# Pocket-based Lead Optimization Strategy for the Design and Synthesis of Chitinase Inhibitors

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Supporting Information

ABSTRACT: Insect chitinases play an indispensable role in shedding old cuticle during molting. Targeting chitinase inhibition is a promising pest control strategy. Of ChtI, a chitinase from the destructive insect pest Ostrinia furnacalis (Asian corn borer), has been suggested as a potential target for designing green pesticides. A 4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate scaffold was previously obtained, and further derivatization generated the lead compound 1 as Of ChtI inhibitor. Here, based on the predicted binding mode of compound 1, the pocket-based lead optimization strategy was applied. A series of analogues was synthesized, and their inhibitory activities against Of ChtI were evaluated. Compound 8 with 6-tert-pentyl showed preferential inhibitory activity with a  $K_i$  value of 0.71  $\mu$ M. Their structure-activity relationships suggested that the compound with larger steric hindrance at the 6-nonpolar group was essential for inhibitory activity due to its stronger interactions with surrounding amino acids. This work provides a strategy for designing potential chitinase inhibitors.

**KEYWORDS:** chitinase, lead optimization, inhibitors, pesticide, Ostrinia furnacalis

# INTRODUCTION

Chitin is a homopolymer of N-acetyl-D-glucosamines (GlcNAc), linked with  $\beta$ -1,4 glycosidic bonds. It is the most important component of insect cuticle but is absent in the vertebrates.<sup>1</sup> Since the cuticle supports the shape of the exoskeleton in insects, it hardly expands with the growth of the insect body, and consequently, multiple molting cycles are required throughout the growth and development of insects.<sup>2</sup> Glycoside-hydrolase family 18 (GH18) chitinase (EC  $(3.2.1.14)^{3,4}$  participates in the molting stage and has the ability to hydrolyze chitin to chitosan oligosaccharides.<sup>5</sup> Therefore, targeting chitinase inhibition suggests a potential strategy for pest management.

Of ChtI is a chitinase derived from Ostrinia furnacalis (Asian corn borer) and is a member of the GH 18, which functions as an essential element in the chitin degradation of the old epidermis during the molting stage. It has been indicated as a potential target for the design of green pesticides. It possesses an open and groove-like cleft in which several binding subsites can be found, labeled as +2, +1, -1, -2, -3, -4, and -5; it also has endo enzymatic activity. A series of aromatic amino acids is located around the active pocket, such as Trp34, Phe61, Trp107, Trp223, and Trp372, and both stacking and hydrophobic interactions are important for the binding between ligand and receptor.<sup>6</sup> To date, few inhibitors against Of ChtI are known. A number of these have been derived from natural products, such as fully deacetylated chitooligosaccharides<sup>7</sup> and phlegmacin B1,<sup>8</sup> while others have been derived via virtual screening.<sup>9</sup> Although these are available, their inhibitory activities are weak, and their structures are complicated, which

makes further optimization more difficult. In summary, it is necessary to develop new, simple, and efficient Of ChtI inhibitors.

Substituted benzothiophene derivatives are essential compounds in agrochemical and pharmaceuticals.<sup>10</sup> These compounds have been reported to have antifungal,<sup>1</sup> antitubercular,<sup>12–14</sup> anti-inflammatory,<sup>15</sup> antimicrobial,<sup>16,17</sup> antianxiety,<sup>18</sup> antibacterial,<sup>19,20</sup> and anticancer<sup>21</sup> efficacy. In our previous work, the 4,5,6,7-tetrahydrobenzo[b]thiophene-3carboxylate derivative was identified as a potential inhibitor class against Of ChtI via structure-based virtual screening. Its further derivatization generated lead compound 1 (Figure 1).<sup>22</sup> Bioassay indicated that compound 1 has an inhibitory effect against Of ChtI. The predicted binding mode suggested a large modification space at its 6-position. Here, in combination with the nature of the amino acids around the active pocket, a



1 (*K*<sub>j</sub> = 1.50 μM)

Figure 1. Structure of lead compound 1.

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Figure 2. Synthetic route of compounds 2–10 and their precursors, 2a–10a. Reagents and conditions: (i) EtOH, morpholine, sulfur, reflux, 5 h; (ii) cyclopentylpropionyl chloride, acetone, reflux, 3–5 h.

pocket-based lead optimization strategy was applied. The obtained results form an expansion of prior work by synthesizing a variety of analogues with nonpolar and polar groups at the 6-position, and by evaluating their inhibitory effects against Of ChtI as well as their structure-activity relationships. By adopting this strategy, the inhibitory activities of the compounds against Of ChtI were effectively improved. This work provides a further perspective for chitinase inhibitors.

## MATERIALS AND METHODS

**Chemicals and Instruments.** All of the reagents were obtained from Innochem Incorporation (Beijing, China). The target compounds were purified via column chromatography on a silica gel 60 (200–300 mesh, Qingdao Haiyang Chemical Co., Qingdao, China). All melting points were determined by an X-4 binocular microscope (Fukai Corporation, Beijing, China) with uncorrected values. The <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of the target compounds were obtained via an AM-300 spectrometer (Bruker, Bremen, Germany) at 300 and 75 MHz, respectively. Deuterochloroform (CDCl<sub>3</sub>) was used as solvent, and tetramethylsilane (TMS) was used as internal standard. High-resolution mass spectrometry (HRMS) data were acquired via a 7.0T FTICR-MS instrument (Varian, Palo Alto, CA).

General Procedure for the Synthesis of Precursors 2a–10a. As shown in Figure 2, to a solution of substituted cyclohexanone (1 equiv) and propyl cyanoacetate (1 equiv) in ethanol, elemental sulfur (1 equiv) and morpholine (1 equiv) were added, and the mixture was stirred for 5 h at 85 °C. After the reaction was completed, the solution was evaporated, dissolved in ethyl acetate, washed in water, dried with anhydrous sodium sulfate, filtered, and ultimately evaporated for purification.

For the precursors 2a - 10a, the materials of substituted cyclohexanone are cyclohexanone, 4-ethyl cyclohexanone, 4-propyl cyclohexanone, 4-isopropyl cyclohexanone, 4-*tert*-butyl cyclohexanone, 4-pentyl cyclohexanone, 4-*tert*-pentyl cyclohexanone, 4-pentyl cyclohexanonexanonexanone, 4-pentyl cyclohexanone, 4-pen

2-Amino-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carboxylic acid propyl ester (**2a**) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 30:1, v/v). White solid; yield 91.44%; mp 84.7–85.7 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  5.99 (s, 2H), 4.17 (t, *J* = 6.5 Hz, 2H), 2.81–2.60 (m, 2H), 2.56–2.34 (m, 2H), 2.01–1.50 (m, 6H), 1.00 (t, *J* = 7.4 Hz, 3H).

2-Amino-6-ethyl-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid propyl ester (3a) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 30:1, v/v). Yellow solid; yield 93.69%; mp 53.6–54.6 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  5.98 (s, 2H), 4.16 (t, J = 6.5 Hz, 2H), 3.01-2.80 (m, 1H), 2.66–2.44 (m, 2H), 2.27–2.00 (m, 1H), 2.00–1.81 (m, 1H), 1.80–1.54 (m, 3H), 1.47–1.24 (m, 3H), 1.00 (t, J = 7.4 Hz, 3H), 0.94 (t, J = 7.4 Hz, 3H).

2-Amino-6-propyl-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carboxylic acid propyl ester (4a) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 30:1, v/v). Yellow solid; yield 79.60%; mp 45.1–46.1 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  6.00 (s, 1H), 4.16 (t, *J* = 6.5 Hz, 2H), 2.99–2.78 (m, 1H), 2.70– 2.44 (m, 2H), 2.24–2.03 (m, 1H), 1.95–1.80 (m, 1H), 1.83–1.61 (m, 3H), 1.50–1.21 (m, 5H), 1.00 (t, J = 7.4 Hz, 3H), 0.92 (t, J = 6.8 Hz, 3H).

2-Amino-6-isopropyl-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carboxylic acid propyl ester (**5a**) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 20:1, v/v). Yellow liquid; yield 71.63%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  5.98 (s, 2H), 4.16 (t, *J* = 6.5 Hz, 2H), 3.06–2.81 (m, 1H), 2.70–2.38 (m, 2H), 2.32–2.14 (m, 1H), 1.98–1.83 (m, 1H), 1.81–1.66 (m, 2H), 1.62–1.43 (m, 2H), 1.40–1.22 (m, 1H), 1.00 (t, *J* = 7.4 Hz, 3H), 0.94 (d, *J* = 6.7 Hz, 3H), 0.91 (d, *J* = 6.6 Hz, 3H).

2-Amino-6-*tert*-butyl-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carboxylic acid propyl ester (**6a**) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 30:1, v/v). Yellow liquid; yield 86.46%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  5.96 (*s*, 2H), 4.17 (t, *J* = 6.5 Hz, 2H), 3.11–2.82 (m, 1H), 2.60–2.37 (m, 2H), 2.35–2.21 (m, 1H), 2.03–1.88 (m, 1H), 1.81–1.63 (m, 2H), 1.54–1.40 (m, 1H), 1.37–1.21 (m, 1H), 1.01 (t, *J* = 7.4 Hz, 3H), 0.92 (*s*, 9H).

2-Amino-6-pentyl-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid propyl ester (7a) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 20:1, v/v). Yellow liquid; yield 88.86%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  5.99 (s, 1H), 4.16 (t, J = 6.5 Hz, 2H), 3.07–2.78 (m, 1H), 2.72–2.41 (m, 2H), 2.24–2.02 (m, 1H), 1.94–1.82 (m, 1H), 1.81–1.64 (m, 3H), 1.44–1.15 (m, 9H), 1.00 (t, J = 7.4 Hz, 3H), 0.89 (t, J = 6.3 Hz, 3H).

2-Amino-6-tertiary-pentyl-4,5,6,7-tetrahydro-benzo[b]thiophene-3carboxylic acid propyl ester (8a) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 20:1, v/v). Yellow liquid; yield 89.67%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  5.99 (s, 2H), 4.16 (t, J = 6.5 Hz, 2H), 3.10–2.84 (m, 1H), 2.59– 2.40 (m, 2H), 2.37–2.24 (m, 1H), 1.97–1.86 (m, 1H), 1.81–1.66 (m, 2H), 1.64–1.51 (m, 1H), 1.37–1.20 (m, 3H), 1.01 (t, J = 7.4 Hz, 3H), 0.86 (s, 3H), 0.84 (s, 3H), 0.83 (t, J = 7.5 Hz, 3H).

2-Amino-6-phenyl-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carboxylic acid propyl ester (**9a**) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 10:1, v/v). Yellow solid; yield 88.15%; mp 60.1–61.1 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.51–7.12 (m, 5H), 6.05 (s, 2H), 4.22 (t, *J* = 6.6 Hz, 2H), 3.15–2.91 (m, 2H), 2.87–2.54 (m, 3H), 2.19–2.03 (m, 1H), 2.00–1.84 (m, 1H), 1.84–1.70 (m, 2H), 1.04 (t, *J* = 7.4 Hz, 3H).

2-Amino-6-methoxyl-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carboxylic acid propyl ester (**10a**) was purified with column chromatography on a silica gel (petroleum ether/ethyl acetate = 8:1, v/v). Yellow liquid; yield 79.04%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  6.02 (s, 2H), 4.16 (t, *J* = 6.6 Hz, 2H), 3.78–3.54 (m, 1H), 3.40 (s, 3H), 3.02–2.61 (m, 3H), 2.57–2.41 (m, 1H), 2.07–1.93 (m, 1H), 1.86–1.61 (m, 3H), 0.99 (t, *J* = 7.4 Hz, 3H).

General Procedure for the Synthesis of the Target Compounds 2–10. As shown in Figure 2, the acetone was used as solvent, and 3-cyclopentylpropionyl chloride (1.5 equiv) was added dropwise to the precursors 2a-10a, respectively (1 equiv), to generate the target compounds 2-10. Then, the mixture was stirred for 3 to 5 h under reflux conditions. After the reaction was completed, the mixture was evaporated for purification.

2-(3-Cyclopentylpropanamido)-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid propyl ester (2) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 50:1, v/v). White solid; yield 90.85%; mp 57.5–58.5 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  11.27 (s, 1H), 4.19 (t, *J* = 6.5 Hz, 2H), 2.81–2.68 (m, 2H), 2.64–2.55 (m, 2H), 2.44 (t, *J* = 11.5 Hz, 2H), 1.87–1.66 (m, 11H), 1.64–1.39 (m, 4H), 1.18–1.04 (m, 2H), 0.99 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  169.74, 166.35, 147.44, 130.19, 126.07, 110.80, 65.75, 39.26, 35.85, 32.07, 31.11, 26.06, 24.78, 23.97, 22.63, 22.54, 21.72, 10.40. HRMS calculated for C<sub>20</sub>H<sub>29</sub>NO<sub>3</sub>S (M+H)<sup>+</sup>: 364.1941, found 364.1939.

2-(3-Cyclopentylpropanamido)-6-ethyl-4,5,6,7-tetrahydro-benzo-[*b*]thiophene-3-carboxylic acid propyl ester (3) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 50:1, v/v). White solid; yield 92.18%; mp 58.8–59.8 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  11.27 (s, 1H), 4.20 (t, *J* = 6.5 Hz, 2H), 2.98–2.85 (m, 1H), 2.78–2.53 (m, 2H), 2.45 (t, *J* = 7.5 Hz, 2H), 2.29–2.13 (m, 1H), 1.99–1.85 (m, 1H), 1.85–1.67 (m, 7H), 1.66–1.42 (m, 5H), 1.41–1.23 (m, 3H), 1.17–1.05 (m, 2H), 1.01 (t, *J* = 7.4 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  169.78, 166.38, 147.56, 130.18, 125.92, 110.64, 65.78, 39.27, 35.86, 35.69, 32.08, 31.12, 29.96, 28.59, 28.31, 25.92, 24.79, 21.73, 11.16, 10.42. HRMS calculated for C<sub>22</sub>H<sub>33</sub>NO<sub>3</sub>S (M + H)<sup>+</sup>: 392.2254, found 392.2244.

2-(3-Cyclopentylpropanamido)-6-propyl-4,5,6,7-tetrahydro-benzo-[*b*]thiophene-3-carboxylic acid propyl ester (4) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 40:1, v/v). White solid; yield 85.42%; mp 64.8–65.8 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  11.27 (s, 1H), 4.22 (t, *J* = 6.5 Hz, 2H), 2.99–2.84 (m, 1H), 2.78–2.55 (m, 2H), 2.46 (t, *J* = 12.1 Hz, 2H), 2.31–2.16 (m, 1H), 1.97–1.85 (m, 1H), 1.84–1.65 (m, 8H), 1.64–1.44 (m, 4H), 1.44–1.24 (m, 6H), 1.18–1.06 (m, 1H), 1.02 (t, *J* = 7.4 Hz, 3H), 0.91 (t, *J* = 6.8 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  169.80, 166.39, 147.57, 130.18, 125.95, 110.67, 65.79, 39.27, 37.85, 35.88, 33.63, 32.09, 31.13, 30.27, 28.96, 25.90, 24.80, 21.73, 19.74, 13.92, 10.42. HRMS calculated for C<sub>23</sub>H<sub>35</sub>NO<sub>3</sub>S (M + H)<sup>+</sup>: 406.2410, found 406.2405.

2-(3-Cyclopentylpropanamido)-6-isopropyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid propyl ester (**5**) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 50:1, v/v). Yellow liquid; yield 86.07%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 11.27 (s, 1H), 4.22 (t, *J* = 6.5 Hz, 2H), 3.06–2.91 (m, 1H), 2.76–2.52 (m, 2H), 2.45 (t, *J* = 7.7 Hz, 2H), 2.41–2.28 (m, 1H), 1.99–1.88 (m, 1H), 1.85–1.69 (m, 7H), 1.66–1.43 (m, 6H), 1.41–1.23 (m, 1H), 1.18–1.07 (m, 2H), 1.02 (t, *J* = 7.4 Hz, 3H), 0.95 (d, *J* = 4.6 Hz, 3H), 0.93 (d, *J* = 4.7 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 169.80, 166.38, 147.59, 130.24, 126.34, 110.61, 65.78, 40.57, 39.28, 35.88, 32.09, 31.65, 31.13, 27.36, 26.47, 26.21, 24.79, 21.73, 19.66, 19.31, 10.42. HRMS calculated for C<sub>23</sub>H<sub>35</sub>NO<sub>3</sub>S (M + H)<sup>+</sup>: 406.2332, found 406.2327.

2-(3-Cyclopentylpropanamido)-6-*tert*-butyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid propyl ester (**6**) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 50:1, v/v). Yellow liquid; yield 85.20%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 11.27 (s, 1H), 4.22 (t, *J* = 6.5 Hz, 2H), 3.13–2.90 (m, 1H), 2.74–2.30 (m, 5H), 2.08–1.94 (m, 1H), 1.86–1.68 (m, 7H), 1.66–1.38 (m, 5H), 1.35–1.22 (m, 1H), 1.17–1.07 (m, 2H), 1.02 (t, *J* = 7.4 Hz, 3H), 0.92 (s, 9H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 169.79, 166.38, 147.63, 130.24, 126.81, 110.51, 65.78, 44.64, 39.28, 35.88, 32.09, 32.05, 31.13, 27.21, 26.93, 25.46, 24.80, 24.16, 21.73, 10.44. HRMS calculated for C<sub>24</sub>H<sub>37</sub>NO<sub>3</sub>S (M + H)<sup>+</sup>: 420.2488, found 420.2483.

2-(3-Cyclopentylpropanamido)-6-pentyl-4,5,6,7-tetrahydro-benzo-[*b*]thiophene-3-carboxylic acid propyl ester (7) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 40:1, v/v). White solid; yield 79.67%; mp 45.5–46.5 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  11.28 (s, 1H), 4.21 (t, *J* = 6.5 Hz, 2H), 3.02– 2.83 (m, 1H), 2.78–2.53 (m, 2H), 2.46 (t, *J* = 8.4 Hz, 2H), 2.30– 2.13 (m, 1H), 1.98–1.85 (m, 1H), 1.84–1.65 (m, 8H), 1.65–1.43 (m, 4H), 1.42–1.20 (m, 9H), 1.21–1.06 (m, 2H), 1.02 (t, *J* = 7.4 Hz, 3H), 0.88 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  169.80, 166.40, 147.56, 130.18, 125.96, 110.66, 65.79, 39.27, 35.88, 35.59, 33.94, 32.09, 31.73, 31.13, 30.32, 29.00, 26.34, 25.92, 24.80, 22.32, 21.73, 13.73, 10.43. HRMS calculated for  $C_{25}H_{39}NO_3S\ (M + H)^+:$  434.2645, found 434.2639.

2-(3-Cyclopentylpropanamido)-6-*tert*-pentyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid propyl ester (**8**) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 40:1, v/v). Yellow liquid; yield 90.03%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 11.27 (s, 1H), 4.22 (t, *J* = 6.5 Hz, 2H), 3.10–2.95 (m, 1H), 2.69–2.29 (m, 5H), 2.02–1.88 (m, 1H), 1.84–1.68 (m, 7H), 1.65–1.44 (m, 5H), 1.41–1.20 (m, 4H), 1.17–1.06 (m, 1H), 1.02 (t, *J* = 7.4 Hz, 3H), 0.86 (s, 3H), 0.85 (s, 3H), 0.82 (t, *J* = 7.4 Hz, 3H), <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 169.81, 166.39, 147.61, 130.26, 126.94, 110.52, 65.79, 42.04, 39.28, 35.89, 34.35, 32.20, 32.09, 31.13, 27.26, 25.07, 24.80, 23.77, 23.65, 23.55, 21.74, 10.45, 7.78. HRMS calculated for C<sub>25</sub>H<sub>39</sub>NO<sub>3</sub>S (M + H)<sup>+</sup>: 434.2723, found 434.2721.

2-(3-Cyclopentylpropanamido)-6-phenyl-4,5,6,7-tetrahydro-benzo-[*b*]thiophene-3-carboxylic acid propyl ester (9) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 40:1, v/v). Yellow liquid; yield 78.03%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 11.33 (s, 1H), 7.50–6.97 (m, 5H), 4.27 (t, *J* = 6.6 Hz, 2H), 3.14–2.67 (m, 5H), 2.50 (t, *J* = 8.5 Hz, 2H), 2.26–2.06 (m, 1H), 2.01–1.88 (m, 1H), 1.89–1.70 (m, 7H), 1.68–1.47 (m, 4H), 1.24–1.10 (m, 2H), 1.05 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 169.95, 166.37, 147.83, 145.46, 130.05, 128.17, 126.53, 126.04, 125.72, 110.69, 65.92, 40.25, 39.31, 35.91, 32.13, 31.81, 31.15, 29.88, 26.48, 24.83, 21.77, 10.45. HRMS calculated for C<sub>26</sub>H<sub>33</sub>NO<sub>3</sub>S (M + H)<sup>+</sup>: 440.2254, found 440.2254.

2-(3-Cyclopentylpropanamido)-6-methoxyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid propyl ester (**10**) was purified by column chromatography on a silica gel (petroleum ether/ethyl acetate = 10:1, v/v). Yellow liquid; yield 85.54%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 11.28 (s, 1H), 4.22 (t, *J* = 6.6 Hz, 2H), 3.80–3.55 (m, 1H), 3.40 (s, 3H), 3.02–2.87 (m, 2H), 2.84–2.58 (m, 2H), 2.46 (t, *J* = 7.4 Hz, 2H), 2.09–1.94 (m, 1H), 1.91–1.67 (m, 8H), 1.63–1.54 (m, 2H), 1.54–1.41 (m, 2H), 1.18–1.07 (m, 2H), 1.02 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 169.95, 166.25, 148.08, 129.77, 123.15, 110.46, 75.09, 65.88, 55.71, 39.26, 35.86, 32.08, 32.02, 31.11, 29.48, 27.26, 24.79, 24.76, 23.70, 21.72, 10.41. HRMS calculated for C<sub>21</sub>H<sub>31</sub>NO<sub>4</sub>S (M + H)<sup>+</sup>: 394.1968, found 394.1964.

Molecular Docking. Molecular docking was performed using Molecular Operating Environment drug-discovery-modeling software (MOE 2018.01).<sup>23</sup> The X-ray crystal structure of Of ChtI with its ligand (GlcNAc)<sub>3</sub> (PDB ID: 3WL1) was used as a template. Prior to docking, the crystal structure was optimized using the Structure Preparation module in MOE, mainly to correct the structure and to prepare macromolecular data for further computational analysis. Following that, molecular docking was conducted using the Dock module in MOE with two rounds of calculation. To generate a large number of poses, the Triangle Matcher method was used first, which generated poses by aligning ligand triplets of atoms on the triplets of alpha spheres in a more systematic way; then the Induce Fit Receptor method, in which the protein side chains are flexible, was used to further refined. GBVI/WSA dG, which estimates the free energy of binding of the ligand from a given pose, was used to score each of the generated poses. Finally, the Fingerprint Cluster method was used to classify the poses according to the binding mode and interaction between ligand and receptor, and similar binding poses were grouped into one class. Representative poses were selected for further study.

**Molecular Dynamics Simulation.** Molecular dynamics (MD) simulations were performed starting with the optimal binding poses obtained from molecular docking using the AMBER 12 software package.<sup>24</sup> The antechamber module was used to generate small molecule templates with generalized AMBER force fields (GAFF).<sup>25</sup> The complex was solvated with TIP3P water models in a 10 Å cubic box,<sup>26</sup> and Na<sup>+</sup> was added to reduce the total number of static charges to zero. Protein parametrization was performed via the tleap module using ff99SB force field. System energy minimization was conducted with both the steepest descent method and the conjugate gradient method. The system was gently annealed from 0 to 298 K, using a Langevin thermostat. The NPT ensemble was performed using a

Berendsen barostat at 1 atmospheric pressure and 298 K at subsequent stages.<sup>27</sup> Finally, a 20 ns molecular dynamics simulation was performed using the PMEMD module.<sup>28</sup>

**Binding Energy Calculation.** Molecular mechanics generalized born surface area (MM-GBSA) calculations were conducted using the AMBER 12 software package. The final equilibrium stage from the molecular dynamics simulation was used for the calculation. Both water and Na<sup>+</sup> were stripped out of the complex, and a series of snapshots (equally spaced at 20 ps intervals) were extracted from the MD trajectory. For each snapshot, the free energy was calculated for complex, protein, and ligand. The binding free energy (*G*) was calculated according to the following formulas, using the MM-GBSA method:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{protein}} - G_{\text{ligand}} \tag{1}$$

For each piece, G was calculated in line with the following formulas:

$$G = G_{\rm gas} + G_{\rm solv} \tag{2}$$

$$G_{\rm gas} = E_{\rm el} + E_{\rm vdw} \tag{3}$$

$$G_{\rm solv} = E_{\rm gb} + E_{\rm surf} \tag{4}$$

Here,  $G_{\rm gas}$  represents the total gas-phase free energy;  $G_{\rm solv}$  represents the solvation free energy;  $E_{\rm el}$  represents the electrostatic energy;  $G_{\rm vdw}$  represents the van der Waals energy;  $E_{\rm gb}$  represents the polar solvation energy; and  $E_{\rm surf}$  represents the nonpolar solvation energy.

Protein Expression and Purification. The expression and purification of Of ChtI was conducted according to previously published literature.<sup>29</sup> Briefly, the recombinant plasmid pPIC9-Of ChtI was electroporated into Pichia pastoris GS115 (Invitrogen, Carlsbad, CA). Then, the cells were grown in BMGY medium (2% peptone, 1% yeast extract, 0.2% biotin, 1.34% yeast nitrogen, 1% glycerol, 10 mL of 20 mM potassium phosphate, and 90 mL of water, at a pH of 6.0) at 30 °C for 24 h. Following this, the mixture was placed into BMMY medium (2% peptone, 1% yeast extract, 0.2% biotin, 1% methanol, 1.34% yeast nitrogen, 50 mL of 20 mM potassium phosphate, and 450 mL of water, at a pH of 6.0) at 30 °C for 72 h. After that, the mixture was centrifuged, and the supernatant was precipitated with 70% ammonium sulfate at 4 °C. The obtained precipitate was dissolved and purified via metal-chelating chromatography with a 1 mL nickel-NTA-Sepharose high-performance column (GE Healthcare, Shanghai, China). The target protein was collected in buffer (20 mM sodium dihydrogen phosphate and 200 mM imidazole, at pH 7.4), SDS-PAGE was used to analyze its purity, and a BCA protein-assay kit (TaKaRa, Beijing, China) was used for quantitation.

**Enzymatic Assay.** Methylumbelliferyl-N,N'-diacetyl- $\beta$ -D-chitobioside (Sigma, Shanghai, China) was used as substrate to determine the enzymatic activity. The test system was 100 mL, which consisted of 2  $\mu$ L of DMSO or chemicals, 88  $\mu$ L of premix of protein and 20 mM sodium phosphate buffer (pH 6.0), and 10  $\mu$ L of substrate. The mixture with DMSO was used as positive control. After a 20 min of incubation at 30 °C, 100  $\mu$ L of 0.5 M Na<sub>2</sub>CO<sub>3</sub> was added to terminate the reaction, and the fluorescence of the released 4-methylumbelliferone was quantitated via microplate reader, using excitation and emission wavelengths of 360 and 450 nm, respectively. Each set of experiments was conducted in triplicate. For the determination of the  $K_i$  value, the variety concentration of chemicals and three substrate concentrations (1, 2, and 4  $\mu$ M) were used. The  $K_i$  values and inhibition types were determined via linear fitting of the data in Dixon plots.

#### RESULTS AND DISCUSSION

**Optimization of Lead Compound and Bioassay for Analogues.** To predict the binding mode, the lead compound 1 was docked into the binding domain of *Of* ChtI, using the DOCK module of MOE, while the X-ray crystal structure of *Of* 

ChtI with its ligand (GlcNAc)<sub>3</sub> (PDB ID: 3WL1) was used as a template. One hundred poses were outputted. These poses were then clustered into different groups according to the fingerprint of the complex, and the classification that contained the most abundant poses was selected for further analysis. In this classification, the poses occupied the subsites from -1 to -3 and were rank-ordered in line with the GBVI/WSA dG generated scores, which provided indications for the accuracies and stabilities of the dockings: a more negative score indicates a more stable interaction. The pose with the lowest score was retained and submitted to the MD simulation to assess the stability of the complex as well as to gain further insight into protein-ligand interactions. After 20 ns of molecular dynamics simulation, the root-mean-square-deviation (RMSD) value on all heavy atoms of the entire simulation was calculated with respect to the starting structure (Figure 3). For the complex,



Figure 3. Time evolution of the RMSD values (calculated on  $\alpha$  carbons) of the complexes between *Of* ChtI and compounds 1–10 analyzed by MD simulations.

an initial increase was observed due to the equilibration of the system. After that, the system was stabilized with an RMSD value around 1.24 Å. The last 2 ns of the simulation were considered as stable and 100 evenly spaced-out snapshots were extracted from these. The MM-GBSA approach was used to calculate the free binding energy and the decomposition free energy between protein and ligand. The output is shown in Table 1.

The dominant conformation of the Of ChtI-compound 1 from the last 2 ns was selected for analysis. The 4,5,6,7tetrahydro-benzo[b]thiophene of compound 1 was found to occupy the subsite -1 and formed an H- $\pi$  interaction with Trp372; the cyclopentane occupied the subsite -3 and formed a H $-\pi$  interaction with Trp34; the carbonyl oxygen atom of amide group formed a hydrogen bond with Trp107; the 3propyl formate formed a hydrophobic interaction with Trp272 and Phe309 (Figure 4A). The presence of these interactions stabilized the binding between Of ChtI and compound 1. Interestingly, during the process of MD simulation, the indole group of Trp107 was quickly deflected and formed a  $\pi - \pi$ stacking effect with the 4,5,6,7-tetrahydro-benzo[b]thiophene of compound 1 (Figure 4B). This further stabilized the binding; however, the existence of this phenomenon still requires further experimental exploration. Furthermore, the decomposition free energy calculations showed that these amino acids contributed strongly to the binding energy and played a key role in the combination (Figure 4C). A hydrophobic cavity (named S1), which was formed by Tyr30, Phe61, Ala188, Met215, Tyr217, and Trp372, was extended near the subsite -1, and the subsite +1 also indicated

	predicted $\Delta G$ (kcal/mol)						
compound	$E_{ m vdw}$	$E_{\rm el}$	$E_{ m gb}$	$E_{ m surf}$	$\Delta G_{ m gas}$	$\Delta G_{ m solv}$	$\Delta G_{ m total}$
1	-50.47	-6.65	18.71	-5.44	-57.13	13.27	-43.86
2	-46.48	-7.60	20.04	-4.91	-54.08	15.12	-38.96
3	-55.75	-7.49	22.45	-6.09	-63.24	16.36	-46.88
4	-54.17	-5.48	19.50	-5.90	-59.64	13.60	-46.04
5	-53.83	-6.60	18.69	-5.64	-60.43	13.05	-47.38
6	-55.98	-8.09	21.21	-6.05	-64.07	15.16	-48.91
7	-55.58	-6.57	22.35	-6.10	-62.15	16.25	-45.90
8	-56.84	-6.32	21.39	-6.43	-63.17	14.96	-48.21
9	-57.81	-4.26	20.55	-6.44	-62.06	14.11	-47.95
10	-38.70	-9.66	23.71	-4.53	-48.36	19.18	-29.18



Figure 4. (A) Interactions between *Of* ChtI and compound 1 shown in 2D diagram. (B) Deflection of Trp107 in the MD simulation. (C) Decomposition free energy of *Of* ChtI with compound 1 calculated by MM-GBSA. (D) Docking-predicted binding mode of compound 1 in the *Of* ChtI active site and the hydrophobic cavities at S1 and S2 regions.

the existence of such a hydrophobic cavity (named S2), which was formed by Tyr149, Pro190, Met215, andTyr217 (Figure 4D). The 6-methyl of compound 1 was located at the mouth of the S1 cavity (Figure 4D). These extended spaces provided favorable conditions for the reasonable optimization of the lead compound 1 at its 6-position. Therefore, an expansion was performed by substituting a variety of nonpolar groups with different steric hindrance at the 6-position of compound 1 to increase the inhibitory activity against *Of* ChtI, while the polar

group served as control. Finally, nine designed compounds were subjected to synthesis and bioassay.

The target compounds were easily and abundantly obtained in line with Figure 2. The reagents and materials that were applied in the synthesis were easy to purchase, and the synthetic route resulted in high atom economy with good utilization of reactant atoms in the end products. The results of the bioassay showed that the inhibitory activities of compounds 3-9 were improved compared to that of lead compound 1 and were also better than that of the control  $(GlcN)_5$ . In particularly, compounds 6, 8, and 9, exhibited  $K_i$  values of 0.98, 0.71, and 0.76  $\mu$ M, respectively (Table 2).

Table 2. Inhibitory Activities of Compounds 1–10 against Of Chtl

aammaumd	$R^1$ —	inhibition rate(	Ki	
compound		10 µM	$2 \mu M$	(µM)
1	-ξ-CH3	91.8±0.6	66.8±0.9	1.50
2	-ۇ-H	86.6±1.9	47.0±6.5	1.65
3	-ۇ-CH₂CH₅	93.3±1.3	69.0±2.5	1.23
4	↓	95.1±0.6	80.2±3.1	1.35
5	-#-<	94.0±0.2	76.3±2.0	1.22
6	-\$~	93.5±2.0	76.4±3.3	0.98
7	$+ \sim \sim$	95.6±0.6	86.9±1.4	1.09
8		94.0±0.7	79.8±1.0	0.71
9	-}	94.6±0.3	76.2±0.8	0.76
10	-ۇ-OCH₃	39.9±2.4	$ND^{a}$	ND <sup>a</sup>
(GlcN)5 <sup>b</sup>	_	$45.9 \pm 0.4^{c}$	$ND^{a}$	$ND^{a}$
	,			

<sup>*a*</sup>Not determined. <sup>*b*</sup>Control compound. <sup>*c*</sup>Determined at 20  $\mu$ M.

Compared to the lead compound 1, the 6-position substituents of compounds 3-9 showed larger steric hindrance and therefore had stronger interactions with the surrounding hydrophobic amino acids. Thus, they had better inhibitory activities. In contrast, the inhibitory activity of compound 2, with a hydrogen atom at 6-position, was slightly weaker than compound 1. However, there was a significant decrease in the inhibitory activity of compound 10 (6-OCH<sub>3</sub>), with inhibition rate of 39.9% at 10  $\mu$ M. In summary, it is beneficial to improve the inhibitory activity that the 6-group is nonpolar and has a large steric hindrance, which also confirms the design idea.

Structure–Activity Relationship Analysis of the Target Compounds. To better understand the different inhibitory activities of the target compounds 2-10, against *Of* ChtI, the molecular docking and MD simulation were performed to study the structure–activity relationships. The

research methods and procedures are consistent with those applied for lead compound 1, as described above. Figure 3 shows the time evolution of the RMSD calculated on all heavy atoms during the simulation time compared to the starting structures. Identical to compound 1, for each complex, an initial increase was observed due to the equilibration of the simulation system, which was followed by a stabilization of the RMSD value around 1.24, 1.43, 1.19, 1.38, 1.34, 1.34, 1.38, 1.43, 1.43, and 1.36 Å with ligands 2-10, respectively. The last 2 ns were considered stable, and from these, the dominant conformations of compounds 2–10 with Of ChtI were selected for analysis. Their binding modes were identical to those of compound 1, which binged at the subsites -1 to -3. Furthermore, the H- $\pi$  interactions of the target compounds with Trp372 and Trp34, hydrogen bond interaction with Trp107, and the hydrophobic interaction with Tyr272 and Phe309 were maintained. During the simulation, the indole group of Trp107 was still quickly deflected to form a  $\pi - \pi$ stacking effect with the 4,5,6,7-tetrahydro-benzo[b]thiophene of the compound. This phenomenon still requires further experimental data.

The position where the 6-group was located extended into S1 or S2 depending on its steric effect. The group with a smaller steric hindrance, such as methyl, ethyl, and methoxyl, tended to extend toward the S1 region; in contrast, the group with larger steric hindrance, such as propyl, isopropyl, tertbutyl, pentyl, tert-pentyl, and phenyl, tended to extend toward the S2 region (Figure 5). All of these formed van der Waals interactions with the surrounding hydrophobic amino acids. The results of the binding free energy calculated by MM-GBSA indicated that the van der Waals interaction played a major role. With the increase of steric hindrance of 6-substituent, the van der Waals interaction showed an increasing trend, and the combined free energy also increased (Table 1). From the perspective of the decomposition of free energy, in addition to the large contribution of Trp372, Trp34, Trp107, Tyr272, and Phe309, the amino acids located in the S1 or S2 region (Phe61, Ala188, Met215, and Tyr217) also contributed greatly. This also indicated that the interactions between the 6-substituent and these amino acids were important. Here, compound 8 was used as an example. The dominant conformations of compounds 8 with Of ChtI indicate H- $\pi$ interactions with Trp372 snd Trp34, hydrogen bond interaction with Trp107, and hydrophobic interaction with Tyr272 and Phe309 (Figure 6). The 6-tert-pentyl extended to the S2 region and formed van der Waals interactions with



Figure 5. (A) Predicted binding modes of compounds 1 (yellow), 2 (blue), 3 (magenta), and 10 (orange) in the Of ChtI active site. (B) Predicted binding mode of compounds 4 (green), 5 (cerulean), 6 (brown), 7 (red), 8 (pink), and 9 (gray) in the Of ChtI active site.



Figure 6. Interactions between *Of* ChtI and compound 8 shown in 2D diagram.

Phe61, Met215, and Try217. Moreover, from the perspective of the decomposition of free energy, these residues contributed greatly (Figure 7).



Figure 7. Decomposition free energy of *Of* ChtI with compound 1 calculated by MM-GBSA.

The present work employed a pocket-based lead optimization strategy for the efficient design of a series of target compounds against *Of* ChtI that have simpler structures than known inhibitors. Furthermore, the synthetic route was easy to perform, and the compounds resulted in high atom economy. The inhibitory activities of compounds 3-9 were found to be relatively stronger than the lead compound 1 as well as the known inhibitor (GlcN)<sub>5</sub>. Structure–activity relationships and MM-GBSA calculations provided insight into their interactions with *Of* ChtI. This work is an example of the integration of computational and experimental studies to obtain potent *Of* ChtI inhibitors. This method can be expanded to similar research to drive pesticide discovery more efficiently.

# ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b00837.

 $K_i$  values of compounds 3–9 against *Of* ChtI; <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of the compounds 2–10 and 2a–10a (PDF)

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#### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

GlcNAc, N-acetyl-D-glucosamine; GH18, glycoside hydrolase family 18; MD, molecular dynamics; MM-GBSA, molecular mechanics generalized born surface area

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