Small-molecule inhibitors of insect chitinases have potential applications for controlling insect pests. Insect group II chitinase (ChtII) is the most important chitinase in insects and functions throughout all developmental stages. However, the possibility of inhibiting ChtII by small molecules has not been explored yet. Here, we report the structural characteristics of four molecules that exhibited similar levels of inhibitory activity against O/ChtII, a group II chitinase from the agricultural pest Asian corn borer Ostrinia furnacalis. These inhibitors were chito-otocaoase (GlCN)₉, dipryrido-pyrimidine derivative (DP), piperidine-thienopyridine derivative (PT), and naphthalimide derivative (NI). The crystal structures of the O/ChtII catalytic domain complexed with each of the four inhibitors at 1.4–2.0 Å resolutions suggested they all exhibit similar binding modes within the substrate-binding cleft; specifically, two hydrophobic groups of the inhibitor interact with +1/+2 tryptophan and a −1 hydrophobic pocket. The structure of the (GlCN)₉ complex surprisingly revealed that the oligosaccharide chain of the inhibitor is orientated in the opposite direction to that previously observed in complexes with other chitinases. Injection of the inhibitors into 4th instar O. furnacalis larvae led to defects in development and pupation. The results of this study provide insights into a general mechanistic principle that confers inhibitory activity against ChtII, which could facilitate rational design of agrochemicals that target ecdysis of insect pests.

Chitinous cuticle forms an exoskeleton that protects insects from environmental stresses and mechanical damage. Periodic ecdysis is necessary for insects to overcome the rigidity of the cuticle during growth and development (1, 2). Because plants, humans, and other mammals lack chitin, interference with or disruption of insect ecdysis might provide an eco-friendly strategy to control insect pests.

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The atomic coordinates and structure factors (codes 6JAV, 6JAW, 6JAX, and 6JAY) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Group II chitinase (ChtII), which is an enzyme indispensable for insect ecdysis at all developmental stages, is a member of the glycoside hydrolase family 18 (GH18). It has a high molecular weight and contains four or five catalytic domains and four to seven chitin-binding domains (3–6). Typically, one or two catalytic domains have substitutions of the proton donor, glutamate, in a conserved catalytic pocket indicating that they may lack catalytic activity, while retaining substrate-binding ability (7). However, two or more catalytic domains in the C-terminal region are presumed to be catalytically active. Recently, two active catalytic domains of O/ChtII (PDB entries 5Y29 and 5Y2A), a ChtII from the lepidopteran pest Ostrinia furnacalis, have been expressed and crystallized (8). The catalytic properties and structural characteristics of O/ChtII suggest that it may carry out the initial decrystallization in the degradation of cuticular chitin. Molting defects have been seen after RNAi silencing of the ChtII gene in several insect species (9–11).

Considering the importance of ChtII, it is an attractive idea to block its activity by small-molecule inhibitors for pest control. Several inhibitors of GH18 chitinases have been isolated from natural resources and their inhibitory mechanisms have been studied (12). Most of these inhibitors fall into two general categories, carbohydrate-based inhibitors and cyclic peptide-based inhibitors. Carbohydrate-based inhibitors mimic the structure of oxazolinium ion intermediates, such as allosamidin and its derivatives (13–15). Cyclic peptide-based inhibitors, such as argifin, argadin, and their derivatives, mimic the carbohydrate-protein interactions (16–21). By virtual screening, other inhibitors were also obtained, including methyloxanthine derivatives (22–24), closantel derivatives (25, 26), berberine (27), styloguanidine (28) and psammaplins (29). These compounds exhibit inhibitory activities at nanomolar to millimolar levels. However, most are derived from natural products and difficult to synthesize and obtain in bulk.

In our previous works, we observed that compounds bearing a large conjugated plane were likely to be potent inhibitors of GH18 chitinase (27, 30). In this study, exploiting our previously reported two constructs of O/ChtII (O/ChtII-C1, residues

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1606–1992: OfChtII-C2, residues 2056–2438) (8), we screened the compound library of our lab which contains a mass of compounds with a large conjugated plane and obtained four potent inhibitors. They possess distinctively different structures but exhibited similar inhibitory activities. In addition, we revealed their binding mechanism by X-ray crystallographic analysis. This work is a first report to provide a starting point for creation of a rational design for inhibitors of ChtII.

Results

Inhibition of OfChtII by small molecules

OfChtII contains two catalytically active domains (OfChtII-C1 and OfChtII-C2) with similar sequences, hydrolase activities, and structural characteristics (8). Using OfChtII-C1 as a representative, we obtained four structurally distinct compounds that showed potent inhibitory activities with $K_i$ values in the micromolar range, including chitoctaose ((GlcN)$_8$), dipyrido-pyrimidine derivative (DP), piperidine-thienopyridine derivative (PT), and naphthalimide derivative (NI) (Fig. 1). These compounds also exhibited similar inhibitory activity toward OfChtII-C2 (Table 1). To gain molecular insights into the principles underlying the mechanism of OfChtII inhibition, we solved the structures of OfChtII-C1 in complex with each of the four inhibitors (Table 2).

Interactions between OfChtII-C1 and (GlcN)$_8$

Fully deacetylated chitooligosaccharide (GlcN), which mimics natural substrates of chitinases, were reported to be chitinase inhibitors (31). As they could be easily derived from chitin, whereas the furan group was inserted into the substrate-binding cleft between $-1$ and $+2$ subsites and stabilized by hydrophobic interactions between inhibitor and protein (Fig. 3A). The dipyrido-pyrimidine moiety stacked with two conserved tryptophan Trp$^{1621}$/Trp$^{1809}$ and formed a sandwich structure, whereas the furan group was inserted into the $-1$ subsite and was well-accommodated in the hydrophobic pocket harboring Trp$^{1621}$/Tyr$^{1803}$/Tyr$^{1856}$/Phe$^{1899}$/Trp$^{1961}$. In addition, water-mediated hydrogen bonds between Trp$^{1621}$ and N2 of the dipyrido-pyrimidine moiety reinforced the binding.

Interactions between OfChtII-C1 and piperidine-thienopyridine derivative

PT inhibits OfChtII efficiently with a $K_i$ value in the low micromolar level. The structure of the complex was obtained and refined to 1.4 Å (Table 2). PT bound along the substrate-binding cleft from $-1$ to $+2$ subsite. The piperidine-thienopyridine moiety stacked well with two conserved tryptophan at subsites $+1$ and $+2$, whereas the chlorobenzene group was located in the hydrophobic pocket at $-1$ subsite. In addition, a

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### Table 1

$K_i$ values of inhibitors for OfChtII-C1 and OfChtII-C2

| Inhibitor     | OfChtII-C1 ($\mu$M) | OfChtII-C2 ($\mu$M) |
|---------------|---------------------|---------------------|
| (GlcN)$_8$    | 38.43               | 32.91               |
| DP            | 1.99                | 1.64                |
| PT            | 1.72                | 1.33                |
| NI            | 2.18                | 2.48                |
Novel group II chitinase inhibitors

Table 2
X-ray data collection and structure-refinement statistics

| PDB entry | (GlcN)₈ | DP | PT | NI |
|-----------|--------|----|----|----|
| 6lAX | 6lAY | 6lAY | 6lAY |
| Space group | P₄₁₂₁₂ | P₄₁₂₁₂ | P₄₁₂₁₂ | P₄₁₂₁₂ |

| Unit-cell parameters | a (Å) | b (Å) | c (Å) | α (°) | β (°) | γ (°) | Wavelength (Å) | Temperature (K) | Resolution (Å) | Unique reflections | Observed reflections | Rmerge | Rfree |
|----------------------|-------|-------|-------|-------|-------|-------|----------------|----------------|---------------|-------------------|-------------------|--------|--------|
| 6lAX | 98.600 | 98.600 | 98.600 | 90.0 | 90.0 | 90.0 | 0.97776 | 100 | 50.0–1.70 (1.73–1.70) | 52,050 | 1,813,545 | 0.0166 | 12.6 (12.2) | 0.010 |
| 6lAY | 98.529 | 98.529 | 98.529 | 90.0 | 90.0 | 90.0 | 0.97776 | 100 | 50.0–1.50 (1.53–1.50) | 75,000 | 2,984,123 | 0.097 | 14.0 (12.8) | 0.007 |
| 6lAV | 94.447 | 94.447 | 94.447 | 90.0 | 90.0 | 90.0 | 0.97930 | 100 | 50.0–1.44 (1.46–1.44) | 85,416 | 2,881,019 | 0.103 | 16.5 (12.1) | 0.010 |
| 6lAW | 94.884 | 94.884 | 94.884 | 90.0 | 90.0 | 90.0 | 0.97930 | 100 | 50.0–2.00 (2.03–2.00) | 32,657 | 1,405,507 | 0.179 | 12.7 (12.6) | 0.010 |

Water molecules 30.75 27.64 29.15 33.37
Protein atoms 17.61 14.90 16.12 23.34
Average B factor (Å²) 20.00 16.84 17.79 24.23
Wilson B factor (Å²) 17.06 12.65 13.19 21.50
Bond angles (°) 1.216 1.008 1.109 0.801
Bond lengths (Å) 0.010 0.007 0.010 0.005
Outliers 0.0 0.0 0.0 0.0
Allowed 1.1 1.6 1.3 1.6
Favored 98.9 98.4 98.7 98.4

Water-mediated hydrogen bond between Asp<sup>1804</sup> and N3 of the pyridine ring further stabilized the binding of the inhibitor (Fig. 3B).

**Interactions between OfChtII-C1 and naphthalimide derivative**

NI is a compound derived from naphthalimide, in which the naphthalimide group is connected with a morpholine group through a small alkyl chain. The structure of the complex was determined at a resolution of 1.9 Å (Table 2). Although they possess different scaffold structures, the inhibitory activity and binding mode of NI toward OfChtII were similar to those of DP and PT (Fig. 3D). The naphthalimide moiety in NI interacted with the tryptophan at subsite +1 and +2 by hydrophobic stacking and the morpholine group pointed to the hydrophobic pocket at the −1 subsite. Besides these common features, specific interactions were also detected, including the morpholine group forming extra polar interactions with Asp<sup>1804</sup>/Trp<sup>1961</sup> (Fig. 3C).

**In vivo activity of the inhibitors**

To test the in vivo activity, the inhibitors were injected into 4th instar, day 1 O. furnacalis larvae. As shown in Fig. 4, 5 days after injection, the control group larvae which were injected with 4% DMSO all survived, whereas 27% of the DP-injected and 17% of the PT-injected larvae died with their bodies shrunk seriously. The new head capsule and cuticle had formed and tanned whereas the old cuticle remained unhydrolyzed, which trapped these larvae and killed them. Twelve days after injection, 93% of the control group larvae molted into normal pupa, whereas nearly 27–36% of experimental group larvae were arrested at the larva stage. Especially for the DP-injected insects, only 30% of the larvae molted into normal pupa and 6% of the larvae molted into abnormal pupa (Fig. 4 and Fig. S3). The trapped larvae could not pupate even 15 days post injection.

**Discussion**

**Fully deacetylated chitooligosaccharides are moderate inhibitors of ChtII**

Because GH18 chitinases employ a substrate-assisted mechanism in which the C2-acetamido group of the −1 sugar acts as the catalytic nucleophile to attack the anomeric carbon (33–36), substrate analogs can be inhibitors of chitinases. In fact, mixed randomly deacetylated chitooligosaccharides with different chain lengths have been reported to inhibit bacterial chitinasin B from Serratia marcescens (SmChiB) (37), and recently we have shown that fully deacetylated chitooligosaccharide (GlcN) exhibited inhibitory activity toward different GH18 chitinases, including group I chitinase (OfChtI) and chitinase h (OfChi-h) from O. furnacalis (31, 32). Moreover, injection of mixed (GlcN)<sub>3</sub>→ into O. furnacalis resulted in the arrest of 85% of the larvae at the larval stage; larvae failed to shed the old cuticle and finally died (31).

Here we found fully deacetylated chitooligosaccharides also showed inhibitory activity toward OfChtII catalytic domains. However, structural comparison of the enzyme-inhibitor complex between OfChtII and other chitinases revealed several differences in the binding mode of the inhibitor (Fig. S2). First, in
the complexes OfChi-h/(GlcN)$_7$ and OfChlI/(GlcN)$_5$, the inhibitor bound along the substrate-binding cleft in the same manner as that observed in substrate-enzyme complexes, in which the nonreducing end of the oligosaccharide chain occupied the minus subsites and the reducing end of oligosaccharide chain lay on the positive subsites. In contrast, in the complex OfChlII-C1/(GlcN)$_8$, the oligosaccharide chain of (GlcN)$_8$ was orientated opposite to that observed in the substrate-enzyme complex. Second, the inhibitor was forced to form a bent conformation in the substrate-binding cleft of OfChi-h or OfChlI.

The GlcN moiety at the $-1$ subsite penetrated into the active pocket and adopted an unfavorable conformation. It was stabilized by a number of polar interactions, particularly hydrogen bonds between the pyranose ring of GlcN$_1$ and glutamate of the catalytic DXDXE motif. For OfChlII-C1/(GlcN)$_8$, the inhibitor maintained a linear conformation and lying along the substrate-binding cleft and preventing interactions between GlcN$_1$ and the active pocket. (GlcN)$_8$ bound to OfChlII-C1 mainly via stacking interactions between sugar rings and aromatic residues from $-3$ to $+2$ subsites. Therefore, fully deacetylated chitooligosaccharides exhibited only moderate inhibitory activities against OfChlII.

**Interactions between OfChlII-C1 and inhibitors showing a common inhibitory feature**

The other three compounds studied were derived from different parent structures but inhibited the OfChlII catalytic domains to the same degree. Exploiting a recently identified crystallizable construct of OfChlII (OfChlII-C1), we determined its structures in complex with different inhibitors.
Novel group II chitinase inhibitors

Intriguingly, we found that the inhibitors bound to the substrate-binding cleft in nearly identical conformations (Fig. 3). Therefore, general principles for O/ChtII inhibition could be deduced. Firstly, hydrophobic interactions between the large hydrophobic groups of the inhibitors and two conserved tryptophans at +1 and +2 subsites secured the binding of the compound within the substrate-binding cleft, specifically the dipyrido-pyrimidine group in DP, the piperidine-thienopyridine group in PT, and the naphthalimide group in NI. The importance of hydrophobic interactions between compound and +1/+2 subsites in inhibitory activity is also observed in other GH18 chitinases. The structures of seven inhibitors in complex with Vibrio harveyi chitinase A (VhChiA), or its mutant, revealed that although they occupied the active site in three different binding modes, at least one inhibitor molecule was clearly defined and restricted almost exclusively to the stacking interactions at +1/+2 subsites (38). Second, several aromatic residues formed a hydrophobic pocket at subsite −1. The small hydrophobic group of inhibitors penetrated into this hydrophobic pocket, providing additional stabilization energy for maintaining their binding conformation. Furthermore, enhanced affinity is achieved if side chains of the inhibitors make other interactions, including water-mediated hydrogen bonds. Based on these observations, we speculate that a compound containing a large hydrophobic group and a small hydrophobic group connected by a linker harboring two to three atoms, would be likely to inhibit O/ChtII. These two hydrophobic groups could form conserved interactions with the +1/+2 tryptophan and the −1 hydrophobic pocket, respectively.

The potential applications of ChtII inhibitors

Because chitin degradation is crucial for arthropod development, small molecule inhibitors against chitinases have potential applications as pesticides. Among the numerous chitinases in insects, ChtII seems to be the most important one for cuticle degradation. Specific knockdown of ChtII transcripts in Tribolium castaneum (TcChtII) prevented embryo hatch, larval molting, pupation, and adult metamorphosis (9). Therefore, blocking the activity of ChtII by small molecule inhibitors is an attractive idea for pest control. In this study, we also evaluated the in vivo bioactivity of these inhibitors. Compared with the control group, nearly 30% of the larvae in three experimental groups were arrested at the 5th instar and failed to molt into pupa. The phenotype is consistent with the results observed in the O/ChtII RNAi experiments, in which the larvae kept in the 1st instar stage because of the defective molt after injecting or feeding specific dsRNA (10). Besides, 37% of the DP-injected and 17% of the PT-injected larvae died during development. These observations indicated that the inhibitors have great potential to agrochemical development. There are still many questions to be solved before using at large scale in an agricultural setting, such as the synthesis, solubility, and transmission of the compounds. However, our results provided a good starting point to develop and modify novel agrochemicals targeting ChtII.

In summary, we first report novel inhibitors of O/ChtII-C1 and the crystal structures of O/ChtII-C1 in complex with different inhibitors. The conserved binding mode among different inhibitors provides critical insights into a general principle underlying inhibitory activity against O/ChtII. Furthermore, in view of the essential function of ChtII in insects, this work will also facilitate rational design of agrochemicals targeting ecdysis of insect pests.

Experimental procedures

Protein expression and purification

O/ChtII-C1 and O/ChtII-C2 were expressed and purified as described previously (8). Briefly, the DNA encoding the target protein with a His6 tag at the N-terminal was cloned into pPIC9 vector and transformed into Pichia pastoris GS115 strain (Invitrogen). Fermentation broth was collected and subjected to ammonium sulfate precipitation. The precipitate was resuspended and purified with a HiTrap FF affinity column (GE Healthcare). The desired protein was eluted with buffer containing 20 mM sodium phosphate (pH 7.4), 0.5 mM sodium chloride, and 250 mM imidazole. The purity of the sample was analyzed by SDS-PAGE. Pure protein was desalted in buffer containing 20 mM Tris (pH 7.5) plus 50 mM NaCl, and concentrated to 10 mg/ml for crystallization.

Inhibition activity assays of chitinase

Chitinase inhibition activities were assayed in end-point experiments using an artificial substrate, 4-methylumbelliferol β-d-N,N’-diacetylchitobioside hydrate (MU-(GlcNAc)$_2$, Sigma-Aldrich) (21, 31). The assay components were incubated in a final volume of 100 μl at 30 °C for 30 min in the presence of 20 mM sodium phosphate buffer (pH 6.0) containing DMSO at a final concentration of 2%, enzyme, inhibitor, and 40 μM MU-(GlcNAc)$_2$. Then enzyme reaction was stopped by addition of 100 μl 0.5 M sodium carbonate solution and fluorescence of the released 4-methylumbelliferone was quantified (excitation 366 nm, emission 445 nm) with a Varioskan Flash microplate reader (Thermo Fisher Scientific). The inhibition constant (K_i) was determined using a similar method at three concentrations of the substrate (40, 20, and 10 μM) and then reciprocal plots of 1/velocity versus inhibitor concentration were constructed. Data analysis was performed with Prism software (GraphPad Software Inc., San Diego, CA).

Crystallization

Crystals were generated at 4 °C by mixing 1 μl reservoir solution with an equal volume of sample and were equilibrated against 600 μl of reservoir solution using the hanging-drop vapor-diffusion method. Native O/ChtII-C1 crystals were grown in the reservoir solution containing 0.2 M sodium chloride, 0.1 M Tris (pH 8.5), and 25% PEG3350. Inhibitor complexes were obtained by soaking the native crystals in mother liquor containing 5–10 mM for each inhibitor overnight prior to cryoprotection with 25% (v/v) glycerol in reservoir solution. Then the crystals were subsequently flash-cooled in liquid nitrogen for storage.

Data collection and structure determination

All diffraction data were collected using the National Facility for Protein Science Shanghai (NFPS) beam line BL18U at
Shanghai Synchrotron Radiation Facility. The diffraction data sets were processed using the HKL-3000 package (39). Structures were determined by molecular replacement with Phaser using native OjChtII-C1 (PDB: 5Y29) as the search model (40). Iterative molecular models were manually built and extended using Coot (41), followed by refinement with PHENIX (42). Structural figures were prepared by PyMOL (Schrödinger, LLC, New York, NY). The data collection and structure refinement statistics are summarized in Table 2.

In vivo activity evaluation of inhibitors

*O. furnacalis* larvae were fed an artificial diet under 70% relative humidity and 16:8 light-dark photoperiod at 26–28°C as described previously (31). The larvae at day 1 of the 4th instar were selected for the microinjection experiments. In the experimental groups, 2 μL of inhibitors (0.5 mM, dissolved in 4% DMSO) were injected into the penultimate abdominal segment of larvae. The larvae injected with 2 μL of 4% DMSO were used as a control group. Each group contained 10 individual larvae with three independent replicates. The treated larvae were reared under identical conditions as described above. Developmental defects and mortality were recorded every day.

**Author contributions**—W. C. and Q. Y. conceptualization; W. C. and Y. Z. software; W. C. and Y. Z. formal analysis; W. C. investigation; W. C. visualization; W. C. and Y. Z. methodology; W. C. writing—original draft; Y. Z. and Q. Y. data curation; Y. Z. and Q. Y. validation; Q. Y. resources; Q. Y. supervision; Q. Y. funding acquisition; Q. Y. project administration; Q. Y. writing—review and editing.

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**Novel group II chitinase inhibitors**
Novel group II chitinase inhibitors

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