Response to enantiomers of (Z3Z9)-6,7-epoxy-octadecadiene, sex pheromone component of *Ectropis obliqua* Prout (Lepidoptera: Geometridae): electroantennagram test, field trapping, and in silico study

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Abstract

Limited information on sex pheromone recognition by tea moths, *Ectropis obliqua* Prout (Lepidoptera: Geometridae), exists for this economically important pest of tea (*Camellia sinensis* L.; Theaceae). Pheromone binding proteins (PBPs), a sub-family of odorant-binding proteins, control transportation of pheromone molecules that may contribute to the discrimination of sex pheromone components. It has been reported previously that *Eobl*PBP1 gene (a pheromone binding protein of *E. obliqua*) is highly expressed in antennae of the male moth. Based on this information, a reliable model of *Eobl*PBP1 was constructed by homology modeling using the enantiomers of Z3Z9-6,7-epo-18:Hy docked into the hydrophobic cavity of the model. Docking results suggested similar binding affinities of this enantiomer to *Eobl*PBP1. However, electroantennogram and field trapping experiments of *E. obliqua* males revealed that response to Z3Z9-(6S,7R)-epo-18:Hy was significantly greater than the opposite configuration, and suggested enantiomeric discrimination could occur on sex pheromone receptors of this species of tea moth.

Key Words: enantiomers; homology modeling; molecular docking; discrimination; *Eobl*PBP1

Resumen

Información limitada existe sobre el reconocimiento de la feromona sexual por la polilla del té, *Ectropis obliqua* Prout (Lepidoptera: Geometridae), una plaga del té de importancia económica (*Camellia sinensis* L.; Theaceae). Las proteínas de unión de feromonas (PBP), una subfamilia de proteínas de unión a odorantes, controlan el transporte de moléculas de feromonas que pueden contribuir a la discriminación de los componentes de las feromonas sexuales. Se ha informado previamente que el gen *Eobl*PBP1 (una proteína de unión a la feromona de *E. obliqua*) se expresa altamente en las antenas de la polilla macho. Basándose en esta información, se construyó un modelo confiable de *Eobl*PBP1 mediante un modelo de homología utilizando los enantiómeros de Z3Z9-6,7-epo-18:Hy acoplado en la cavidad hidrófoba del modelo. Los resultados de acoplamiento sugirieron afinidades de unión similares de este enantiómero a *Eobl*PBP1. Sin embargo, el electroantenograma y los experimentos de captura de campo de machos de *E. obliqua* revelaron que la respuesta a Z3Z9-(6S, 7R)-epo-18:Hy fue significativamente mayor que la configuración opuesta y sugirió que podría existir discriminación enantiomérica en los receptores de feromonas sexuales de esta especie de polillas del té.

Palabras Clave: enantiómeros; modelo de homología; acoplamiento molecular; discriminación; *Eobl*PBP1

The tea geometrid, *Ectropis obliqua* Prout (Lepidoptera: Geometridae) is a serious defoliator of tea bushes, *Camellia sinensis* L. (Theaceae), in southeastern China. Outbreak populations of this phytophagous pest can completely defoliate a tea bush, thereby causing severe reduction of tea production with considerable economic damage (Hazarika et al. 2009; Zhang et al. 2014). Heavy application of synthetic pesticides are still widely used to control this pest, which can result in undesirable residues on tea leaves and soil causing adverse health and environmental effects (Hazarika et al. 2001; Ye et al. 2014). The use of mating disruption strategies that employ area-wide application of sex pheromones to manage *E. obliqua* populations has become a promising alternative to pesticide applications (Hazarika et al. 2009). Attempts to identify sex pheromones of *E. obliqua* began 30 yr ago, but the components still have not been accurately identified (Li et al. 1988; Yao et al. 1991; Liu et al. 1994). Yang et al. (2015) examined the bioactive compounds extracted from female sex pheromone glands of *E. obliqua* and identified them as Z3Z9-6,7-epo-18:Hy (major component) and Z3Z6Z9-18:Hy (secondary component) (Fig. 1A). These researchers also reported on the behavioral responses of male moths of this species to these components in wind-tunnel and field bioassays.

Insect sex pheromones are hydrophobic molecules, so odorant binding proteins are needed to transport these molecules across the hydrophilic antennal lymph to olfactory receptor neurons located in male sensilla (Gómez et al. 2008). Odorant binding proteins are a group of small, soluble proteins in the sensillum lymph and are regarded as a first-layer filter to differentiate odorant molecules. Based on amino acid sequences and structural characteristics, lepidopteran odorant binding proteins can be divided into several groups, including pheromone

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Fig. 1. Sex pheromone components of *Ectropis obliqua* Prout. (A) sex pheromone components; (B) enantiomers of Z3Z9-6,7-epo-18:Hy.

binding proteins (PBPs) and general odorant binding proteins (Pelosi & Maida 1995). Pheromone binding proteins mainly bind to insect sex pheromones, and are thought to be involved in pheromone reception processes (Sun et al. 2013a; Jin et al. 2014) while general odorant binding proteins are believed to detect plant volatiles and sex pheromones (Liu et al. 2015a). Although several crystalline structures of pheromone binding proteins have been characterized, cloning, expression, and identification of their component parts have proven to be expensive and time consuming. Homology modeling, based on comparison of target and templates, provides a promising alternative to predict protein structures (Venthur et al. 2014). The modeled structures of pheromone binding proteins have been reported in several Lepidoptera insects such as *Bombyx mori* L. (Lepidoptera: Bombycidae) (Gräter et al. 2006), *Lymantria dispar* L. (Lepidoptera: Lymantriidae) (Honson et al. 2003), *Spodoptera litura* F. (Lepidoptera: Noctuidae) (Liu et al. 2012), and so on. Furthermore, the structures of pheromone binding proteins, prediction of binding site, clarification of binding mode, and interactions of protein-ligands can be performed by molecular docking and molecular dynamics (Mutis et al. 2014).

Recently, 2 enantiomers of the major sex pheromone components produced by female *E. obliqua*: Z3Z9-(6R,7S)-epo-18:Hy (1) and Z3Z9-(6S,7R)-epo-18:Hy (2) (Fig. 1B) have been synthesized successfully in a previous study by the current authors including electroantennagram responses (Yu et al. 2017). Moreover identification, characterization of odorant binding proteins, and their expression patterns from *E. obliqua* also have been accomplished (Sun et al. 2017). The *Eobl*PBP1 protein previously has been found to be highly expressed in antennae of male moths, and has provided us an opportunity to understand the sex pheromone recognition in this species (Ma et al. 2016a). We report here on results from electroantennograms and field trap collections to examine the attractiveness of Z3Z9-6,7-epo-18:Hy enantiomers of female *E. obliqua* to males. Furthermore, a homology modeling method was used to construct structures of *Eobl*PBP1 protein and molecular docking used to investigate the binding affinities of the different sex pheromone enantiomers.

Materials and Methods

INSECTS

Moths were collected by sweep net from Dong-zhi County (30.1000°N, 117.0200°E), Anhui Province, People's Republic of China, and identified as *E. obliqua* (Yang et al. 2015). Insects were maintained in a plastic jar (240 mL) covered with nylon mesh and reared at 25 °C, 60% to 70% RH, and a 14:10 (L:D) photoperiod. Eggs collected from females were very carefully transferred to another jar, and maintained in dry conditions until hatching. Hatched larvae were reared on fresh tea leaves under ambient environmental conditions in our laboratory. After pupation, pupae were sexed and maintained on moist sand (10:1 sand:water) until emergence. Adults that emerged were maintained separately in 240 mL plastic jars(Fukang Plastic Co. Ltd., Dongguan, Guangdong, China) with 10% honey soaked in cotton ad libitum.

CHEMICALS

Z3Z6Z9-18:Hy (a triene, chemical purity > 98%) was prepared from linolenic acid as described previously by Liu et al. (1994). A mixture of 3 corresponding racemic monoepoxydienes of this triene was prepared by oxidation with m-chloroperbenzoic acid. Flash chromatography was performed on silica gel to obtain racemic Z3Z9-6,7-epo-18:Hy containing hexane:benzene (5:1). Enantiomers of Z3Z9-6,7-epo-18:Hy were synthesized with chemical purity > 95% and *ee* > 80% from *cis*-2-butene-1,4-diol as previously reported by Yu et al. (2017).

ELECTROANTENNAGRAM MEASUREMENT

Electroantennagram recordings were conducted with an EAG 2014 system (Syntech Co., Hilversum, Netherlands) consisting of a probe/micromanipulator (MP-15), a data acquisition interface box (serial IDAC-2), and a stimulus air controller (CS-55). Before electroantennagram measurements, the system was cleaned using an active charcoal purified stream of air. Male *E. obliqua* antennae were freshly excised from 1-d-old individuals, and mounted onto an antenna holder in the electroantennagram probe electrogel (Spectro 360, Parker Laboratory, Princeton, New Jersey). A continuous air stream (flow rate, 2 L per min) created by the stimulus air controller was allowed to flow toward the antenna. Epoxide racemate and enantiomers of Z3Z9-6,7-epo-18:Hy were dissolved in n-hexane to produce 1 mg per mL solutions. It has been reported that (Z)-3-hexenol can elicit stable electroantennagram responses to antenna of male *E. obliqua* (Sun et al. 2014). Therefore, 10 µL (Z)-3-hexenol solution (0.005µg per µL) was used as the reference stimuli and 10 µL of n-hexane used as the solvent control (Luo et al. 2017). Ten µL of each test solution was placed on a small strip of qualitative filter paper (0.5 × 2.5 cm, fast-speed type; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and allowed to evaporate for 30 s. At this time, the strip was introduced into a Pasteur pipette (Jiechen Instruments Co. Ltd., Shanghai, China) (15 cm long) with its tip inserted into the tubing (8 mm in diam) up to 8 cm before the exit of the continuous air stream. The base of the pipette was connected to the pulsed air stream from the stimulus air controller. Stimuli with the test chemicals were provided to the insect antennae by puffing

pulsed air for 0.3 s (flow rate, 2 L per min) at the base of the pipette. Each recording was repeated 6 times with different antennae in the following sequence: n-hexane, reference compound, test compound (3 times), reference compound, and n-hexane. Control-adjusted electroantennagram responses of the stimuli were presented as proportional responses relative to the reference stimuli (Zhang et al. 2014). Mean data were analyzed using a 1-way analysis of variance (ANOVA) followed by LSD multiple comparison test. Differences were considered significant at $P < 0.05$.

FIELD EXPERIMENTS

Field trapping experiments were conducted in a tea orchard in Xian-Ning (29.8700°N, 114.2800°E), Hubei, China, during May 2016. A pheromone-baited water pan trap was used in all field experiments (Fig. 2). Traps were baited with synthetic compounds in a 4:6 ratio of triene; 6,7-epoxide enantiomers were injected into a green rubber septa (6 mm OD; Pherobio Technology Co., Ltd., Beijing, China) that was suspended at 1.3 m from ground surface and spaced 10 m apart. Pheromone compounds were dissolved in distilled hexane to produce 1 mg per mL solutions and 10 µL of total volume injected into a blank septa. Septa injected with distilled hexane were used as a control. All septa were stored at 4 °C until use. Each treatment had 8 replicates, and trap positions within each replicate were randomized. Trap catches were checked the next d. Statistical significance of the differences between treatments was assessed by 1-way ANOVA, followed by Duncan multiple range test ($P < 0.05$).

ALIGNMENT AND HOMOLOGY MODELING

A BLAST search (Altschul et al. 1990) of the amino acid sequence of *Eobl*PBP1 was conducted against the current Protein Data Bank database (https://www.rcsb.org/) to find structural templates. In this case, the crystalline structure of *Bmor*PBP (Protein Data Bank ID: 1DQE) at a resolution of 1.8 Å was chosen as the template for *Eobl*PBP1. Multiple sequence alignment was generated using the DNAMAN 8 software program (Lynnon Biosoft, San Ramon, California, USA). Using sequence alignments and the software template, Modeller 9.18 (Šali & Blundell 1993) was used to construct a 3-dimensional structure of *Eobl*PBP1 using a comparative protein modeling method (i.e., homology modeling). Ten candidates were generated by Modeller and the structure with the lowest DOPE (Discrete Optimized Protein Energy) score was chosen as a solution. To refine the protein, the relax module of program Rosetta was used to give final structure of the target protein (Das & Baker 2008).

MOLECULAR DOCKING

The structures of female *E. obliqua* sex pheromone components, Z3Z9-6,7-epo-18:Hy (6S,7R- and 6R,7S- enantiomer), were created and optimized using chemBio3D Ultra 13 (PerkinElmer, Billerica, Massachusetts, USA). Molecules were applied with MM2 forcefield and saved in a Protein Data Bank format. The Protein Data Bank files were uploaded to AutoDockTools (The Scripps Research Institute, La Jolla, California, USA) and saved as PDBQT format for docking. The AutoDockTools

Fig. 2. Field trial of *Ectropis obliqua* Prout. (A) Water pan trap; (B) male moths captured.

software was used also to assign polar hydrogens and add Gasteiger charges to the protein, then the structure saved in PDBQT file format. After preparation of protein and ligands, structures were docked into the model of *Eobl*PBP1 using the AutoDock Vina 1.1.2 software (The Scripps Research Institute, La Jolla, California, USA). A docking grid with a size of 18 Å × 18 Å × 18 Å was used, and the grid center was set at 7.332 (X-axis), 22.972 (Y-axis), 3.629 (Z-axis). Free energy scores for ligands were calculated, and the lowest energy of the ligand-protein complexes was selected for analysis.

Results

In the laboratory electroantennagram bioassays, male *E. obliqua* antennal response to 6S,7R-enantiomer was at the same level as epoxide racemate, but significantly greater than responses to the 6R,7S-enantiomer ($P < 0.05$) (Fig. 3). The results suggest that (6R,7S)-enantiomer may not be the effective configuration.

In the field trapping experiment, the number of *E. obliqua* males captured in water pan traps was significantly greater for the 6S,7R-enantiomer (blend A) compared with 6R,7S-enantiomer (blend B) and control ($P < 0.05$) (Fig. 4).

Electroantennagram and field trapping results suggested a chiral discrimination of 1 or more particular proteins at the molecular level. In order to understand the interaction between sex pheromone molecules and the protein, structural information of *Eobl*PBP1 was needed. Currently, there are no NMR or X-ray data for *Eobl*PBP1. Therefore, based on the templates and our alignment results, the 3D model of *Eobl*PBP1 was successfully generated. After refinement, the Verify Score of models by Profiles-3D was 65.13 (theoretical score 66.0542 and 29.7244) for *Eobl*PBP1, implying that overall quality of the predicted *Eobl*PBP1 structure was reliable. In addition, the Ramachandran plot of *Eobl*PBP1 revealed that 95.8% of the residues were in the favored region, 4.2% of the residues were in the allowed region, and none were in the disallowed region (Fig. 5C).

Results of the predicted 3D structure showed that *Eobl*PBP1 is compact and a stable globular protein that contains 6 α-helical domains: α1 (residues Glu6–Glu26), α2 (residues Ile32–Asn38), α3 (residues Pro49–Leu62), α4 (residues His76–His82), α5 (residues Asp86–Ser103), and α6 (residues Arg109–Leu127) (Fig. 5B). All 6 antiparallel α-helices form a compact hydrophobic binding pocket. Although there still are several hydrophilic amino acids inside the binding cavity, Thr117 and Arg121 interacted with the epoxy ring of ligands. Not surprisingly, 3 pairs of disulphide bridges were found in the structure that was confirmed to be a common feature of insect pheromone binding proteins.

We found no discrimination between *Eobl*PBP1 and ligands in our docking experiments, although the results showed that orientation and conformation of Z3Z9-6,7-epo-18:Hy enantiomers were clear. Two enantiomers were anchored in the same binding cavity with opposite epoxy ring orientation (Fig. 6C), surrounded by hydrophobic amino acids. No

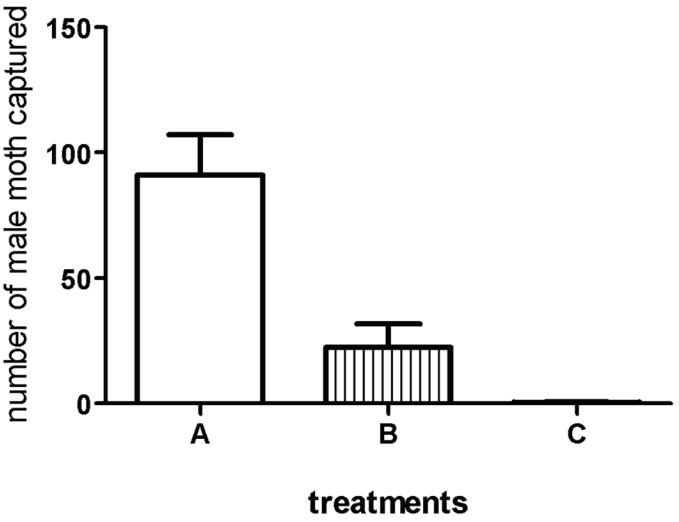

Fig. 4. Pan trap catches of male *Ectropis obliqua* Prout baited with binary blends of racemic and enantiomers of Z3Z9-6,7-epo-18:Hy with triene in Xian-Ning County, China. (A) Z3Z9-6S,7R-epoxy-18:H (6 µg) + Z3Z6Z9-18:H (4 µg); (B) Z3Z9-6R,7S-epoxy-18:H (6 µg) + Z3Z6Z9-18:H (4 µg); (C) blank lure (control). Data are mean ± SD (n = 8) of male *E. obliqua* trap catches in Xian-Ning County, China, May 2016 by 3 different combinations compared with water pan trap.

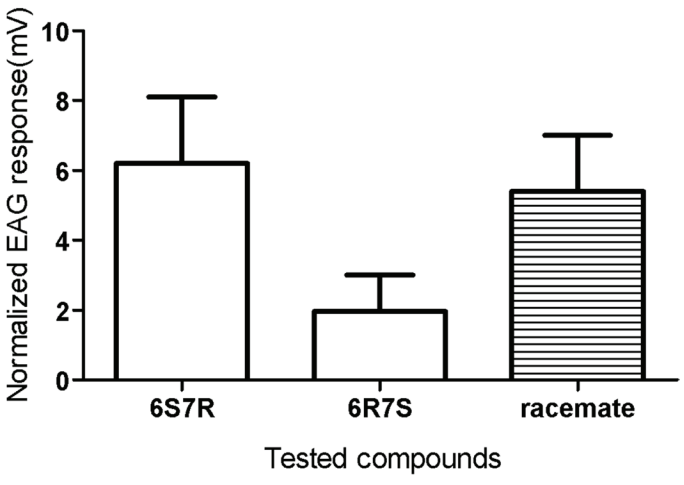

Fig. 3. Electroantennagram responses of male *Ectropis obliqua* Prout antenna to racemate and enantiomers of Z3Z9-6,7-epo-18:Hy. Data are mean ± SD (n = 6) of male *E. obliqua* antennal responses by 2 enantiomers and racemate of Z3Z9-6,7-epo-18:Hy: Z3Z9-6S,7R-epoxy-18:H, Z3Z9-6R,7S-epoxy-18:H, racemate.

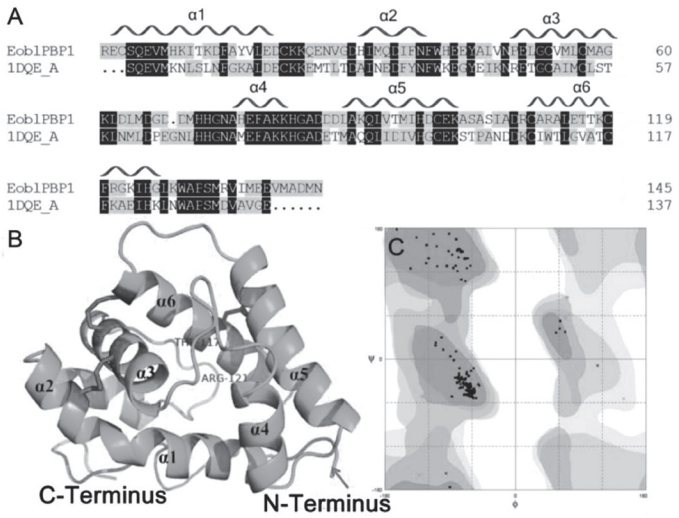

Fig. 5. Modeled 3D structure and validation of *Eobl*PBP1. (A) Sequence alignment of *Eobl*PBP1 and template 1DQE_A. α-helices are displayed as squiggles. Identical residues are highlighted in white letters with deep blue background. (B) Overall structure of the *Eobl*PBP1. Three disulfide bonds are in red. N-terminus, C-terminus, and α-helices are labeled. Two potential key residues: Thr117 and Arg 121 are in orange. (C) Ramachandran plot of *Eobl*PBP1.

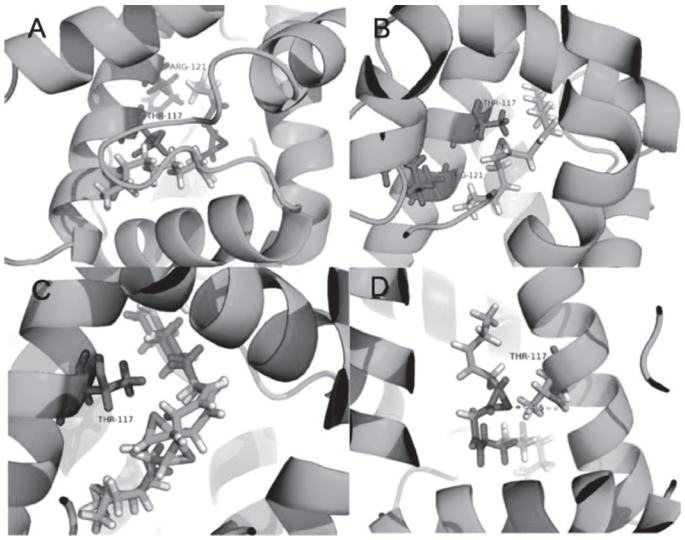

Fig. 6. Results of docking Z3Z9-6S,7R-epoxy-18:H and Z3Z9-6R,7S-epoxy-18:H into predicted model of *Eobl*PBP1. (A) Orientation of Z3Z9-6R,7S-epoxy-18:H in protein *Eobl*PBP1. Supposed key residues are in red and labeled. (B) Orientation of Z3Z9-6S,7R-epoxy-18:H in protein *Eobl*PBP1. Supposed key residues are in red and labeled. (C) Alignment of 2 enantiomers in different binding conformation. (D) The distance between oxygen in epoxy ring (Z3Z9-6S,7R-epoxy-18:H) and hydroxyl hydrogen in Thr 117 is labeled in yellow dotted line.

expected H-bonds were formed between Thr 117, Arg 121, and ligands (Figs. 6A, B). Van der Waals and hydrophobic interactions were important linkages between *Eobl*PBP1 and epoxide ligands. When Z3Z9-6S,7R-epoxy-18:H was docked into the protein, the distance of oxygen in the epoxy ring and hydroxyl hydrogen in Thr 117 was found to be 4.5 Å (Fig. 6D). Moreover, interaction energies between ligands and *Eobl*PBP1 confirm that Z3Z9-6S,7R-epoxy-18:H and Z3Z9-6R,7S-epoxy-18:H have similar binding affinity, though Z3Z9-6R,7S-epoxy-18:H has a lower energy value and is predicted to bind better than the opposite configuration (Table 1).

Discussion

We found that the 6R,7S-enantiomer consistently produced a lower response and captured fewer male *E. obliqua*; this was caused by the presence of a "wrong" enantiomer. In this case, enantio-enriched but not enantio-pure Sharpless asymmetric epoxidation had occurred. Previously, epoxidation has been reported to account for 5% or more "incorrect" enantiomer synthesis (Katsuki & Sharpless 1980). We believe that a lower response of the 6R,7S-enantiomer could have been caused by the presence of trace amounts of 6S,7R-enantiomer. Mori (2007) investigated the stereochemistry-bioactivity relationships among pheromones and classified them into 10 categories. Based on our electroantennagram and field trapping results with that of Kenji's investigation (Mori 2007), we could assume that 6S,7R-enantiomer is bioactive, and its opposite enantiomer does not inhibit or promote a response in male *E. obliqua*. This is the most common relationship with about 60% of chiral pheromones belonging to this group. Pheromones in this category can be used as their racemates in practical applications (Mori 2007). Recently, Z3,Z9-6,7-epoxy-18:H and Z3,Z6,Z9-18:H also has been identified as a component of sex pheromone in *Ectropis grisescens* Warren (Lepidoptera: Geometridae) (Ma et al. 2016b). In a related study, Z3,Z9-6,7-epoxy-19:H was identified as a key substance which caused premating isolation of the 2 sibling moths: *E. grisescens* and *E. obliqua* (Luo et al. 2017). All of the reports were based on the racemate of Z3,Z9-6,7-epoxy-18:H. Considering the important role chirality plays, it is possible that these 2 sibling moths use different bioactive enantiomers as components of their sex pheromones that serve as an avenue of reproductive isolation.

Table 1. Binding affinities of different enantiomers.

Ligands	Affinity(kcal per mol)
Z3Z9-6R,7S-epoxy-18:H	−6.3
Z3Z9-6S,7R-epoxy-18:H	−6.7

In 2 recent silico studies by Sun et al. (2013b) and Liu et al. (2015b), male moths of *E. obliqua*, *Eobl*PBP1 was proven to be specifically expressed (and dramatically detected) in their antennae, and shared a similar tissue expression pattern with pheromone binding protein genes in other moths. These results suggested a clear implication of male *E. obliqua* attractiveness in response to female-produced sex pheromones of this species (Sun et al. 2017). Therefore, a predicted 3D model could be built and used to investigate the binding mode of the different epoxide enantiomers.

In conclusion, silico interaction between ligands and *Eobl*PBP1 should be confirmed by further X-ray crystallography studies. We believe that the knowledge we generated regarding the molecular interactions of *Eobl*PBP1 supports the use of these sex pheromones for pest management in the field. Complementary experimental assays, such as competitive binding assays and site-directed mutagenesis, could validate the results obtained in our experiments to facilitate further research into the olfaction mechanism of *E. obliqua* males. Such investigations would serve to support alternative pest control strategies through design and development of novel pheromone analogs, as well as inhibitors of pheromone degradation.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (31200490), National Modern Agriculture Technology System (CARS-23), and Anhui Major Demonstration Project for Leading Talent Team on Tea Chemistry and Health. The senior author expresses his appreciation for the time spent at Mie University, Japan, with best wishes to Hirokata Katsuzaki and his team.

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