JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.7b05491 • Publication Date (Web): 01 Jan 2018

Just Accepted

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Design, Synthesis and Antifungal Activities of 3-Acyl Thiotetronic Acid Derivatives: New Fatty Acid Synthase Inhibitors

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ABSTRACT. Emerging fungal phytodiseases are increasingly becoming a food security threat. Twenty-six new 3-acylthiotetronic acid derivatives were designed, synthesized, characterized, and evaluated for activities against *Valsa mali*, *Curvularia lunata*, *Fusarium graminearum* and *Fusarium oxysporum* f. sp. *lycopersici*. Among the 26 compounds, **6f** was the most effective against *V. mali*, *C. lunata*, *F. graminearum* and *F. oxysporum* f. sp. *lycopersici* with median effective concentrations (EC_{50}) of 4.1, 3.1, 3.6 and 4.1 μg/mL, respectively, while the corresponding EC_{50} were 0.14, 6.7, 22.4, and 4.3 μg/mL of the fungicide azoxystrobin, 4.2, 41.7, 0.42 and 0.12 μg/mL of the fungicide carbendazim, and >50, 0.19, 0.43, >50 μg/mL of the fungicide fluopyram. The inhibitory potency against *V. mali* fatty acid synthase agreed well with the *in vitro* antifungal activity. The molecular docking suggested that the 3-acylthiotetronic acid derivatives targeted the C171Q KasA complex. The findings help understanding the mode of action and design and synthesis of novel potent fungicides.

Keywords: Thiotetronic acid; Fatty acid synthase; Antifungal activity; Fungicide

INTRODUCTION

Natural products have long been used as pesticides and have often served as a source of inspiration in the discovery of commercial synthetic pesticides as they could aid in identification of new mode of action and be used as lead structures to derive new potent pesticides.[1-7] The heterocyclic cores of tetronic acids (2,4-furanodione), thiotetronic acid (2,4-thiophenedione) and tetramic acids (pyrrolidine-2,4-dione) have attracted significant attention over the years due to their occurrence as naturally bioactive products isolated from bacteria, molds, algae, fungi, lichens and sponges (Figure 1),[8] which display a wide range of biological functions. Tetronic acids are antioxidant,[9] antiepileptic,[10] antitumor,[11] anti-inflammatory,[12,13] and insecticidal.[14] Tetramic acids exhibit antibacterial,[15] antiviral,[16] anticancer,[17] and herbicidal activities.[18] Thiolactomycin is a unique thiolactone and specifically inhibits biosynthesis of fatty acids and mycolic acid, by which exhibits anti-mycobacterial activities.[19, 20]

Fatty acid synthesis in fungi is compartmentalized in the large multifunctional fatty acid synthases (FAS). FAS function as molecular assembly lines and increase the time of synthesis by channeling substrates between the active sites and achieving high local concentrations of intermediates.[21-23] In fatty acid synthesis, extension of a growing fatty acyl chain by two carbons at a time is initiated by the coupling of a malonylated acyl carrier protein (ACP) with a thioester linked acyl group at the active site of β-ketoacyl synthase (KAS).[24] There are two tandem KAS-type enzymes, KasA and KasB. KasA specifically elongates palmitoyl-CoA to C38-42 monounsaturated acyl chains, whereas KasB extends up to 54 carbons on average in the presence of KasA to produce longer chain multi unsaturated hydrocarbons.[19, 25] In previous studies, the

acylated enzyme intermediate was mimicked by a C171Q KasA variant in which Gln-171 forms hydrogen bonds with the KasA oxyanion hole.[26-28] Considering the importance of fatty acids in fungal survival, this acyl enzyme mimic could be particularly important for the design of new anti-fungal agents to inhibit KAS by binding preferentially to the acyl-enzyme.

In our efforts to screen for agricultural fungicides from natural products, attempts have been made to test whether modulation of the five membered ring systems could lead to high fungicidal activity. Based on the proven potencies of tetramic acid, tetronic acid, thiotetronic acid natural products and the mode of action of thiotetronic acid antibiotics inhibiting fatty-acid synthases,[19,20] 26 novel thiotetronic derivatives were designed and synthesized targeting KasA and KasB to inhibit the biosynthesis of fatty acids and mycolic acid and to reveal the structure and fungicidal activity information. In addition, docking simulations were performed on the basis of the X-ray crystallographic structure of the C171Q KasA to explore the binding mode of the compounds in the active site.

MATERIALS AND METHODS

Chemicals. Azoxystrobin (98%) and carbendazim (98%) were purchased from J&K Chemical Ltd. (Shanghai, China). Fluopyram (99%) was purchased from Anpel Laboratory Technologies, Inc. (Shanghai, China). Azoxystrobin, carbendazim and fluopyram are commercial fungicides and were used as positive controls. The other reagents were all purchased from J&K Chemical and were analytically or chemically pure. All solvents and liquid reagents were dried by standard methods in advance and distilled prior to use.

Fungi. The plant pathogenic fungi, *Valsa mali*, *Curvularia lunata*, and *Fusarium graminearum*, and *Fusarium oxysporum* f. sp. *lycopersici* were kindly provided by the Department of Plant Pathology, Anhui Agricultural University, China. These fungi were grown on potato dextrose agar (PDA) plates at 28 °C and maintained at 4 °C with periodic sub-cultivations.

Instruments. ^{1}H NMR and ^{13}C NMR spectra were recorded on an Agilent 600M DD2 (Agilent Technologies Co. Ltd., Shanghai, China) spectrometer at 25 °C and referenced to tetramethylsilane (Me_4Si). Chemical shifts were reported in ppm (δ) using the residual solvent line as an internal standard. The purity was determined on an Agilent LC-MS Symmetry 3 under a gradient elution of 5-90% aqueous acetonitrile containing 0.05% trifluoacetic acid (TFA) over 8 min (10 min run time) at a flow rate of 2 mL/min. The column used was a 50 mm x 4.6 mm i.d., 5 μM, C18 column (Agilent). High resolution mass spectrometry (HRMS) data were obtained on a Varian QFT-ESI instrument (Varian, Inc., Alto Palo, CA). Melting points were determined on a XT4 MP apparatus (Taike Corp., Beijing, China) and were un-corrected. Analytical thin-layer chromatography (TLC) was performed on glass-backed silica gel GF254 sheets (Yantai Jiangyou Silica Gel Development Co. Ltd., China). All compounds were detected at 254 nm or 365 nm.

Synthesis. The synthetic route of the target compounds **5a-5d**, **6a-6h**, **7a-7e** and **8a-8i** was outlined in Figure 2 using thioacetic acid as a starting material.

Synthesis of 2-(acetylthio)acetic acid, **2**. A mixture of *p*-dioxane (200 mL) and Et_3N (161 g, 1.58 mol) was chilled in an ice-water bath to ca. 10 °C. To this solution thioacetic acid (120 g, 1.58 mol) was added dropwise while the temperature was maintained between 10-15 °C. The

93 resulting brick red solution of thiolacetic acid and triethylamine (Et_3N) was added dropwise to a
94 solution of bromoacetic acid (220 g, 1.58 mol) in *p*-dioxane (500 mL) with vigorous stirring. A
95 resulting precipitate of triethylamine hydrobromide slowly formed. During the course of addition,
96 the reaction mixture became warm and was stirred at ambient temperature for 16 h. The reaction
97 mixture was filtered to remove Et_3NHBr. The filtrate was evaporated and the residue was
98 dissolved in saturated aqueous K_2CO_3. The aqueous K_2CO_3 was washed twice with diethyl ether
99 (Et_2O). The aqueous phase was separated and acidified cautiously to pH 3 by the dropwise
100 addition of concentrated HCl. The product was extracted several times into Et_2O. The combined
101 Et_2O extract was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated. The
102 residue was distilled under reduced pressure (1 Torr), and the fraction boiling between 118 and
103 122 °C was collected to afford pure 2-(acetylthio)acetic acid, **2**, as a pale yellow oil. Yield, 90%;
104 yellow oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 3.76 (s, 2H), 2.43 (s, 3H). ^{13}C NMR (150 MHz,
105 $CDCl_3$) δ: 193.9, 174.7, 31.3, 30.1.

106 *Synthesis of 3-acetyl-4-hydroxythiophen-2(5H)-one*, **4**. Oxalyl chloride (5.44 g, 55.5 mmol,
107 1.5 equiv) was added dropwise over 1 h at 0 °C to a solution of 2-(acetylthio)acetic acid **2** (5.00
108 g, 37 mmol) and DMF (0.38 mL, 4.82 mmol) in anhydrous CH_2Cl_2 (100 mL). The mixture was
109 stirred at ambient temperature for 8 h. After solvent removal, the crude oil was distilled at 7 mbar
110 to give pure 2-(acetylthio)acetic chloride as an orange oil. The acyl chloride (5.65 g, 37 mmol)
111 was then added to a solution of toluene (80 mL) and cooled to 0 °C by means of an ice bath.
112 Methyl acetoacetate sodium salt (15.33 g, 111 mmol) was then added dropwise, while the
113 temperature was maintained between 5-10 °C for 3 h. The final slurry was washed with 1 M

aqueous HCl (3 × 100 mL). The toluene layer was extracted with dilute Na_2CO_3 (3 × 100 mL) aqueous solution. To the aqueous phase was added NaOH (2.40 g, 60 mmol). The solution was kept overnight. The aqueous phase was then acidified cautiously to pH 3 by the dropwise addition of concentrated HCl. The precipitated crude product was extracted with Et_2O several times. The combined Et_2O extract was concentrated *in vacuo* and purified by silica gel column chromatography (EtOAc/MeOH, 15:1, v/v) (R_f 0.4) to give the pure intermediate **4**. Yield, 67%; white solid; m.p. 82-83 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 4.17 (s, 2H), 2.42 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 197.0, 194.7, 193.2, 110.8, 35.4, 25.4; HRMS [ESI] *m/z*: calcd for ([M-H]$^-$): 156.9960; found: 156.9965.

General procedure for the synthesis of compounds, **5a-5d**.

To a solution of 158 mg (1 mmol) of the intermediate **4**, PPh_3 (314 mg, l.2 mmol) and the corresponding alcohol (1.2 mmol) were dissolved in 10 mL of dry THF, followed by addition of 0.19 ml (1.2 mmol) of diisopropyl azodicarboxylate in 5 mL of THF. The reaction was stirred overnight at room temperature and then concentrated *in vacuo*. The residue was purified by a silica gel column with hexane/EtOAc (2:1, v/v) as the eluant. The crude product was then recrystallized from hexane to give **5a-5d**.

3-Acetyl-4-ethoxythiophen-2(5H)-one, **5a**. Yield, 56%; green solid; m.p. 82-83 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 4.33 (q, J = 6.9 Hz, 2H), 4.01 (s, 2H), 2.45 (s, 3H), and 1.51 (t, J = 6.9 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 193.8, 193.5, 192.2, 118.1, 67.8, 31.4, 30.8, and 14.8; HRMS [ESI] *m/z*: calcd for ([M+H]$^+$), 187.0429; found: 187.0432.

3-Acetyl-4-isopropoxythiophen-2(5H)-one, **5b**. Yield, 60%; green solid; m.p. 76-78 °C; ^{1}H

NMR (600 MHz, $CDCl_3$) δ: 4.84 (m, 1H), 4.02 (s, 2H), 2.43 (s, 3H), and 1.45 (d, J = 6.1 Hz, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 193.5, 192.0, 182.7, 118.3, 75.8, 31.2, 30.9, and 22.7; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 201.0585; found: 201.0592.

3-Acetyl-4-propoxythiophen-2(5H)-one, **5c**. Yield, 73%; green solid; m.p. 72-73 °C； ^1H NMR (600 MHz, $CDCl_3$) δ: 4.21 (t, J = 6.4 Hz, 2H), 4.01 (s, 2H), 2.44 (s, 3H), 1.91 – 1.84 (m, 2H), and 1.06 (t, J = 7.4 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 193.5, 192.1, 183.9, 117.9, 73.3, 31.4, 30.8, 22.6, and 10.2; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 201.0585; found: 201.0592.

3-Acetyl-4-(benzyloxy)thiophen-2(5H)-one, **5d**. Yield, 45%; green solid; m.p. 94-95 °C; ^1H NMR (600 MHz, $CDCl_3$) δ: 7.40 (dt, J = 14.0, 7.4 Hz, 5H), 5.34 (s, 2H), 4.03 (s, 2H), and 2.43 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 193.8, 192.3, 183.1, 134.0, 129.1, 127.2, 118.8, 110.0, 73.1, 31.7, and 30.9; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 249.0585; found: 249.0587.

General Procedure for the Preparation of Compounds **6a-6h**

A solution of 1.58 g (10 mmol) of 3-acetyltetrahydrothiophene-2,4-dione, **4**, 11 mmol of the corresponding aromatic aldehyde, and 30 mg of *p*-toluenesulfonic acid in 50 mL of toluene was heated under reflux until water no longer separated as an azeotrope (10-12 h, TLC). The mixture was cooled to room temperature. The precipitate (3-acetyl-5-benzylidene derivatives), **6a-6h,** was filtered off and recrystallized from MeOH/EtOAc.

3-Acetyl-5-benzylidene-4-hydroxythiophen-2(5H)-one, **6a**. Yield, 42%; yellow solid; m.p. 151-152 °C; ^1H NMR (600 MHz, DMSO-d_6) δ: 7.81 (s, 1H), 7.65 (d, J = 7.6 Hz, 2H), 7.50 (t, J = 7.5 Hz, 2H), 7.45 (t, J = 7.3 Hz, 1H), and 2.47 (s, 3H), ^{13}C NMR (150 MHz, DMSO-d_6) δ: 195.9,

187.1, 186.0, 134.2, 131.1 (2C), 130.8, 130.7, 129.7 (2C), 127.3, 108.2, and 26.7; HRMS [ESI] *m/z*: calcd for ([M-H]$^-$), 245.0272; found: 245.0275.

3-Acetyl-4-hydroxy-5-(4-nitrobenzylidene)thiophen-2(5H)-one, **6b**. Yield, 65%; yellow solid; m.p. 204-205 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.28 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H), 7.69 (s, 1H), and 2.39 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 193.2, 186.2, 185.9, 147.1, 141.6, 135.1, 131.3 (2C), 124.6, 124.5 (2C), 106.8, and 27.7; HRMS HRMS [ESI] *m/z*: calcd for ([M-H]$^-$), 290.0123; found: 290.0128.

3-Acetyl-5-(4-fluorobenzylidene)-4-hydroxythiophen-2(5H)-one, **6c**. Yield, 53%; yellow solid; m.p. 181-183 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.79 (s, 1H), 7.71 (dd, J = 8.6, 5.6 Hz, 2H), 7.35 (t, J = 8.8 Hz, 2H), and 2.46 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 195.6, 186.9, 186.0, 164.0, 162.4, 133.5, 131.0, 129.4, 127.4, 116.9, 116.7, 108.0, and 26.8; HRMS [ESI] *m/z*: calcd for ([M-H]$^-$), 263.0178; found: 263.0180.

3-Acetyl-4-hydroxy-5-(4-methoxybenzylidene)thiophen-2(5H)-one, **6d**. Yield, 56%; yellow solid; m.p. 186-187 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.79 (s, 1H), 7.63 (d, J = 8.7 Hz, 2H), 7.08 (d, J = 8.8 Hz, 2H), 3.81 (s, 3H), and 2.46 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 187.1, 185.9, 161.5, 133.3, 131.2, 126.8, 115.4, 108.1, 56.0, and 27.1; HRMS [ESI] *m/z*: calcd for ([M-H]$^-$), 275.0378; found: 275.0381.

3-Acetyl-5-(4-chlorobenzylidene)-4-hydroxythiophen-2(5H)-one, **6e**. Yield, 45%; yellow solid; m.p. 172-174 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.73 (s, 1H), 7.65 (d, J = 8.5 Hz, 2H), 7.55 (d, J = 8.5 Hz, 2H), and 2.44 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 195.0, 186.7, 186.0, 135.0, 133.4, 132.5, 129.7, 129.1, 128.4, 107.8, and 27.0; HRMS [ESI] *m/z*: calcd for

177 ([M-H]$^-$), 278.9883; found: 278.9886.

178 *3-Acetyl-4-hydroxy-5-(4-(trifluoromethyl)benzylidene)thiophen-2(5H)-one*, **6f**. Yield, 55%;

179 yellow solid; m.p. 162-164 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.84–7.79 (m, 4H), 7.71 (s,

180 1H), and 2.40 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 193.8, 186.5, 186.0, 138.9, 132.8,

181 131.1, 129.3, 126.3, 126.0, 110.0, 107.2, and 27.5; HRMS [ESI] *m/z*: calcd for ([M-H]$^-$),

182 313.0146; found: 313.0149.

183 *4-((4-Acetyl-3-hydroxy-5-oxothiophen-2(5H)-ylidene)methyl)benzonitrile*, **6g**. Yield, 54%;

184 yellow solid; m.p. 214-215 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.91 (d, J = 8.2 Hz, 2H), 7.76

185 (d, J = 8.2 Hz, 2H), 7.70 (s, 1H), and 2.42 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 194.0,

186 186.4, 186.0, 139.3, 133.2, 133.1 (2C), 126.3, 119.0, 111.5, 107.2, and 27.2; HRMS [ESI] *m/z*:

187 calcd for ([M-H]$^-$), 270.0225; found: 271.0230.

188 *3-Acetyl-4-hydroxy-5-(4-methylbenzylidene)thiophen-2(5H)-one*, **6h**. Yield, 57%; yellow

189 solid; m.p. 182-183 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.72 (s, 1H), 7.53 (d, J = 7.5 Hz, 2H),

190 7.31 (d, J = 7.4 Hz, 2H), 2.43 (s, 3H), and 2.33 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 186.0,

191 187.0, 140.6, 131.8, 131.1 (2C), 130.3 (2C), 129.8, 27.3, and 21.5; HRMS [ESI] *m/z*: calcd for

192 ([M-H]$^-$), 259.0429; found: 259.0431.

193 *General procedure for the synthesis of* **7a-7e**

194 A stirred solution of 1 mmol of **6b** or **6d**, 1.2 mmol of the corresponding alcohol and 314 mg

195 (l.2 mmol) of PPh_3 was dissolved in 10 mL of dry THF, followed by addition of 0.19 mL (1.2

196 mmol) of diisopropyl azodicarboxylate in 5 mL of THF. The reaction mixture was stirred

197 overnight at room temperature and then concentrated *in vacuo*. The residue was purified by a

198 silica gel column eluted with hexane/EtOAc (6:1, v/v). The crude product was then recrystallized
199 from hexane to give **7a-7e**.

200 *3-Acetyl-4-methoxy-5-(4-methoxybenzylidene)thiophen-2(5H)-one*, **7a**. Yield, 65%; yellow
201 solid; m.p. 103-104 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.58 (s, 1H), 7.50 (d, J = 8.7 Hz, 2H),
202 6.96 (d, J = 8.8 Hz, 2H), 4.04 (s, 3H), 3.86 (s, 3H), and 2.58 (s, 3H). ^{13}C NMR (150 MHz,
203 $CDCl_3$) δ: 196.4, 189.5, 175.3, 161.2, 132.6 (2C), 131.3, 127.0, 124.2, 116.7, 114.7 (2C), 63.4,
204 55.5, and 31.6; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 291.0691; found: 291.0693.

205 *3-Acetyl-4-ethoxy-5-(4-nitrobenzylidene)thiophen-2(5H)-one*, **7b**. Yield, 57%; yellow solid;
206 m.p. 156-158 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.28 (d, J = 8.8 Hz, 2H), 7.68 (d, J = 8.8 Hz,
207 2H), 7.61 (s, 1H), 4.25 (q, J = 7.0 Hz, 2H), 2.58 (s, 3H), and 1.49 (t, J = 7.0 Hz, 3H). ^{13}C NMR
208 (150 MHz, $CDCl_3$) δ: 196.3, 188.2, 172.7, 147.6, 140.5, 132.2, 130.8 (2C), 127.3, 124.1 (2C),
209 117.6, 72.8, 31.6, and 15.2; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 320.0592; found: 320.0594.

210 *3-Acetyl-5-(4-methoxybenzylidene)-4-propoxythiophen-2(5H)-one*, **7c**. Yield, 62%; yellow
211 solid; m.p. 104-106 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.58 (s, 1H), 7.56–7.48 (m, 2H),
212 7.02–6.93 (m, 2H), 4.11 (t, J = 6.4 Hz, 2H), 3.85 (s, 3H), 2.56 (s, 3H), 1.91 – 1.80 (m, 2H), and
213 1.05 (t, J = 7.4 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 196.4, 189.5, 174.7, 161.1, 132.6 (2C),
214 131.0, 127.0, 124.6, 116.8, 114.6 (2C), 78.2, 55.5, 31.5, 23.1, and 10.4; HRMS [ESI] *m/z*: calcd
215 for ($[M+H]^+$), 319.1004; found: 319.1006.

216 *3-Acetyl-4-butoxy-5-(4-nitrobenzylidene)thiophen-2(5H)-one*, **7d.** Yield, 58%; yellow solid;
217 m.p. 108-109 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.28 (d, J = 8.6 Hz, 2H), 7.68 (d, J = 8.7 Hz,
218 2H), 7.59 (s, 1H), 4.18 (t, J = 6.4 Hz, 2H), 2.58 (s, 3H), 1.88 – 1.79 (m, 2H), 1.57 – 1.45 (m, 2H),

and 1.00 (t, J = 7.4 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 196.3, 188.2, 173.0, 147.6, 140.5, 132.3, 130.8 (2C), 127.2, 124.1 (2C), 117.5, 76.7, 31.6 (2C), 19.1, and 13.7; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 348.0905; found: 347.0908.

3-Acetyl-4-(benzyloxy)-5-(4-nitrobenzylidene)thiophen-2(5H)-one, **7e**. Yield, 63%; yellow solid; m.p. 101-102 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.27 (d, J = 8.8 Hz, 2H), 7.65 (d, J = 8.7 Hz, 2H), 7.59 (s, 1H), 7.42 (t, J = 6.4 Hz, 3H), 7.38 – 7.33 (m, 2H), 5.29 (s, 2H), and 2.45 (s, 3H). ^{13}C NMR (150MHz, $CDCl_3$) δ: 196.3, 188.2, 172.4, 147.6, 140.4, 134.3, 132.2, 130.9 (2C), 129.9, 129.2, 129.0 (2C), 128.3 (2C), 128.1, 127.8, 124.1 (2C), 118.3, 77.7, and 31.4; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 382.0749; found: 382.0752.

General procedure for the synthesis of Compounds **8a-8i**

A mixture of **6a-6d** (1 mmol), the corresponding aromatic amine (1.1 mmol), TsOH (30 mg) in 40 mL methanol was heated at 65 °C for 4 h. After cooling, the precipitated product was filtered and recrystallized from a mixture of methanol and chloroform (1:1) to afford **8a**-**8i**.

5-Benzylidene-3-(1-(4-(trifluoromethyl)phenylamino)ethylidene) thiophene-2,4(3H,5H)-dione , **8a**. Yield, 80%; yellow solid; m.p. 202-203 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 13.76 (s, 1H), 7.76 (d, J = 8.8 Hz, 3H), 7.60 (d, J = 7.8 Hz, 2H), 7.45 (t, J = 7.6 Hz, 2H), 7.37 (d, J = 7.9 Hz, 3H), and 2.66 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.7, 189.2, 168.8, 138.8, 134.5, 130.5, 130.3, 129.4, 129.0, 128.5, 127.0, 126.1, 125.9, 124.4, 122.6, 102.3, and 16.9. HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 390.0775; found: 390.0778.

5-Benzylidene-3-(1-(4-methoxyphenylamino)ethylidene)thiophene-2,4(3H,5H)-dione, **8b**. Yield, 75%; yellow solid; m.p. 182-184 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 13.76 (s, 1H), 7.73 (s,

1H), 7.59 (d, J = 7.6 Hz, 2H), 7.44 (t, J = 7.7 Hz, 2H), 7.35 (t, J = 7.4 Hz, 1H), 7.13 (d, J = 8.6 Hz, 2H), 6.98 (d, J = 8.7 Hz, 2H), 3.85 (s, 3H), and 2.57 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.5, 189.2, 169.4, 159.4, 134.8, 130.4, 130.0, 129.1, 128.8, 128.1, 127.7, 126.8, 114.9, 101.6, 55.6, and 16.8; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 352.1007; found: 352.1010.

5-(4-Nitrobenzylidene)-3-(1-(phenylamino)ethyl)thiophene-2,4(3H,5H)-dione, **8c**. Yield, 78%; yellow solid; m.p. 254-256 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 13.79 (s, 1H), 8.27 (d, J = 8.4 Hz, 2H), 7.70 (t, J = 9.3 Hz, 3H), 7.51 (t, J = 7.4 Hz, 2H), 7.44 (t, J = 7.2 Hz, 1H), 7.24 (q, J = 7.8 Hz, 2H), and 2.63 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.0, 187.8, 169.6, 147.1, 141.2, 135.3, 134.7, 130.7, 129.8, 128.7, 125.6, 125.2, 124.5, 124.0, 101.3, and 16.9; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 369.0909; found: 369.0911.

5-(4-Fluorobenzylidene)-3-(1-(4-(trifluoromethyl)phenylamino)ethylidene)thiophene-2,4(3H, 5H)-dione, **8d**. Yield, 85%; m.p. 181-183 °C; yellow solid; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 14.05 (s, 1H), 7.76 (d, J = 8.1 Hz, 2H), 7.71 (d, J = 9.3 Hz, 1H), 7.61 – 7.51 (m, 2H), 7.36 (t, J = 7.5 Hz, 2H), 7.13 (t, J = 8.5 Hz, 2H), and 2.66 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.6, 188.8, 168.9, 162.2, 138.8, 132.4, 132.1, 130.8, 129.2, 127.2, 127.0, 125.9, 116.2, 116.0, 102.2, and 16.9; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 408.0681; found: 408.0685.

5-(4-Fluorobenzylidene)-3-(1-(phenylamino)ethylidene)thiophene-2,4(3H,5H)-dione, **8e**. Yield, 88%; yellow solid; m.p. 175-176 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 13.89 (s, 1H), 7.70 (d, J = 9.5 Hz, 1H), 7.60 – 7.53 (m, 2H), 7.49 (t, J = 7.3 Hz, 2H), 7.41 (t, J = 7.2 Hz, 1H), 7.22 (d, J = 7.5 Hz, 2H), 7.13 (t, J = 8.3 Hz, 2H), and 2.62 (d, J = 30.8 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.5, 188.9, 169.2, 162.1, 135.5, 132.3, 132.1, 131.0, 129.8, 128.4, 126.6, 125.6,

116.1, 115.9, 101.6, and 16.8; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 340.0807; found: 340.0810.

5-(4-Fluorobenzylidene)-3-(1-(4-fluorophenylamino)ethylidene)thiophene-2,4(3H,5H)-dione, **8f**. Yield, 73%; yellow solid; m.p. 175-177 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 13.80 (s, 1H), 7.68 (d, J = 13.4 Hz, 1H), 7.56 (s, 2H), 7.19 (dd, J =10.3 Hz, 4H), 7.12 (s, 2H), and 2.58 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.5, 188.8, 169.5, 162.5, 132.3, 132.1, 131.5, 131.0, 129.4, 127.5, 126.8, 116.8, 116.0, 101.7, and 16.7; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 358.0713; found: 358.0716.

5-(4-Fluorobenzylidene)-3-(1-(4-methoxyphenylamino)ethylidene)thiophene-2,4(3H,5H)-dione, **8g**. Yield, 65%; yellow solid; m.p. 164-165 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 13.73 (s, 1H), 7.67 (d, J = 13.4 Hz, 1H), 7.63 – 7.46 (m, 2H), 7.12 (t, J = 9.2 Hz, 4H), 6.97 (d, J = 8.1 Hz, 2H), 3.84 (s, 3H), and 2.58 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.4, 188.8, 169.4, 163.7, 162.0, 159.4, 133.3, 132.1, 131.1, 129.7, 128.1, 127.0, 126.8, 126.4, 116.1, 115.9, 114.9, 101.5, 55.6, and 16.7; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 370.0913; found: 370.0915.

5-(4-Methoxybenzylidene)-3-(1-(4-methoxyphenylamino)ethylidene)thiophene-2,4(3H,5H)-dione, **8h**. Yield, 79%; yellow solid; m.p. 156-157 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 13.95 (s, 1H), 7.71 (d, J = 8.8 Hz, 1H), 7.55 (t, J = 9.8 Hz, 2H), 7.48 (t, J = 7.7 Hz, 2H), 7.40 (t, J = 7.4 Hz, 1H), 7.21 (t, J = 7.0 Hz, 2H), 6.97 (d, J = 8.6 Hz, 2H), 3.85 (s, 3H), and 2.65 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.6, 189.4, 169.0, 160.4, 135.6, 132.2, 132.0, 129.7, 128.3, 127.9, 127.4, 127.2, 125.8, 125.6, 114.4 (2C), 101.8, 55.4, and 16.8; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 382.1113; found: 382.1116.

5-(4-Methoxybenzylidene)-3-(1-(phenylamino)ethylidene)thiophene-2,4(3H,5H)-dione , **8i**.

Yield, 86%; yellow solid; m.p. 190-192 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 13.80 (s, 1H), 7.69 (s, 1H), 7.55 (d, J = 7.1 Hz, 2H), 7.13 (d, J = 6.4 Hz, 2H), 6.97 (s, 4H), 3.85 (s, 6H), and 2.59 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 189.6, 189.3, 169.2, 160.4, 159.3, 132.2, 128.2, 127.7, 127.5, 127.3, 127.0, 126.8, 114.8, 114.4, 101.7, 55.6, 55.4, and 16.7; HRMS [ESI] *m/z*: calcd for ($[M+H]^+$), 352.1007; found: 352.1009.

Bioassays. Fungicidal activities of **5a-5d**, **6a-6h**, **7a-7e** and **8a-8i** were tested *in vitro* against *V. mali*, *C. lunata*, *F. graminearum*, and *F. oxysporum* f. sp. *lycopersici*. Their inhibition (%) was determined according to the mycelium growth rate method. The test fungi grown on PDA medium slants were subcultured for 48 h in Petri dishes prior to testing and used for inoculation of fungal strains on PDA plates. The test compound was completely dissolved in 0.5 mL of DMSO and then added to 9.5 mL of sterile water. The resulting solution was mixed with 90 mL of sterile molten PDA to obtain the final concentrations of 50 μg/mL for the activity screening test. PDA with different compounds was poured into 90 mm Petri dishes (15 mL/dish), on which a 5 mm thick and 6 mm diameter disc of fungus cut from previously subcultured Petri dishes was placed at the center of the semisolid medium after the medium in the plate was partially solidified. The final concentration of DMSO was 0.5%, which was proven to have no significant effect on the growth of target fungi. DMSO alone and the widely used commercial fungicides azoxystrobin, fluopyram, carbendazim (50 μg/mL) dissolved in DMSO were used as a solvent control and positive controls, respectively. The DMSO concentration in both cases was 0.5% in the culture medium. The dishes were kept in an incubator at 28 °C for 72 h. Each experiment was carried out in triplicate. After the mycelia fully covered the control group Petri dishes, the

303 diameters (in mm) of fungal colony in the treatment Petri dishes were measured with a caliper in
304 three different directions. The growth inhibition percentages were calculated according to the
305 following formula and expressed as means ± standard deviations (SD).

306 Inhibition rate (%) = $[(d_c - d_0) - (d_s - d_0)]/(d_c - d_0) \times 100$

307 where d_0 is the diameter of the fungus cut; d_c is the diameter of a fungal colony in the solvent
308 control plates; and d_s is the diameter of a fungal colony in the compound-treated plates.

309 Based on the results of *in vitro* antifungal activity, highly active compounds were further
310 determined for their median effective concentrations (EC_{50}) according to the same method
311 described above. The stock solution was mixed with the autoclaved PDA medium to prepare a
312 set of media containing 0.625, 1.25, 2.5, 5, 10, and 20 μg/mL of the test compound, while the
313 media containing 0.0781, 0.312, 1.25, 5, 20, and 50 μg/mL of the positive control fungicide.
314 Similarly, 0.5% DMSO in culture medium was used as a solvent control. Each test was
315 performed in triplicate. EC_{50} values and their confidence intervals at 95% probability (95% CI)
316 were calculated with DPS software ver. 7.05 (Hangzhou Ruifeng Information Technology Co.,
317 Ltd., Hangzhou, China).

318 **Fatty Acid Synthase Activity Assay.** FAS activity was spectrophotometrically determined
319 by measuring the malonylcoenzyme A (CoA)–dependent oxidation of nicotinamide adenine
320 dinucleotide phosphate reduced form (NADPH) according to the method of Nepokroeff *et al*.[29]
321 Mycelia of *V. mali* were inoculated at 0.05 $OD_{600\ nm}$ and grown on a reciprocal shaker (180 rpm,
322 25 °C) for 5 d in Sabouraud maltose broth (SMB) medium that contained different
323 concentrations (1.25, 2.5, 5, 10, 20, and 50 μg/mL) of **6a**, **6c**, **6e**, **6f**. Mycelia were harvested by

vacuum filtration and disrupted in liquid nitrogen using a mortar and pestle. The resultant powder was resuspended to 10% w/v in mitochondrial extraction buffer (10 mM of $KHPO_4$ pH 7.2, 10 mM of KCl, 10 mM of $MgCl_2$, 0.5 M of sucrose, 0.2 mM of EDTA, 2 mM of PMSF). The extract was centrifuged twice at 5000*g*, 4 °C and 10 min, and intact mitochondria were then pelleted at 10000*g* for 20 min at 4 °C and resuspended in the same buffer. The supernatant was used to evaluate the FAS activity at 340 nm (before/after malonyl-CoA). The assay solution without malonyl-CoA served as a background for the assay. Each sample was assayed in triplicate.

X-ray Diffraction. Single crystals of $C_{15}H_{13}NO_5S$ (**7b**) were grown from MeOH. A suitable crystal was selected and mounted on a SuperNova, Dual, Cu at zero, AtlasS2 diffractometer (Rigaku Oxford Diffraction, Tokyo, Japan). The crystal was kept at 290 (2) K during data collection. Using Olex2,[30] the structure was solved with the ShelXT[31] structure solution program using Direct Methods and refined with the ShelXL[32] refinement package using Least Squares minimization.

Molecular Modeling. Molecular docking of **6f** to the C171Q KasA enzyme was performed with the software YetiX 8.3.[33] The enzyme domain with an active conformation (PDB code: 4c6u) was used as the receptor structure for the docking experiments. **6f** was constructed using BioX 4.6 [34] and the atomic partial charges were calculated by AmberTools. The docking modes were optimized by the directional Yeti force field.[35]

RESULTS AND DISCUSSION

345 **Chemistry.** The synthetic route for **5a-5d**, **6a-6h**, **7a-7e** and **8a-8i** was outlined in Figure 2.
346 As an initial material, thioacetic acid **1** was reacted with bromoacetic acid to prepare
347 acetothioacetic acid **2**.[36] Treatment of acetothioacetic with oxalyl chloride followed by Claisen
348 condensation with methyl acetoacetate sodium salt produced acetylthio diketoester **3**.[37]
349 Hydrolysis of acetylthio diketoester formed γ-mercapto diketoester, followed by cyclization and
350 acidification to provide 3-acetyl-4-hydroxy-*5H*-furan-2-ones **4**. The target compounds **6a-6h**
351 were synthesized via the reaction of **4** with the corresponding substituted benzaldehydes in
352 toluene under reflux. The compounds **5a-5d** and **7a-7e** were prepared via the Mitsunobu reaction
353 of **4** and **6a-6e** with appropriate alcohols. The compounds **8a-8i** were synthesized by the
354 nucleophilic reaction of **6a-6e** with an amine.
355 **Crystal Structure of 7b**. The crystal data of **7b** were presented in Table 1. Figure 3 shows a
356 perspective view of **7b** with the atomic labelling system.[38] The result demonstrated that **6a-6e**
357 adopt a *Z*-configuration rather than *E*-conformation in the formation of double bond by
358 dehydration of aldol reaction products. However, crystal structures of **6a-6e** could not be
359 obtained in the present study.
360 **Antifungal Activity.** Table 2 shows the *in vitro* antifungal activity of **5a-5d**, **6a-6h**, **7a-7e**
361 and **8a-8i** at the concentration of 50 μg/mL against the fungi *V. mali, C. lunata, F. graminearum,*
362 *and F. oxysporum* f. sp. *Lycopersici.* All compounds possess antifungal activities in various
363 degrees against each of the tested fungi.
364 The commercial fungicides azoxystrobin, carbenzazim and fluopyram were used as positive
365 controls to compare the potency of the synthetic chemicals because they are commonly used to

366 control the four fungal pathogens. Azoxystrobin is a systemic and broad-spectrum fungicide. It
367 prevents production of ATP by binding tightly to Complex III of the mitochondrial electron
368 transport chain.[39] Carbendazim is also a broad-spectrum fungicide. It probably binds to tubulin
369 and suppresses microtubule assembly dynamic, although the exact mechanism of action is
370 unclear.[40] Fluopyram inhibits the succinate dehydrogenase and is a broad spectrum fungicide
371 registered for use in USA in 2012 and in the EU in 2013.[41] To each fungus, at the concentration
372 of 50 μg/mL, some compounds were more active than the positive control of azoxystrobin.
373 Carbenzazim showed high activity against *V. mali, C. lunata* and *F. graminearum* except *F.*
374 *oxysporum* f. sp. *Lycopersici, while* fluopyram showed higher potency to *C. lunata* and *F.*
375 *graminearum* than *V. mali* and *F. oxysporum* f. sp. *lycopersici.* In order to further explore the
376 antifungal potential and structure-activity relationship, the compounds with inhibition >50% at
377 50 μg/mL were further examined to determine their EC_{50} against the four fungal strains (Table
378 3).

379 Tests of fungicidal activity (Table 2) indicated that at the concentration of 50 μg/mL, **4** and
380 **5a-5d** showed low inhibitions (approximately < 35%) against *V. mali, C. lunata, F. graminearum,*
381 *and F. oxysporum* f. sp. *lycopersici*. The compounds **6a-6h** exhibited high activities (> 70%
382 inhibition at 50 μg/mL), with EC_{50} of 3.1−20.3 μg/mL against *V. mali*. In contrast, **6a**, **6b**, **6c** and
383 **6e**, **6f** at 50 μg/mL inhibited greater than 83% against *V. mali*, while the inhibitions by
384 azoxystrobin, fluopyram and carbendazim were 81.6%, 6.0% and 97.9%, respectively. For *C.*
385 *lunata*, some of **6a-6h** with EC_{50} of 3.1−42.2 μg/mL showed higher activity than azoxystrobin
386 (EC_{50} = 6.7 μg/mL) and carbendazim (EC_{50} = 41.2 μg/mL) against *C. lunata*. Fluopyram showed

387 the highest potency to *C. lunata* with an EC_{50} of 0.18 μg/mL. Considering *F. graminearum*,
388 **6a-6h** had moderate potencies (35%-70% inhibition) at 50 μg/mL, with EC_{50} of 3.1−46.6 μg/mL.
389 **6e** showed the highest activity with an EC_{50} of 3.1 μg/mL, while EC_{50} of fluopyram and
390 carbendazim were 0.43 and 0.42 μg/mL, respectively. For *F. oxysporum* f. sp. *lycopersici*, **6a-6h**
391 exhibited high activities (> 70% inhibition at 50 μg/mL), with EC_{50} of 4.1−16.0 μg/mL. **6f**
392 showed the highest activity with an EC_{50} value of 4.1 μg/mL, being similar to azoxystrobin
393 (EC_{50} = 4.3 μg/mL) and 33 fold higher than carbendazim (EC_{50} = 0.123 μg/mL). Table 1 shows
394 that at a concentration of 50 μg/mL, **7a-7d** exhibited low (< 35%) to moderate inhibitions
395 (35%-70%) against the target fungi. It was noteworthy that **7e** was highly active (73.4%
396 inhibition at 50 μg/mL) against *V. mali*, whereas **8a-8i** had weak inhibitions (approximately
397 <35%) against *V. mali, C. lunata, F. graminearum and F. oxysporum* f. sp. *lycopersici.*

398 Tables 2 and 3 show that the type and position of substituents on the 3-acylthiotetronic acid
399 had significant effects on the activity. The general trend is that the presence of benzylidene at the
400 5-position increased the activity against the four target fungi, while 4-alkoxy substituents, **5a-5d**,
401 did not improve the activity in comparison with a hydroxyl moiety. Further optimization of **6**, by
402 alkylation of 3-acylthiotetronic acid at 4-hydroxy and condensation of 3-acetyl with amine of
403 3-acylthiotetronic acid, led to a decrease of the activity against the four fungi. Studies on the
404 antifungal activities of 3-acylthiotetronic acid derivatives could provide useful information for
405 evaluating the structure-activity relationship. The 4-hydroxyl and 3-acetyl groups played an
406 important role in the fungicidal activity.

407 Comparison of **6a** and **6b-6h**, which contained different substituents on the benzene ring,

indicated that the presence of a fluorine atom (**6c**) or a trifluoromethyl group (**6f**) led to higher activity than **6a** against *V. mali*, *C. lunata*, *F. graminearum*, while showing similar activities against *F. oxysporum* f. sp. *lycopersici*. This result suggested that the presence of 4'-F and CF_3 improved the activity. The methyl-substitution and methoxy-substitution reduced the activities against all four fungi. The presence of -CN group decreased the activities against *V. mali*, *C. lunata*, *F. graminearum*. The introduction of -Cl atom improved the activity against both *F. graminearum* and *C. lunata*, but had weak influence on the activity against *V. mali* and *F. oxysporum* f. sp. *lycopersici*.

Inhibition of Fungal Fatty Acid Synthase. The synthetic compounds possess the scaffold of thiolactone as thiolactomycin does, which inhibits fatty-acid synthases. The compounds **6a**, **6c**, **6e** and **6f** had EC_{50} of 4.4, 3.1, 4.5, and 4.1 μg/mL, respectively, against *V. mali.* They were selected and tested against FAS *in vitro*. As demonstrated in Figure 4, **6a**, **6c**, **6e** and **6f** strongly inhibited FAS with an half maximum inhibition concentration (IC_{50}) of 6.1, 5.3, 6.2 and 4.9 μg/mL, respectively, and showed the similar inhibition profile as that using the mycelium growth inhibition assay *in vitro*. The results proved the hypothesis and design of thiotetronic acid derivatives as FAS inhibitors.

Molecular docking. Figure 5 shows the binding pose and features of **6f** with the target protein. First, the hydroxyl group on the thiophene ring may form two hydrogen bonds with the nitrogen atoms from the imidazole of His311 and His345, while another hydrogen bond may be formed between the 3-acyl group with the nitrogen atom of His345. Therefore, 4-hydroxy group and 3-acyl group of thiophene are likely the important part of the pharmacophore. Secondly, a

π-π stacking probably occurs between the thiophene ring and the benzyl ring of Phe404, which plausibly explains the activity enhancement by the introduction of benzylidene at 5-position on the thiophene ring. Thirdly, the hydrophobic benzyl trifluoride group may reside deeply at the hydrophobic pocket, which provides a strong hydrophobic interaction. Additionally, a π-π stacking between the benzyl trifluoride group and the benzyl group of Phe402 further stabilized the interaction. Such specific binding features reasonably made **6f** a potent inhibitor to the target.

In summary, a series of novel 3-acylthiotetronic acid derivatives were designed and synthesized. Those compounds exhibited good to excellent fungicidal activities against the four fungi. The compound **6f** showed the highest fungicidal activity against the four tested fungal pathogens with EC_{50} of 3.1-4.1 μg/mL. The EC_{50} of the commercial fungicides azoxystrobin, carbenzazim and fluopyram ranged between 0.12 and >50 μg/mL against *V. mali, C. lunata, F. graminearum, and F. oxysporum* f. sp. *lycopersici*. **6f** and its analogs are potent FAS inhibitors. **6f** is non-selective to the four fungal species, whereas the commercial fungicides azoxystrobin, carbenzazim and fluopyram is selective to one or two of the four tested fungal species. The results revealed that the compounds are potent fungicide candidates, which could be further optimized and developed as lead compounds.

Supporting Information

Characterization data, ^{1}H NMR, ^{13}C NMR spectra for products **5a-5d**, **6a-6h**, **7a-7e, 8a-8i** and X-Ray diffraction data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

Funding

This project was supported in part by the National Key Research and Development Program of China (No. 2016YFD0200205) and the Public Sector (Agriculture) Research Program of China (201303088-11).

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

P.L. and R.H. conceived and designed the experiments; Y.C. performed the experiments; Z.Z. performed the molecular docking. T.S. and X.W. analyzed the data; P.L. wrote the paper. Q.L. interpreted the data and revised the manuscript. J.X. revised the manuscript.

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578 **FIGURE CAPTIONS**

579 **Figure 1.** Structures of tenuazonic acid, RK-682, and thiolactomycin showing the cores of

580 tetramate, tetronate, and thiotetronate

581 **Figure 2.** Synthesis of **5a-5d**, **6a-6h, 7a-7e** and **8a-8i**

582 **Figure 3.** X-ray single crystal structure of **7b**

583 **Figure 4.** Dose-response curve of **6a**, **6c**, **6e** and **6f** on fungal FAS inhibition

584 **Figure 5.** Predicted docking poses of **6f** in complex with C171Q Kas A enzyme (PDB: 4c6u)

585

586 **Table 1.** Crystal data and structure refinement for **7b**

Empirical formula	$C_{15}H_{13}NO_5S$
Formula weight	319.32
Temperature/K	290(2)
Crystal system	triclinic
Space group	P-1
a/Å	8.1146(2)
b/Å	13.3325(5)
c/Å	14.3250(4)
α/°	81.416(3)
β/°	89.944(2)
γ/°	77.457(2)
Volume/Å^3	1495.06(8)
Z	4
ρ_{calc}g/cm^3	1.419
μ/mm^{-1}	2.146
F(000)	664.0
Crystal size/mm^3	0.280 × 0.250 × 0.220
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	6.872 to 142.584
Index ranges	$-6 \le h \le 9$, $-16 \le k \le 14$, $-15 \le l \le 17$
Reflections collected	9811
Independent reflections	5628 [R_{int} = 0.0249, R_{sigma} = 0.0301]
Data/restraints/parameters	5628/0/401
Goodness-of-fit on F^2	1.035
Final R indexes [I>=2σ (I)]	R_1 = 0.0420, wR_2 = 0.1155
Final R indexes [all data]	R_1 = 0.0473, wR_2 = 0.1233
Largest diff. peak/hole / e Å^{-3}	0.17/-0.23

587 **Table 2**. Antifungal activities of the target compounds at 50 μg/mL

Comp	Average inhibition (%) ± SD (n = 3)			
	V. mali	*C. lunata*	*F. graminearum*	*F. oxysporum* f. sp. *lycopersici*
5a	5.8 ± 0.2	7.4 ± 0.1	4.3 ± 3.8	6.2 ± 0.9
5b	6.5 ± 0.1	18.9 ± 0.5	8.6 ± 0.9	5.6 ± 3.0
5c	7.3 ± 0.1	8.3 ± 2.0	11.1 ± 0.0	4.9 ± 1.7
5d	5.4 ± 0.1	11.1 ± 2.6	17.9 ± 3.1	3.7 ± 1.5
6a	90.4 ± 0.2	91.2 ± 0.8	72.8 ± 0.9	79.0 ± 0.9
6b	84.7 ± 0.5	88.7 ± 1.5	63.9 ± 0.8	79.6 ± 1.5
6c	83.7 ± 0.3	92.5 ± 3.1	67.9 ± 0.9	80.2 ± 0.9
6d	73.1 ± 0.3	83.7 ± 3.9	47.5 ± 0.9	74.1 ± 0.0
6e	83.8 ± 0.8	93.7 ± 1.8	69.1 ± 0.9	80.8 ± 0.9
6f	85.7 ± 0.3	92.5 ± 0.0	68.0 ± 0.9	79.6 ± 1.5
6g	78.7 ± 0.5	76.7 ± 3.6	54.4 ± 0.0	74.1 ± 0.0
6h	77.4 ± 1.2	73.0 ± 5.0	66.7 ± 1.5	79.0 ± 0.9
7a	47.4 ± 0.4	33.5 ± 3.6	27.2 ± 0.9	31.5 ± 1.5
7b	13.7 ± 0.0	7.40 ± 0.1	3.1 ± 0.9	11.11 ± 0.0
7c	24.3 ± 0.5	20.8 ± 0.4	4.3 ± 0.9	8.0 ± 0.9
7d	30.0 ± 0.0	24.9 ± 0.4	1.85 ± 0.0	13.0 ± 1.5
7e	73.4 ± 1.1	58.6 ± 0.6	49.4 ± 0.9	57.0 ± 0.9
8a	23.6 ± 0.2	19.9 ± 0.8	4.9 ± 0.9	8.6 ± 0.9
8b	7.6 ± 0.5	14.1 ± 0.3	4.9 ± 0.9	4.9 ± 0.9
8c	6.0 ± 0.3	11.9 ± 0.4	11.1 ± 0.0	10.5 ± 0.9
8d	6.9 ± 0.3	10.5 ± 0.2	3.7 ± 0.0	9.9 ± 1.7
8e	14.6 ± 0.1	25.0 ± 4.3	1.9 ± 0.0	8.0 ± 0.9
8f	15.7 ± 0.8	18.1 ± 3.0	9.9 ± 0.9	1.2 ± 0.9
8g	4.1 ± 0.3	10.4 ± 3.6	35.2 ± 0.0	31.5 ± 0.0
8h	3.5 ± 0.3	9.2 ± 0.6	6.2 ± 0.9	9.9 ± 0.9
8i	54.1 ± 2.1	10.9 ± 0.6	6.2 ± 0.9	4.3 ± 0.9
4	20.8 ± 0.5	2.7 ± 0.0	32.7 ± 0.0	12.6 ± 0.0
Azoxystrobin	81.6 ± 0.0	54.7 ± 0.6	69.1 ± 0.0	79.8 ± 0.0
Fluopyram	6.0 ± 1.2	97.7 ± 0.0	97.8 ± 0.8	41.6 ± 0.7
Carbendazim	97.9 ± 0.4	100 ± 0.0	100 ± 0.9	58.0 ± 0.9

588 **Table 3.** EC_{50} values (μg/mL) of **6a~6h** and **7e** against *V. mali*, *C. lunata*, *F. graminearum* and *F.*

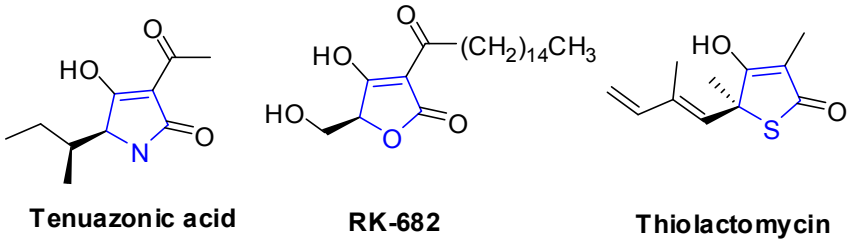
589 *oxysporum* f. sp. *lycopersici*

Fungi	Compd [a]	Toxicity regression equation *	R^2	EC_{50} (μg/mL)	95% CI [b] of EC_{50}
V. mali	**6a**	y=1.611x-1.041	0.837	4.43	2.30-9.89
	6b	y=1.303x-1.201	0.954	8.35	6.76-10.8
	6c	y=1.417x-0.688	0.906	3.06	1.73-5.19
	6d	y=1.364x-1.260	0.982	8.38	6.85-10.7
	6e	y=1.799x-1.172	0.988	4.48	3.86-5.22
	6f	y=1.328x-0.817	0.970	4.12	3.41-5.02
	6g	y=1.675x-1.233	0.837	5.44	2.84-13.8
	6h	y=0.919x-1.203	0.826	20.3	8.68-42.0
	7e	y=1.564x-1.981	0.959	18.5	14.5-25.6
	Azoxystrobin	y=0.583+0.304	0.916	0.30	0.034-0.872
	Fluopyram			>50.0	
	Carbendazim	y=1.876x-1.173	0.908	4.22	1.13-14.4
C. lunata	**6a**	y=1.252x-1.586	0.971	18.5	13.8-27.7
	6b	y=1.478x-2.164	0.948	29.1	19.0-57.7
	6c	y=1.602x-1.476	0.953	8.40	6.00-12.9
	6d	y=1.535x-2.123	0.947	24.2	18.2-36.3

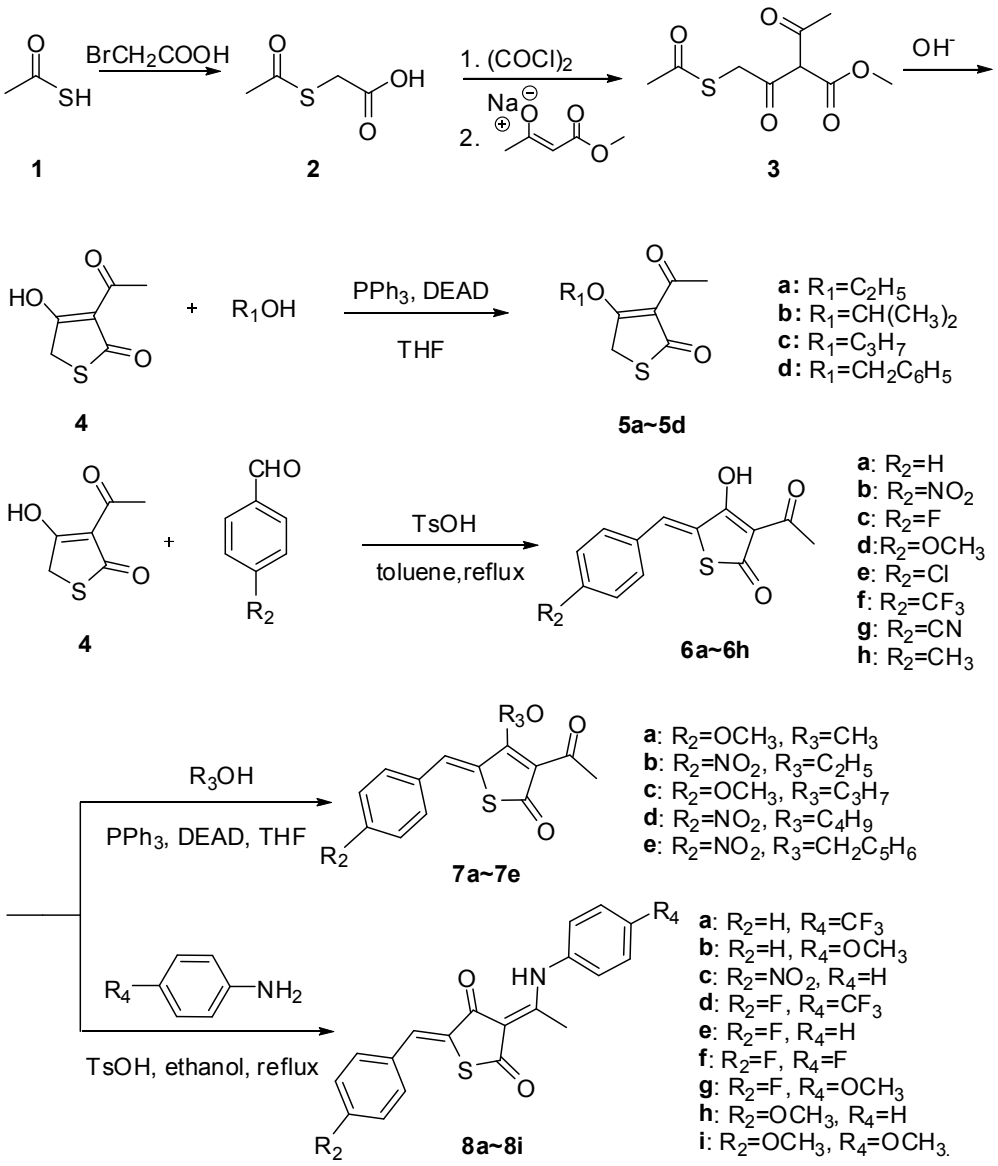
	6e	y=1.515x-1.091	0.975	5.30	4.43-6.31
	6f	y=0.844x-0.411	0.923	3.10	2.27-4.09
	6g	y=1.175x-1.909	0.933	42.2	26.7-87.8
	6h	y=0.985x-1.152	0.814	14.8	6.73-18.2
	Azoxystrobin	y=0.055x-0.366	0.896	6.70	4.61-11.8
	Fluopyram	y=0.891x+0.669	0.974	0.178	0.112-0.259
	Carbendazim	y=1.425x-2.308	0.842	41.7	19.8-48.6
F. graminearum	**6a**	y=0.996x-0.935	0.989	8.70	6.63-12.2
	6b	y=0.835x-0.939	0.978	13.3	9.29-22.6
	6c	y=0.873x-0.635	0.965	5.30	4.05-7.36
	6d	y=0.967x-1.279	0.965	21.1	14.4-37.2
	6e	y=0.731x-0.355	0.979	3.10	2.15-4.25
	6f	y=0.708x-0.396	0.940	3.60	2.57-5.17
	6g	y=0.796x-0.935	0.980	14.9	10.1-27.1
	6h	y=1.471x-1.539	0.978	11.1	9.05-14.3
	7e	y=1.254x-2.093	0.965	46.6	34.2-71.5
	Azoxystrobin	y=0.467x-0.632	0.946	22.4	9.05-30.7
	Fluopyram	y=0.780x+0.287	0.925	0.428	0.093-1.11
	Carbendazim	y=1.703x+0.641	0.947	0.420	0.166-0.995
F. oxysporum f.	**6a**	y=1.394x-1.227	0.950	7.60	5.32-12.2

sp. *lycopersici*	**6b**	y=1.468x-1.274	0.981	7.40	6.13-9.11
	6c	y=1.782x-1.780	0.971	10.0	8.42-12.9
	6d	y=1.233x-1.486	0.989	16.0	12.1-23.3
	6e	y=1.643x-1.467	0.971	7.80	6.59-9.47
	6f	y=1.549x-0.943	0.994	4.10	3.44-4.82
	6g	y=1.363x-1.359	0.949	9.90	6.82-17.2
	6h	y=1.630x-1.759	0.981	12.0	9.88-15.2
	7e	y=1.865x-1.799	0.960	9.20	6.57-14.6
	Azoxystrobin	y=1.086x-0.693	0.975	4.30	4.35-4.39
	Fluopyram			> 50.0	
	Carbendazim	y=1.885x+6.723	0.907	0.123	0.096-0.358

590 [a] Average of three replicates; [b] confidence intervals at 95% probability.


HO
O
N
O
Tenuazonic acid
HO
O
(CH2)14CH3
HO
O
O
RK-682
HO
S
O
Thiolactomycin

591 **Figure 1**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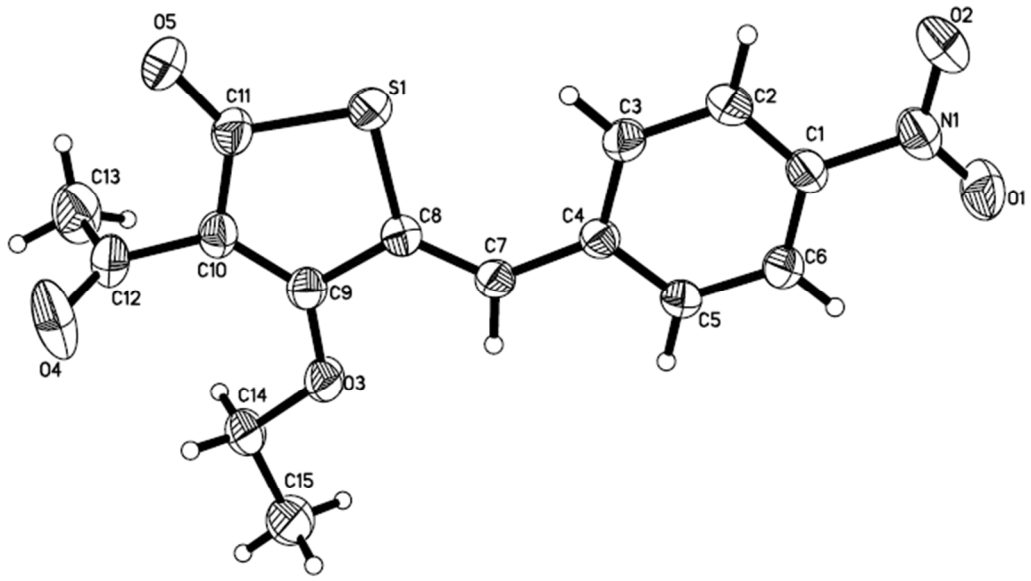
1. (COCl)2
2.
OH-
1
2
3
4
R1OH
PPh3, DEAD
THF
5a~5d
a: R1=C2H5
b: R1=CH(CH3)2
c: R1=C3H7
d: R1=CH2C6H5
TsOH
toluene,reflux
6a~6h
a: R2=H
b: R2=NO2
c: R2=F
d:R2=OCH3
e: R2=Cl
f: R2=CF3
g: R2=CN
h: R2=CH3
R3OH
PPh3, DEAD, THF
7a~7e
a: R2=OCH3, R3=CH3
b: R2=NO2, R3=C2H5
c: R2=OCH3, R3=C3H7
d: R2=NO2, R3=C4H9
e: R2=NO2, R3=CH2C5H6
TsOH, ethanol, reflux
8a~8i
a: R2=H, R4=CF3
b: R2=H, R4=OCH3
c: R2=NO2, R4=H
d: R2=F, R4=CF3
e: R2=F, R4=H
f: R2=F, R4=F
g: R2=F, R4=OCH3
h: R2=OCH3, R4=H
i: R2=OCH3, R4=OCH3.

592 **Figure 2**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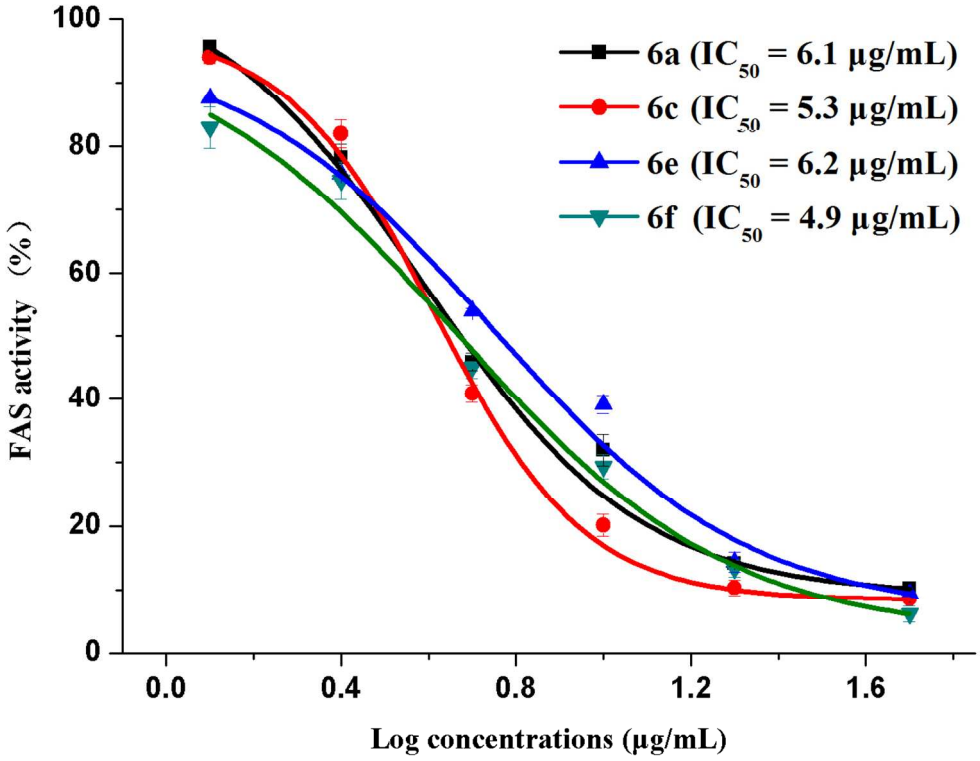
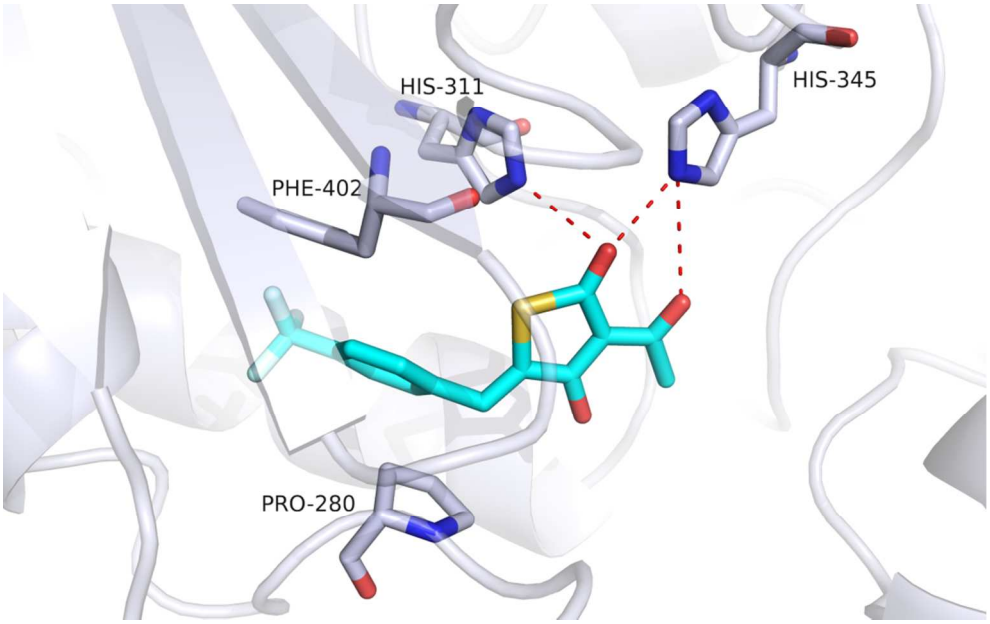
C2
C1
N1
O2
O1

593 **Figure 3**


100
80
60
40
20
0
FAS activity （%）
0.0
0.4
0.8
1.2
1.6
Log concentrations (μg/mL)
6a (IC50 = 6.1 μg/mL)
6c (IC50 = 5.3 μg/mL)
6e (IC50 = 6.2 μg/mL)
6f (IC50 = 4.9 μg/mL)

594 **Figure 4**



595 **Figure 5**

596 **ToC Graphic**