discovery process is to enlarge the number of druggable targets as well as to deeply understand the molecular and cellular mechanism of action of current agrochemicals. With a known target, multiple techniques such as crystallography, computational modeling, biochemistry and binding kinetics can be used to analyze the target interaction with drugs, enabling efficient target-based drug design.[3] However, target validation of current drugs remains the major challenge for target-based drug discovery, especially for the agrochemical discovery. The definite mechanism of most agrochemicals are unclear, which results in so few targets that can be used for target-based molecular design.

Bacterial plant pathogens, especially the top 10,[4] severely infect a number of economically important crops and seriously reduce the crop yield. The bacterial outbreaks are generally difficult to control due to lack of effective bactericides and the increasing resistance problem, thereby emphasizing an urgent demand for the discovery of new antibacterial agents. The sulfone reagents represent promising biologically active privileged structures that can react covalently with the active sites

of certain proteins.[5-7] Among them, the drug candidate **0** (jiahuangxianjunzuo, in registration stage) and its analogs have been reported to show potent efficacy against those diseases[8-12] that are extremely difficult to control, such as *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Xanthomonas axonopodis* pv. *citri* (*Xac*). However, no detailed target analysis of this bactericide has been conducted so far in living systems.

Recently, an unbiased chemical proteomic strategy termed "activity-based protein profiling" (ABPP)[13-15] has been used as a powerful method for the target deconvolution of bioactive molecules. ABPP used activity-site directed probes to profile the function state of enzymes directly in native biological systems, which has been developed for multiple enzyme classes, such as cysteine proteases, serine hydrolases, phosphatases and kinases.[16] In order to investigate the interactions between **0** and its protein targets in bacteria, we designed a series of alkyne-functionalized probes **1**–**6** (Figure 1A) and systematically evaluated their reactivity and selectivity directly in living bacterial cells. As expected, minor change in a common structure resulted in a selective labeling event of ~45 kDa protein. All probes containing an alkyne handle were used for the modification with a reporter group (e.g., biotin) via the 1,3-dipolar Huisgen cycloaddition (click chemistry, CC) after cell penetration and lysis.[17,18] Labeled proteins are separated by SDS gels, visualized by streptavidin blot, and subsequently identified by mass spectrometry (Figure 1B).[19]

2. Materials and methods

2.1 Instruments and Chemicals

^{1}H and ^{13}C NMR spectral analysis were performed on a JEOL-ECX 500 NMR spectrometer using $CDCl_3$ or DMSO-d_6 as the solvent and TMS as an internal standard. High resolution mass spectra (HRMS) was conducted on an LTQ Orbitrap (Thermo Scientific). Imaging analysis was conducted on the the ChemiDoc XRS+ System (Bio-Rad). All reagents from Chinese Chemical Reagent Company were reagent-grade and used without further purification.

2.2 General Synthetic Procedures for the Intermediates 1−6

The intermediates **1−4** were prepared according to previously reported methods.[8] Thioether derivatives **4** were prepared by esterification, hydrazidation, cyclization and thioetherification reactions. To a solution of **4** (0.03 mol) in 50 mL CCl_4 was added N-bromosuccinimide (0.036 mol) and a catalytic amount of azobisisobutyronitrile (AIBN, 0.003 mol). The mixture was refluxed for 48 h and then the CCl_4 was evaporated to obtain the crude product, which was further washed with saturated aqueous $NaHCO_3$ and dried over Na_2SO_4. The organic layer was evaporated and the residue was purified by silica gel column chromatography to obtain the intermediates **5a−5f** with yields of 43−77%. To a solution of intermediate **5** (5 mmol) and propargyl alcohol (5.1 mmol) in 20 mL dried DMF, 60% sodium hydride (6 mmol) was added. The reaction was stirred for 2 h under the ice bath. The mixture was quenched by adding saturated NH_4Cl (aq) and extracted by ethyl acetate. The organic layer was washed with saturated NH_4Cl for two times and dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography to obtain the intermediates **6a−6f** with yields of

43−66%.

2.3 General Synthetic Procedures for the probes 1−6

To a stirred solution of intermediate **6** (5 mmol) in 15 mL CH_2Cl_2 was added m-CPBA (15 mmol). The reaction was stirred for 2 h and the mixture was filtered. After evaporation, the obtained crude product was purified by silica gel column chromatography with ethyl acetate/petroleum ether (v/v = 1:6) to give the pure probes **1**–**6** with yields of 40−70%. The representative data for probe **1** are presented below.

2-(methylsulfonyl)-5-(4-fluoro-3-((prop-2-yn-1-yloxy)methyl)phenyl)-1,3,4-oxadiazole **(**Probe **1).** Yield 70%; white solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (dd, *J* = 6.6, 2.2 Hz, 1H, Ph-6-H), 8.10 (m, 1H, Ph-2-H), 7.25 (t, *J* = 8.9 Hz, 1H, Ph-5-H), 4.74 (s, 2H, Ph-CH_2O-), 4.30 (d, *J* = 2.4 Hz, 2H, -OCH_2-C≡CH), 3.53 (s, 3H, -SO_2CH_3), 2.52 (t, *J* = 2.4 Hz, 1H, -C≡CH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.8, 164.4, 162.4, 162.2, 129.8, 129.5, 127.1, 118.5, 116.9, 78.9, 75.5, 64.5, 58.2, 43.0; HRMS (ESI) calculated for $C_{13}H_{11}FN_2O_4S$ $[M+H]^+$ m/z 311.0496, found 311.0493.

2.4 General Synthetic Procedures for negative probe (NP)

Sodium hydroxide (120 mg, 3 mmol) dissolved in 5 mL distilled water was added to a solution of probe **1** (465 mg, 1.5 mmol) in 5 mL THF. The reaction was stirred at RT for 2 h. The mixture was acidified with dilute HCl to pH 4−5 and extracted with 20 mL CH_2Cl_2 for two times. The organic layer was evaporated and the crude product was purified by silica gel column chromatography with ethyl acetate/petroleum ether (v/v = 1:6) to give the pure NP.

5-(4-fluoro-3-((prop-2-yn-1-yloxy)methyl)phenyl)-1,3,4-oxadiazol-2-ol (NP).

Yield 85%; white solid; ^{1}H NMR (500 MHz, DMSO-D_6) δ 12.60 (s, 1H, -OH), 7.84 (d, *J* = 6.8 Hz, 1H, Ph-6-H), 7.79 (m, 1H, Ph-2-H), 7.39 (t, *J* = 8.8 Hz, 1H, Ph-5-H), 4.64 (s, 2H, Ph-CH_2O-), 4.26 (d, *J* = 2.4 Hz, 2H, -OCH_2-C≡CH), 3.51 (t, *J* = 2.4 Hz, 1H, -C≡CH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.6, 161.5, 154.7, 154.6, 128.0, 127.5, 126.3, 120.2, 116.4, 79.0, 75.3, 64.6, 58.0; HRMS (ESI) calculated for $C_{12}H_9N_2O_3F$ $[M-H]^-$ m/z 247.0513, found 247.0523.

2.5 *In Vitro* Antibacterial Bioassay

In this study, the antibacterial activities of target probes against *Xoo* and *Xac* were screened by a turbidimeter test. About 40 μL of liquid nutrient broth (1 g of yeast powder, 3 g of beef extract, 10 g of glucose, 5 g of peptone, and 1 L of distilled water, pH 7.0−7.2) containing *Xoo* or *Xac* was added to 5 mL of nutrient broth (NB) media containing the test compounds as experimental groups and DMSO as a blank control. The inoculated test tubes were incubated at 28 ± 1 °C with a constant shake at 180 rpm until the bacteria reached logarithmic growth phase. The growth of the culture was monitored on a microplate reader by measuring the optical density at 600 nm (OD_{600}). The *in vitro* inhibition rates I (%) are calculated by the formula described below, where C represents the corrected absorbance value (OD_{600}) of the DMSO NB media and T represents the corrected absorbance value (OD_{600}) of the compounds treated NB media.

$$\text{Inhibition rate } I\,(\%) = (C - T) / C \times 100$$

The half-maximal inhibitory concentration (IC_{50}) was determined from the equation via software SPSS 17.0. Each experiment was repeated three times.

144 **2.6 Cell culture**

145 *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99A and *Xanthomonas axonopodis*
146 pv. *citri* (*Xac*) strain *Xac* 29−1 cells were cultured in liquid nutrient broth medium
147 (3.0 g beef extract, 5.0 g peptone, 1.0 g yeast powder, 10.0 g glucose, added into 1 L
148 distilled water, pH 7.0−7.2) at 28 °C for 12 h. Cells were cultivated in a constant
149 temperature shaker with a speed of 180 rpm.

150 **2.7 Preparation of proteomes for *in vitro* experiments**

151 *Xoo* or *Xac* cells were cultured in 1 L liquid nutrient broth medium at 28 °C until the
152 OD_{600} reached 0.6 and then harvested by centrifugation at 6000 rpm. The bacterial
153 cell pellet was washed with PBS three times, resuspended in 40 mL PBS (pH 7.2)
154 buffer and lysed by sonication with a VCX150 (Sonics) instrument under ice cooling.
155 The cell mixture was separated by centrifugation at 12000 rpm for 45 min and the
156 protein concentration of supernatant was determined by the Bradford assay, which
157 was further adjusted to 1 mg/mL by dilution with PBS and stored at −80 °C until use.

158 **2.8 *In vitro* Labeling of Bacterial Proteomes**

159 Proteome samples (44 μL of 1 mg/mL protein in PBS) were treated with 1 μL desired
160 concentrations of probe **1** or **2** for 2 h at 25 °C. For competition experiments with
161 compound **0**, proteomes (43 μL) were first incubated with various concentrations of **0**
162 (1 μL) for 2 h at 25 °C, followed by addition of probe **1** (5 μM) or **2** (50 μM) for
163 another 2 h. After incubation, 1 μL biotin-azide reagent (200 μM) was added,
164 followed by 1 μL freshly prepared sodium ascorbate solution (5 mM). Samples were
165 gently vortexed, and the cycloaddition was initiated by the addition of 3 μL

166 $CuSO_4$/BTTAA mixture. A 50 mM BTTAA solution was preincubated with 50 mM
167 $CuSO_4$ solution (2:1 v/v) before use. The reaction mixtures were then incubated at 37
168 °C for 2 h in the dark. After click chemistry, 50 μL 2× SDS loading buffer was added
169 into the mixture to stop the reaction, which was further heated at 95 °C for 10 min. 10
170 μg protein per lane was loaded and separated by SDS-PAGE.

171 **2.9 *In situ* labeling of living cells**

172 *Xoo* or *Xac* cells were grown to OD_{600}=0.6 in liquid nutrient broth medium at 28 °C.
173 The growth media was removed by centrifugation at 6000 rpm, followed by washing
174 with PBS for three times. The cell pellet was resuspended in 990 μL PBS and treated
175 with various concentrations of probes (10 μL) or equal DMSO control at 25 °C for 2 h.
176 In the case of competitive ABPP experiments, cells were resuspended in 980 μL PBS
177 and preincubated with various concentrations of **0** (10 μL) at 25 °C for 2 h, and then
178 treated with probe **1** (5 μM) or **2** (50 μM) at 25 °C for another 2 h. After incubation
179 with probes, the supernatant was separated by centrifugation and excessive probes
180 were further removed by washing the cell pellet with PBS for three times. The cell
181 pellet was lysed by sonication on ice in 100 μL PBS, followed by centrifugation at
182 12000 rpm for 30 min. Protein concentration of supernatant was determined by
183 Bradford assay and diluted to 1 mg/mL in PBS. The follow-up steps were performed
184 as described above.

185 **2.10 Streptavidin blot**

186 Proteins on the SDS-gel were transferred onto a PVDF membrane at 4 °C for 60 min.
187 The membrane was further blocked in 5% nonfat dried milk solution for 2 h at room

temperature. After washing with PBST (PBS with 0.05% Tween 20), the membrane was incubated with streptavidin-horseradish peroxidase (Sangon Biotech) (with 1:1000 dilution in 5% nonfat dried milk PBST solution) for 2 h at 4 °C, and detected by ECL western blotting detection reagents (Bio-Rad). The PVDF membranes were scanned using the ChemiDoc XRS+ System (Bio-Rad).

2.11 Biotin-streptavidin enrichment experiments

After the *in situ* labeling experiments as described above, the cell pellet was resuspended with PBS and lysed by sonication on ice. The supernatant was separated by centrifugation and the protein concentration was measured by Bradford assay, which was further adjusted to the same concentration. To a 950 μL supernatant, 10 μL biotin-azide (200 μM), 10 μL sodium ascorbate solution (5 mM) and 30 μL premixed $CuSO_4$/BTTAA solution were added, followed by incubation at 37 °C for 2 h. The reaction was then quenched by addition of 4 mL pre-chilled acetone. After incubation at –80 °C overnight, the mixture was subsequently centrifuged at 12000 rpm for 15 min to obtain precipitated proteins. The supernatant was removed and precipitate was washed with pre-chilled MeOH thrice. The pellet was air dried and resuspended in 1 mL 0.2% SDS by sonication. Subsequently, the supernatant was incubated with 1 mL prewashed streptavidin beads (Promega) for 2 h. After enrichment, beads were washed with 1% SDS/PBS, 6 M urea and PBS. 50 μL 2×SDS loading buffer was added to the beads, which was further heated at 95 °C for 10 min to release the target proteins from the beads. The supernatant was collected by centrifugation and subjected to a preparative SDS-gel. The enriched bands were visualized by coomassie

blue staining.

2.12 In-gel digestion analysis and target identification

The protein band on the SDS-gel was excised and cut into 1 mm^2 pieces using a razor blade. The gel pieces were washed with ddH_20, 100 mM ammonium bicarbonate and dehydrated with 100% CH_3CN, which were further reduced by 10 mM DTT for 45 min at 37 °C with gentle shaking. Then the gel pieces were alkylated by 55 mM iodoacetamide for 1 hour at 37 °C in the dark. For trypsin digestion, 0.5 μg trypsin (Promega) in 100 μL 25 mM ammonium bicarbonate was added and the reaction was incubated at 37 °C overnight. The tryptic peptides were further separated by centrifugation and analyzed using a Nano-Liquid Chromatogram coupled with Triple TOF 5600 MS (AB SCIEX). The mass spectrometry data were searched using the MaxQuant software (v.1.5.2.8) against the corresponding *Xanthomonas* proteome of the uniprot database. For protein modifications, *N*-terminal acetylation and oxidation on methionine were chosen as variable modification and carbamidomethylation on cysteine as fixed modification. Mass tolerances of the precursor and fragment ions were set to 6 and 20 ppm. The false discovery rates for peptide and protein identification were set to 1%.

2.13 Western blot

After the enrichment experiment by streptavidin beads, proteins on the SDS-gel were transferred onto a PVDF membrane, which was further blocked in 5% nonfat dried milk solution for 2 h. After washing with PBST, the membrane was incubated with primary rabbit polyclonal anti-DLST antibody (Wuhan GeneCreate Biological

232 Engineering Co., Ltd., 1:2000 dilution in 5% milk in PBST) for 2 h at 4 °C, followed
233 by PBST washing. Subsequently, goat anti-rabbit IgG HRP-conjugated secondary
234 antibody (Bio-Rad, 1:2,000 dilution in 5% nonfat dried milk PBST solution) was add
235 and the membrane was incubated at 4 °C for 2 h. Finally, the membrane was washed
236 with PBST and signals were detected by ECL western blotting detection reagents
237 (Bio-Rad) using ChemiDoc XRS+ System (Bio-Rad).

238 **2.14 Cloning and recombinant expression of DLST (uniprot: A0A0K0GL90)**

239 The DLST gene (sucB) with NdeI/XhoI restriction enzyme sites was cloned into the
240 pet-28a (+) plasmid vector by ligation independent cloning using the following
241 primers:

242 DLST-pET-28a (+) Forward: cgcggcagccatatggccaccgaagttaaagttcc

243 DLST-pET-28a (+) Reverse: tggtggtgctcgagttacagaccaaacagcatac

244 DLST-pET-28a (+) construct was transformed into *E. coli* BL21 cells for expression
245 and selected on LB agar plates with a 50 μg/ml concentration of kanamycin. Cells
246 were incubated with kanamycin (50 μg/ml) at 37 °C until an OD_{600} of 0.6. Then
247 isopropyl *β*-D-thiogalactopyranoside (IPTG, 500 μM) was added to induce protein
248 expression. Bacteria were harvested and the cell pellet was then resuspended in lysis
249 buffer, followed by sonication (30% power, 30 min) on cie. The mixture was
250 separated by centrifugation and the supernatant was loaded on a pre-equilibrated 5 mL
251 HisTrap™ HP column (GE Healthcare). DLST was purified via a linear gradient
252 elution with buffer A (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 50 mM imidazole, 5%
253 glycerol, 1 mM DTT) and buffer B (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 400

mM imidazole, 5% glycerol, 1 mM DTT) using an Äkta purifier system (GE Healthcare). Fractions containing DLST were pooled, concentrated and digestion by thrombin for 4 h, followed by further purification with HisTrap™ column. Finally, fractions containing DLST were concentrated and adjusted to a concentration of 1 mg/mL for *in vitro* labeling experiments and then stored at −80 °C.

2.15 *In vitro* labeling of recombinant DLST

50 ng recombinant DLST (in 44 μL PBS) was treated with desired concentration of probe **1** (1 μL) and the reaction was incubated at 25 °C for 2 h. In competition experiment, 50 ng recombinant DLST was pretreated with different concentrations of **0** at 25 °C for 2 h, followed by addition of 5 μM probe **1** for another 2 h. The followed steps were described as above.

2.16 ATP content detection

Then 5 ml of each cell culture (OD_{600}=0.2) were incubated with desired concentration of **0** at 28 °C for 2 h with the speed of 180 rpm. After incubation, the cells were harvested by centrifugation and washed with PBS. The pellet was resuspended with lysis buffer from the ATP detection kit and lysed by sonication (30% power) on ice. The supernatant was separated by centrifugation and ATP content was determined by the luciferin-luciferase bioluminescence assay kit following the kit literature (Beyotime, China).

3. Results and discussion

3.1 Antibacterial activity test of probes 1−6

To unravel the biological targets of **0**, we introduced a short alkyne tag to its benzene

ring to synthesize a series of sulfone probes (Scheme 1). Also, we designed a negative probe for control experiments, which was synthesized by probe **1** under an intense base condition (Scheme 2). To test whether the structural modifications influence the antibacterial activity of all probes, we determined their individual half-maximal inhibitory concentration (IC_{50}) against *Xoo* and *Xac* (Table 1) by a turbidimeter test, as described previously.[8] As expected, all probes showed good antibacterial activity against *Xoo* and *Xac,* suggesting that the addition of the clickable alkyne tag does not interfere with drug antibacterial activity. Notably, the antibacterial activity of probe **1** (IC_{50}=3.58 μM) against *Xoo* was similar to **0** (IC_{50}=3.51 μM), and the antibacterial activity of probe **2** (IC_{50}=7.06 M) against *Xac* was slightly better than **0** (IC_{50}=8.59 μM). Probes **1** and **2** perfectly keep the antibacterial activity of **0**, which is a prerequisite for ABPP target discovery. Meanwhile, the NP showed a dramatic drop in potency (IC_{50}>500 μM) both in *Xoo* and *Xac* compared with **0**, thereby implying that the sulfonyl group may be critical for its antibacterial activity.

3.2 Target analysis in *Xoo* and *Xac* by probes 1 and 2

We next tested whether these probes are cell permeable and can label enzymes in living pathogens. In this study, 5 μM probes **1**–**6** and 50 μM probes **1**–**4** were incubated with *Xoo* and *Xac* cells for 2 h, respectively. The subsequent cell lysis and CC with biotin-azide were followed by SDS-PAGE analysis. Analysis of the labeled proteins by streptavidin blot detection showed a major band at ~45 kDa (Figure S1) both in *Xoo* and *Xac*, thereby indicating that these probes can enter the cells. All probes exhibited similar target preferences, thereby indicating that protein labeling is

298 primarily driven by the scaffold rather than the attached modifications. In accordance
299 with their activity, probe **1** showed a higher reactivity with p45 than probes **2**–**6** in
300 *Xoo* cells, and probe **2** outbalanced others in the *Xac* cells. Therefore, we used probes
301 **1** and **2** to conduct all subsequent studies as a representative example.

302 To evaluate the reactivity of the probes, probe **1** was used to treat *Xoo* cellular
303 lysates and living cells and probe **2** for *Xac* labeling experiments with different
304 concentrations. Both *in vitro* and *in situ* studies of *Xoo* have showed that the labeling
305 intensity of p45 increased over the concentration of probe **1**, which indicated a
306 dose-dependent manner with p45 (Figure 2A). An intense labeling of this band could
307 be observed as low as 2.5 μM, thereby indicating a high-affinity binding of p45 with
308 probe **1**. Moreover, probe **1** demonstrated a distinct selectivity with p45 in *Xoo* cells.
309 As shown in Figure 2A, labeling of *Xoo* lysates resulted in a much higher background
310 and relative weaker labeling intensity compared with *Xoo* cells at the same
311 concentration of probe **1**, thereby emphasizing the significance of *in situ* labeling as a
312 precondition for specific target identification. Consistent with probe **1**, probe **2** also
313 showed good target selectivity for p45 at low concentration in *Xac* labeling
314 experiments. Both *in vitro* and *in situ* studies of *Xac* have revealed a dose-dependent
315 manner with p45 and even in a high concentration of 100 μM, p45 was identified as
316 an undoubted major target (Figure 2B).

317 To test whether the probes specifically interact with p45, we conducted *in vitro*
318 and *in situ* competitive experiments with the pretreatment of **0**. By treating *Xoo* cells
319 with **0** at the indicated concentrations for 2 h before the addition of 5 μM of probe **1**,

we measured an approximate IC_{50} value of 50 μM for **0** labeling of p45 (Figure 2C). Preincubation with 100 μM **0** resulted in a total loss of labeling signals, thereby indicating that **0** and the corresponding probe compete for the same binding sites. Consistent with the *in situ* labeling, we also found that pretreatment with **0** in *Xoo* cellular lysates abolished this labeling event with a similar potency to that detected in living cells (Figure S2A). By contrast, p45 again appeared to be a specific target both in *Xac* cells and lysates because its labeling signal was gradually reduced in the presence of an excess of **0** (Figure 2D, Figure S2B). Taken together, p45 is the specific and main target of **0** both in *Xoo* and *Xac*.

To visualize the rate of the labeling reaction, *Xoo* and *Xac* cells were incubated with sulfone probes for various incubation times. The cells were lysed by sonication, separated by centrifugation at 12000 rpm and protein concentration was measured by the Bradford assay. After CC reaction with biotin-azide and SDS-PAGE analysis, the labeled proteins were detected by streptavidin blot. As a result, we found a detectable signal appeared within 5 min, indicating that the sulfone probes can be used for the rapid detection of p45 in living cells. In addition, the labeling intensity of p45 increased over time, which emphasizes that the probe binds to p45 in a time-dependent manner (Figures 3A).

3.3 Reactive mode of sulfone probes with p45

As described above, the sulfone probes **1-6** showed good antibacterial activity against *Xoo* and *Xac,* while the NP probe almost showed no antibacterial activity. We speculated that the sulfonyl group is critical for the labeling ability of the probes. To

test this hypothesis, 10 μM probe **1** and NP were used to treat *Xoo* cells. Furthermore, 50 μM probe **2** and NP were used for *Xac* cells, followed by CC reaction and streptavidin blot. Interestingly, the probe **1** and **2** strongly labeled p45 and no labeling event was detected by NP even at the concentration of 50 μM (Figure 3B), indicating that the oxadiazole ring was covalently attacked by the nucleophilic residues of protein targets and sulfonyl group served as a leaving group. This result was in accordance with the reactivity of sulfone reagents as reported previously, thereby emphasizing the crucial role of sulfonyl group for labeling protein targets.[5-7] When the sulfonyl group is substituted, the probe cannot react with the nucleophilic residue of target proteins and the corresponding antibacterial activity was significantly decreased.

3.4 Target identification by MS analysis and validation by western bolt

After these initial experiments, we next aimed to identify the p45 protein by a chemical proteomics approach. We treated *Xoo* and *Xac* cells with 100 μM probe **1** and 200 μM probe **2** for 2 h, respectively, followed by lysis and CC reaction. The target proteins were affinity purified by streptavidin beads, separated by SDS-PAGE and stained by coomassie blue (Coo). As a result, the probes **1** and **2** strongly purified the p45 both in *Xoo* and *Xac* cells (Figure 3C and 3D). The p45 band was then diced and subjected to in-gel trypsin digestion.[20,21] The resulting peptides were analyzed by LC–MS/MS, and dihydrolipoamide S-succinyltransferase (DLST) was identified as the likely target of sulfone probes (Table 2, Table S1). As expected, the intensity of p45 in probe **1** treated group is much higher than DMSO control. The predicted

364 molecular weight of DLST is 42.3 kDa, which is in accordance with the major
365 labeling band detected in all labeling experiments.

366 To further validate the MS results, we implemented target enrichment by a
367 pull-down experiment that was then applied to western blot analysis[22] using the DLST
368 antibody. For anti-DLST western blot, the enriched target protein was prepared in the
369 identical way as biotin-streptavidin enrichment experiments as described above. After
370 SDS-PAGE, proteins were transferred onto a PVDF membrane, blocked with 5%
371 non-fat dried milk solution and incubated with anti-DLST antibody, followed by the
372 incubation with goat anti-rabbit IgG HRP-conjugated secondary antibody. The signals
373 were detected by ECL western blotting detection reagents. As a result, an intense
374 labeling band appeared in the probe-treated sample but not in the DMSO control,
375 indicating the purified p45 by probes **1** and **2** was DLST (Figure 3E and 3F). These
376 results have confirmed DLST as the target of probe **1** in *Xoo* and probe **2** in *Xac*.

377 **3.5 *In vitro* interaction of probe 1 with recombinant DLST**

378 For additional validation experiments, recombinantly expressed DLST (from *Xoo*)
379 was labeled by probe **1** in a dose-dependent manner, and no labeling event was
380 detected by NP (Figure 4A). The labeling of DLST was markedly decreased with a
381 preincubation of excess **0**, thereby suggesting a specific interaction of the DLST with
382 probe **1** (Figure 4B). For sensitivity detection experiment, a series of concentrations of
383 recombinant DLST was incubated with probe **1** for 2 h. After CC reaction and
384 streptavidin blot, we showed that probe **1** is highly sensitive for DLST with a
385 detection limit of 3.1 nM (Figure 4C). Furthermore, the labeling of DLST was

completely blocked by preincubation with phenylmethylsulfonyl fluoride (PMSF) (Figure S3), which is a known active-site inhibitor of serine proteases,[23] thereby indicating that probe **1** may covalently bind to the serine residues. Among these results, we have unambiguously confirmed that DLST is the target of probe **1** in cells.

3.6 Function study of DLST by 0

DLST is the structural and catalytic core of the 2-oxoglutarate dehydrogenase complex (KGDHC),[24] which catalyzes the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA and acts as a key regulatory enzyme of energy production in the tricarboxylic acid (TCA) cycle.[25] To date, efficacious and selective DLST inhibitors are unavailable and the functional studies of DLST were mostly conducted by genetic approaches.[26-28] To determine whether the inhibition of DLST will alter the energy production in cells, we treated *Xoo* cells with various concentrations of **0** and determined the ATP content by luciferin-luciferase bioluminescence assay. As expected, the ATP content was decreased with the increase of **0** and resulted in more than 40% decrease at the concentration of 10 IC_{50} as compared with the DMSO control (Figure 4D). When DLST was inhibited, the energy production was interrupted, thereby indicating that the compound **0** may serve as an effective tool for the function study of DLST in bacteria.

In summary, we have developed a series of cell-permeable probes for target identification of bactericide **0** in living cells. Through chemical proteomic strategies, we validated DLST as an unprecedented target, which was also the first reported target of this novel structure. As a central enzyme in the TCA cycle, targeting this

energy regulator may, therefore, represent an attractive strategy for antibacterial agent discovery. To date, the function of DLST remains largely unknown and was studied mostly by genetic approaches due to lack of effective inhibitors. For the first time, we reported the covalent DLST inhibitor, the drug candidate **0**, which may serve as a useful tool for the function study of DLST in cells. Taken together, we anticipate target deconvolution of this sulfone-based antibacterial reagent may offer a guidance for the discovery of novel agrochemicals.

Abbreviations Used

ABPP, activity-based protein profiling; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*; *Xac*, *Xanthomonas axonopodis* pv. *citri*; CC, click chemistry; AIBN, azobisisobutyronitrile; m-CPBA, 3-Chloroperbenzoic acid; DLST, dihydrolipoamide S-succinyltransferase; BTTAA, 2-(4-((bis((1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid

Acknowledgment

We acknowledge financial support by the Key Technologies R&D Program (2014BAD23B01), National Natural Science Foundation of China (21877021, 21662009), the Research Project of Chinese Ministry of Education (213033A, 20135201110005).

Supporting Information

Supplementary data including Figure S1–S3, Table S1, Synthesis and characterization data for other target compounds, ^{1}H NMR, ^{13}CNMR, and HRMS spectra, in the online version, at https://pubs.acs.org/journal/jafcau.

Conflict of interest

The authors declare no conflict of interest.

References

(1) Lamberth, C.; Jeanmart, S.; Luksch, T.; Plant, A. Current Challenges and Trends in the Discovery of Agrochemicals. *Science* **2013**, *341*, 742-746.

(2) Sparks, T. C.; Lorsbach, B. A. Perspectives on the agrochemical industry and agrochemical discovery. *Pest Manag. Sci.* **2017**, *73*, 672-677.

(3) Croston, G. E. The utility of target-based discovery. *Expert Opin. Drug Discov.* **2017**, 12, 427-429.

(4) Mansfield, J.; Genin, S.; Magori, S.; Citovsky, V.; Sriariyanum, M.; Ronald, P.; Dow, M.; Verdier, V.; Beer, S. V.; Machado, M. A.; Toth, I.; Salmond, G.; Foster, G. D. Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol. Plant Pathol.* **2012**, *13*, 614-629.

(5) Zhang, D.; Devarie-Baez, N. O.; Li, Q.; Lancaster, J. R.; Xian, M. Methylsulfonyl Benzothiazole (MSBT): A Selective Protein Thiol Blocking Reagent. *Org. Lett.* 2012, 14, 3396-3399.

(6) Chen, X.; Wu, H.; Park, C. M.; Poole, T. H.; Keceli, G.; Devarie-Baez, N. O.; Tsang, A. W.; Lowther, W. T.; Poole, L. B.; King, S. B.; Xian, M.; Furdui, C. M. Discovery of Heteroaromatic Sulfones As a New Class of Biologically Compatible Thiol-Selective Reagents. *ACS Chem. Biol*. **2017**, *12*, 2201-2208.

(7) Matos, M. J.; Oliveira, B. L.; Martínez-Sáez, N.; Guerreiro, A.; Cal, P. M. S. D.; Bertoldo, J.; Maneiro, M.; Perkins, E.; Howard, J.; Deery, M. J.; Chalker, J. M.; Corzana, F.; Jiménez-Osés, G.; Bernardes, G. J. L. Chemo- and Regioselective Lysine Modification on Native Proteins. *J. Am. Chem. Soc*. **2018**, *140*, 4004-4017.

(8) Xu, W. M.; Han, F. F.; He, M.; Hu, D. Y.; He, J.; Yang, S.; Song, B. A. Inhibition of Tobacco Bacterial Wilt with Sulfone Derivatives Containing an 1,3,4-Oxadiazole Moiety. *J. Agric. Food Chem*. **2012**, *60*, 1036-1041.

(9) Li, P.; Yin, J.; Xu, W. M.; Wu, J.; He, M.; Hu, D. Y.; Yang, S.; Song, B. A. Synthesis, Antibacterial Activities, and 3D-QSAR of Sulfone Derivatives Containing 1, 3, 4-Oxadiazole Moiety. *Chem. Biol. Drug Des.* **2013**, *82*, 546-556.

(10) Li, P.; Shi, L.; Yang, X.; Yang, L.; Chen, X. W.; Wu, F.; Shi, Q. C.; Xu, W. M.; He, M.; Hu, D. Y.; Song, B. A. Design, synthesis, and antibacterial activity against rice bacterial leaf blight and leaf streak of 2,5-substituted-1,3,4-oxadiazole/thiadiazole sulfone derivative. *Bioorg. Med. Chem. Lett*. **2014**, *24*, 1677-1680.

(11) Gao, M. N.; Yu, L.; Li, P.; Song, X. P.; Chen, Z.; He, M.; Song, B. A. Label-free quantitative proteomic analysis of inhibition of *Xanthomonas axonopodis* pv. *citri* by the novel bactericide Fubianezuofeng. *Pestic. Biochem. Physiol*. **2017**, *138*, 37-42.

(12) Li, P.; Hu, D. Y.; Xie, D. D.; Chen, J. X.; Jin, L. H.; Song, B. A. Design, Synthesis, and Evaluation of New Sulfone Derivatives Containing a 1,3,4-Oxadiazole Moiety as Active Antibacterial Agents. *J. Agric. Food Chem*. **2018**, *66*, 3093-3100.

(13) Evans, M. J.; Cravatt, B. F. Mechanism-Based Profiling of Enzyme Families. *Chem. Rev.* **2006**, *106*, 3279-3301.

(14) Barglow, K, T.; Cravatt, B. F. Activity-based protein profiling for the functional

477 annotation of enzymes. *Nat. Methods* **2007**, *4*, 822-827.

478 (15) Cravatt, B. F. Wright, A. T.; Kozarich, J. W. Activity-Based Protein Profiling:
479 From Enzyme Chemistry to Proteomic Chemistry. *Annu. Rev. Biochem.* **2008**, *77*,
480 383-414.

481 (16) Sanman, L. E.; Bogyo, M. Activity-Based Profiling of Proteases. *Annu. Rev.*
482 *Biochem*. **2014**, *83*, 249-273.

483 (17) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. A Stepwise
484 Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of
485 Azides and Terminal Alkynes. *Angew. Chem., Int. Ed*. **2002**, *114*, 2708-2711.

486 (18) Speers, A. E.; Adam, G. C.; Cravatt, B. F. Activity-Based Protein Profiling in
487 Vivo Using a Copper(I)-Catalyzed Azide-Alkyne [3 + 2] Cycloaddition. *J. Am.*
488 *Chem. Soc*. **2003**, *125*, 4686-4687.

489 (19) Zheng, B.; DeRan, M.; Li, X.; Liao, X.; Fukata, M.; Wu, X. 2-Bromopalmitate
490 Analogues as Activity-Based Probes To Explore Palmitoyl Acyltransferases. *J.*
491 *Am. Chem. Soc.* **2013**, *135*, 7082-7085.

492 (20) Shevchenko, A.; Tomas, H.; Havlis, J.; Olsen, J. V.; Mann, M. In-gel digestion
493 for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc*.
494 **2006**, *1*, 2856-2860.

495 (21) Qian, Y.; Schürmann, M.; Janning, P.; Hedberg, C.; Waldmann, H.
496 Activity-Based Proteome Profiling Probes Based on Woodward's Reagent K with
497 Distinct Target Selectivity. *Angew. Chem., Int. Ed*. **2016**, *55*, 7766-7771.

498 (22) Chen, Y. C.; Backus, K. M.; Merkulova, M.; Yang, C.; Brown, D.; Cravatt, B. F.;

499 Zhang, C. Covalent Modulators of the Vacuolar ATPase. *J. Am. Chem. Soc*. **2017**,
500 *139*, 639-642.
501 (23) Böttcher, T.; Sieber, S. A. β-Lactones as Privileged Structures for the Active-Site
502 Labeling of Versatile Bacterial Enzyme Classes. *Angew. Chem., Int. Ed.* **2008**, *47*,
503 4600-4603.
504 (24) Knapp, J. E.; Mitchell, D. T.; Yazdi, M. A.; Ernst, S. R.; Reed, L. J.; Hackert, M.
505 L. Crystal structure of the truncated cubic core component of the Escherichia coli
506 2-oxoglutarate dehydrogenase multienzyme complex. *J. Mol. Biol*. **1998**, *280*,
507 655-668.
508 (25) Anderson, N. M.; Mucka, P.; Kern, J. G.; Feng, H. The emerging role and
509 targetability of the TCA cycle in cancer metabolism. *Protein Cell* **2018**, *9*,
510 216-237.
511 (26) Yang, L.; Shi, Q.; Ho, D. J.; Starkov, A. A.; Wille, E. J.; Xu, H.; Chen, H. L.;
512 Zhang, S.; Stack, C. M.; Calingasan, N. Y.; Gibson, G. E.; Beal, M. F. Mice
513 deficient in dihydrolipoyl succinyl transferase show increased vulnerability to
514 mitochondrial toxins. *Neurobiol. Dis*. **2009**, *36*, 320-330.
515 (27) Keßler, M.; Berger, I. M.; Just, S.; Rottbauer, W. Loss of dihydrolipoyl
516 succinyltransferase (DLST) leads to reduced resting heart rate in the zebrafish.
517 *Basic Res. Cardiol*. **2015**, 110:14.
518 (28) Anderson, N. M.; Li, D.; Peng, H. L.; Laroche, F. J. F.; Mansour, M. R.; Gjini, E.;
519 Aioub, M. Helman, D. J.; Roderick, J. E.; Cheng, T. Harrold, I.; Samaha, Y.;
520 Meng, L.; Amsterdam, A.; Neuberg, D. S.; Denton, T. T.; Sanda, T.; Kelliher, M.

521 A.; Singh, A.; Look, A. T.; Feng, H. The TCA cycle transferase DLST is
522 important for MYC-mediated leukemogenesis. *Leukemia* **2016**, 30, 1365-1374.

Figure captions

Scheme 1. Synthetic route of **0**-derived probes.

Scheme 2. Synthetic route of negative probe.

Figure 1. Design of **0**-derived probes. (A) Chemical structures of **0** and its clickable analogs. (B) Detection and identification of probe-labeled proteome by the ABPP strategy.

Figure 2. *In vitro* and *in situ* labeling of bacterial pathogens by the **0**-derived probes. (A) Dose response of *in vitro* and *in situ* labeling revealed the potential engagement of probes **1** and **2** to an unknown target of ~45 kDa (p45) both in *Xoo* and *Xac* (B). Coo, Coomassie blue staining shows protein loading. (C) Dose-dependent blockade of labeling of p45 by pretreatment with **0** in *Xoo* and *Xac* cells (D). A–D: gel analysis by streptavidin blot.

Figure 3. Kinetic analysis, covalent reaction and target identification of DLST in *Xoo* and *Xac*. (A) Time-course experiments revealed a time-dependent manner with p45. (B) *In situ* labeling of *Xoo* and *Xac* by probes **1**–**2** and NP indicated a crucial role of sulfonyl group for the probes' labeling ability. A–B: gel analysis by streptavidin blot. (C) Streptavidin pull-down experiment by probe **1** enriched p45 in *Xoo* cells. (D) Streptavidin pull-down experiment by probe **2** enriched p45 in *Xac* cells. C–D: gel analysis by coomassie blue staining. Immunoblot analysis using an antibody confirmed the protein pulled down by probe **1** (E) and probe **2** (F) was DLST. E–F: gel analysis by western blot.

Figure 4. Labeling of recombinant DLST and its function study by **0**. (A)

546 Dose-dependent labeling of recombinant DLST with probe **1**. (B) Increasing
547 concentrations of **0** prevented labeling of recombinant DLST with probe **1**. (C)
548 Labeling of different concentrations of recombinant DLST indicated a high sensitivity
549 with probe **1**. A–C: gel analysis by streptavidin blot. (D) Equant *Xoo* cells were
550 treated with the concentrations of 0–10 IC_{50} of **0**, resulting in a dramatic decrease of
551 ATP content. Error bars represent s.d. in three independent replicates.

553 **Table 1.** Antibacterial Activities of **0** and **0**-derived probes against *Xoo* and *Xac in*

554 *vitro*.

compound	*Xoo*			*Xac*		
	regression equation	r	IC_{50} (μM)	regression equation	r	IC_{50} (μM)
0	y = 7.64x + 5.53	0.98	3.51 ± 0.04	y = 4.39x + 3.61	0.99	8.59 ± 0.16
Probe **1**	y = 10.40x + 4.51	0.99	3.58 ± 0.03	y = 3.75x + 2.24	0.97	17.53 ± 0.10
Probe **2**	y = 7.53x + 1.90	0.98	7.95 ± 0.09	y = 2.55x + 4.08	0.99	7.06 ± 0.25
Probe **3**	y = 28.44x – 0.92	0.99	5.51 ± 0.03	y = 1.93x + 3.73	0.97	15.60 ± 0.14
Probe **4**	y = 27.40x – 12.27	0.99	13.94 ± 0.03	y = 2.34x + 3.62	0.98	12.63 ± 0.33
Probe **5**	y = 4.19x + 4.33	0.99	3.99 ± 0.08			
Probe **6**	y = 4.80x + 3.05	0.98	6.80 ± 0.16			
NP			>500			>500

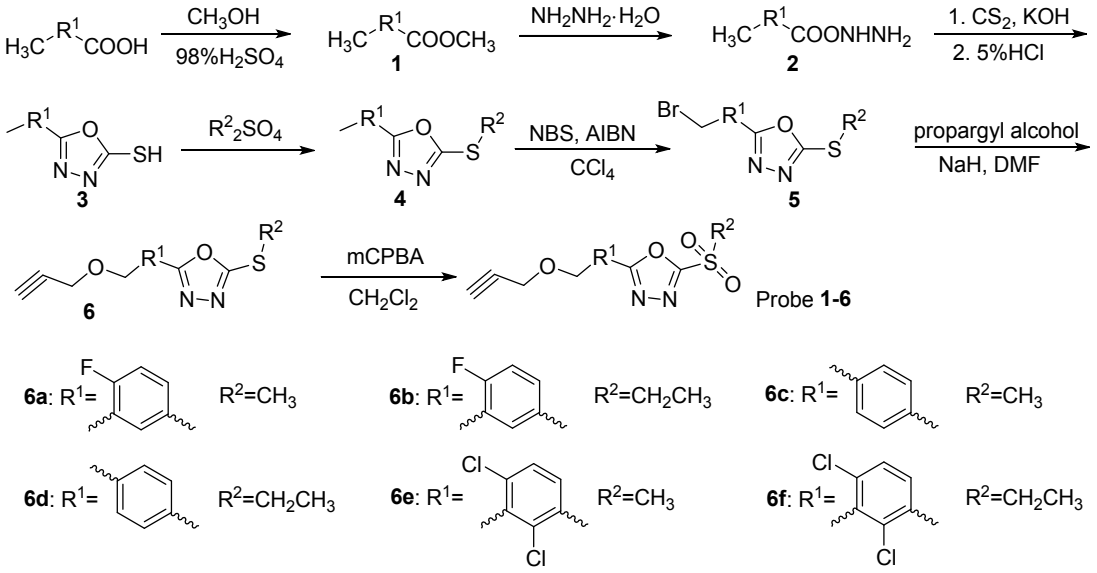
556 **Table 2.** Results of gel-based protein identification of p45 band of *Xoo* labeled with

557 100 μM probe **1**.

Uniprot accession	Protein name	MW (kDa)	Intensity			
			DMSO (1)	DMSO (2)	Probe **1** (1)	Probe **1** (2)
A0A0K0GL90	dihydrolipoamide S-succinyltransferase	42.1	5.97E+04	6.07E+04	6.47E+06	6.52E+06

559 **Figures**

560 **Scheme 1**



561

563 **Scheme 2**

564

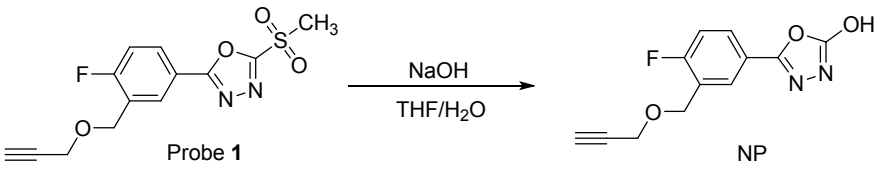
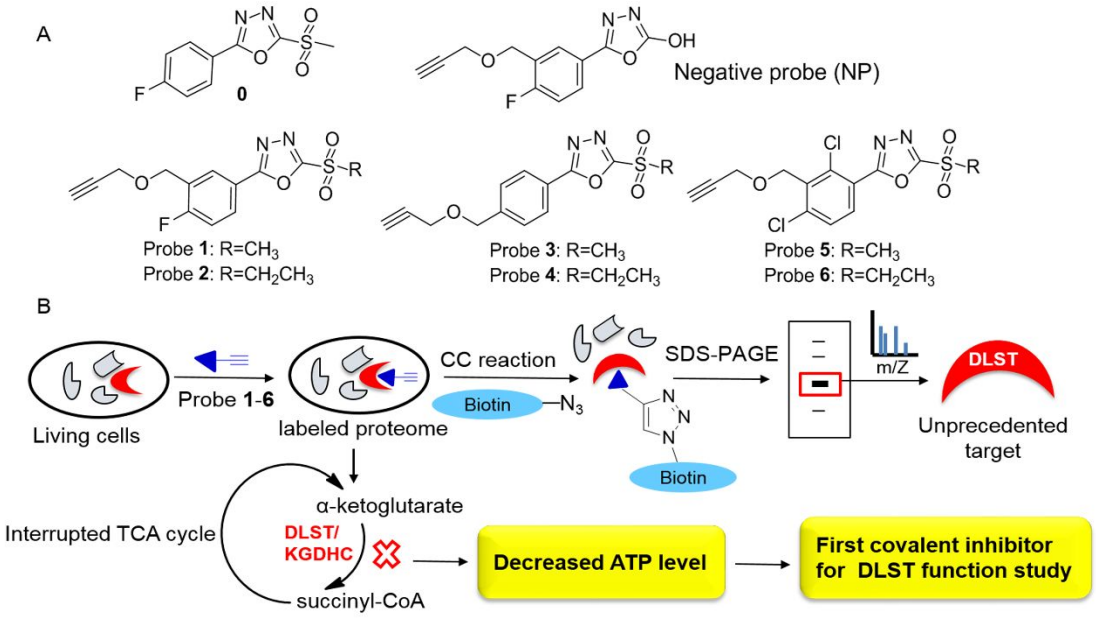
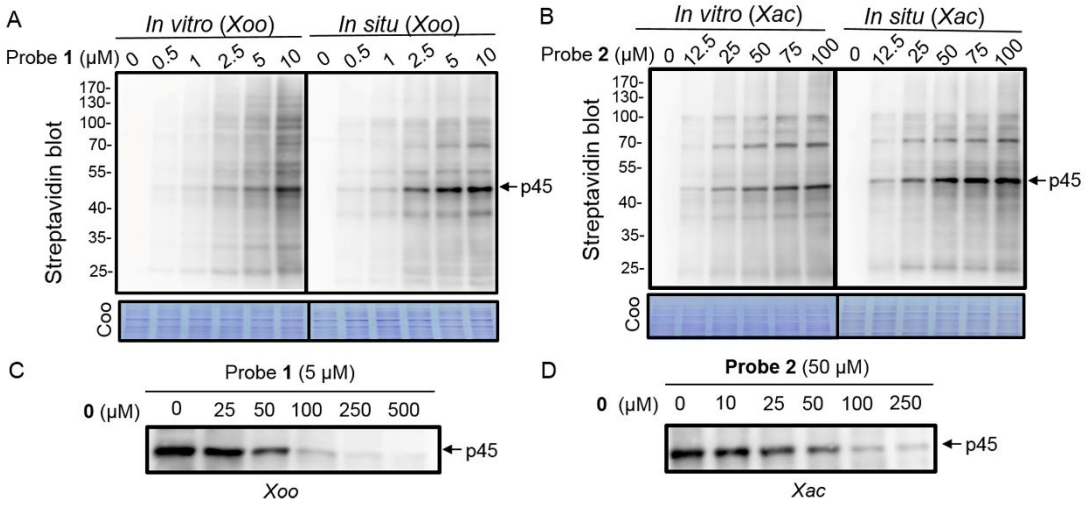


Figure 1

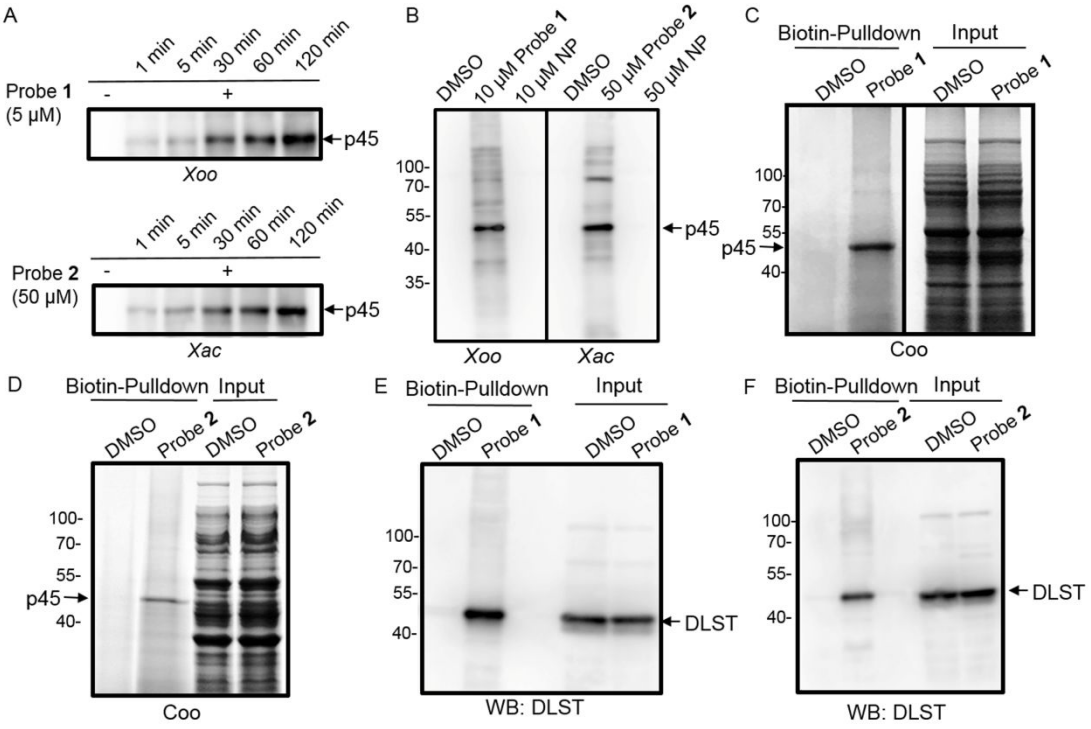

A
0
Negative probe (NP)
Probe 1: R=CH3
Probe 2: R=CH2CH3
Probe 3: R=CH3
Probe 4: R=CH2CH3
Probe 5: R=CH3
Probe 6: R=CH2CH3
B
Living cells
Probe 1-6
labeled proteome
CC reaction
Biotin
N3
SDS-PAGE
m/Z
DLST
Unprecedented target
Biotin
α-ketoglutarate
Interrupted TCA cycle
DLST/ KGDHC
succinyl-CoA
Decreased ATP level
First covalent inhibitor for DLST function study

568 **Figure 2**



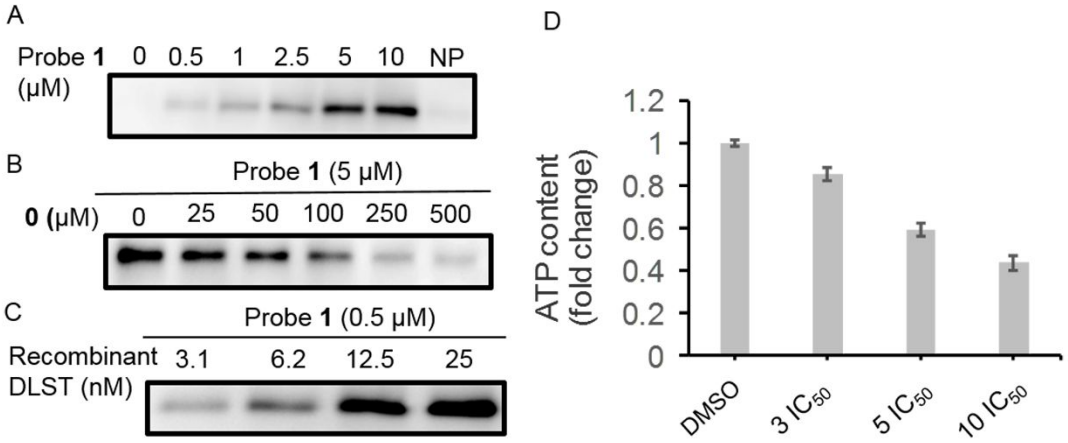
569

571 **Figure 3**



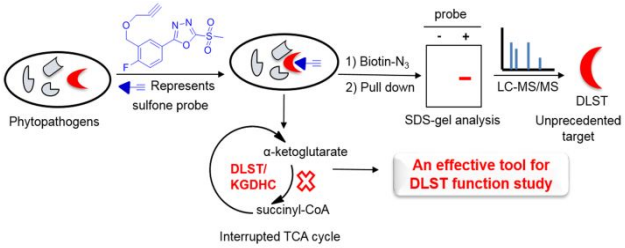
572

574 **Figure 4**



575

577 **Table of Contents Graphic**



578