Design and Synthesis of Fluorophore-Tagged Disparlure Enantiomers to Study Pheromone Enantiomer Discrimination in the Pheromone-Binding Proteins from the Gypsy Moth, Lymantria dispar

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Abstract
Fluorescent analogues of the gypsy moth sex pheromone (+)-disparlure (1) and its enantiomer (−)-disparlure (ent-1) were designed, synthesized, and characterized. The fluorescently labelled analogues 6-FAM (+)-disparlure and 1a 6-FAM (−)-disparlure were prepared by copper-catalyzed azide-alkyne cycloaddition of disparlure alkyne and 6-FAM azide. These fluorescent disparlure analogues 1a and ent-1a were used to measure disparlure binding to two pheromone-binding proteins from the gypsy moth, LdisPBP1 and LdisPBP2. The fluorescence binding assay showed that LdisPBP1 has a stronger affinity for 6-FAM (−)-disparlure ent-1a, whereas LdisPBP2 has a stronger affinity for 6-FAM (+)-disparlure 1a, consistent with findings from previous studies with disparlure enantiomers. The 6-FAM disparlure enantiomers appeared to be much stronger ligands for LdisPBPs, with binding constants (Kd) in the nanomolar range, compared to the fluorescent reporter 1-NPN (which had Kd values in the micromolar range). Fluorescence competitive binding assays were used to determine the displacement constant (Ki) for the disparlure enantiomers in competition with fluorescent disparlure analogues binding to LdisPBP1 and LdisPBP2. The Ki data show that disparlure enantiomers can effectively displace the fluorescent disparlure from the binding pocket of LdisPBPs and, therefore, occupy the same binding site.

Keywords Fluorescein · Fluorophore-tagged disparlure enantiomers · Enantioselective synthesis · Cis-epoxide · Fluorescence binding assays · Click reaction

Introduction
Pheromones are detected by moths using male-specific receptor neurons located on the antennae (Krieger and Breer 1999), which transform a chemical signal into a neuronal signal. Antennae of male moths are covered in sensory hairs (sensilla), which are innervated with dendrites of olfactory sensory neurons. Pores in the cuticle of the hairs allow the diffusion of pheromones to the interior, where they first interact with pheromone-binding proteins (PBPs) in the lymph and next with pheromone receptors (PRs). The latter are embedded in the dendritic end of pheromone sensory neurons and are bathed in sensillum lymph (an aqueous solution rich in PBPs, fatty acids, and ions; Nardella et al. 2015). PRs are ion channels that open upon binding of the pheromone, causing a slow depolarization of the dendritic membrane (Sato et al. 2008; Wicher et al. 2008). The PBPs bind pheromones and other compounds reversibly and selectively, in order to keep them in the lymph.

In the gypsy moth, Lymantria dispar, two PBPs have been identified, LdisPBP1 and LdisPBP2 (Vogt et al. 1989). Both proteins share 50% identity and belong to the insect odorant-binding protein (OBP) family with a long C-terminus. The LdisPBP1 and LdisPBP2 contain 143 and 145 amino acid residues, respectively. Insect PBPs have helical structures, stabilized by three conserved disulfide bridges. Furthermore, PBPs access two structural forms, the A form (usually seen under acidic conditions) (Terrado et al. 2020) and the B form (often found under basic conditions) (Sandler et al. 2000).

Previously, we have shown that (+)-disparlure (1), the gypsy moth sex pheromone, and its enantiomer
(−)-disparlure (ent-1) (Fig. 1) bind strongly to LdisPBP1 and LdisPBP2, with opposite enantioselectivity. LdisPBP1 has higher affinity for (−)-disparlure (ent-1), whereas LdisPBP2 has higher affinity for (+)-disparlure (1) (Plettner et al. 2000; Yu and Plettner 2013). This remarkable enantiomeric discrimination by the gypsy moth PBPs depends on non-covalent interactions between these proteins and the pheromone when these molecules collide. Analysis of the sequences of LdisPBPs has revealed the presence of phenylalanine amino acid residues that are highly conserved among lepidopteran PBPs, in particular, Phe12, Phe36, Phe76, Phe119 (PBP1), and Phe120 (PBP2). These residues interact with the hydrophobic region (hydrocarbon chains) of the ligands when the PBP is in the B form (Sandler et al. 2000; Honson et al. 2003; Sanes and Plettner 2016). Furthermore, the binding site residues that vary between LdisPBPs were found to be: Asn35, Ala73, Leu91, and Ala135 in LdisPBP1, whereas in LdisPBP2 these residues were substituted with Asp35, Thr73, Ile91, and Leu136 (Sanes and Plettner 2016). Our recent NMR and molecular docking studies have demonstrated that the disparlure enantiomers adopt different conformations and orientations in the internal and external binding pockets of LdisPBP1 and LdisPBP2 (Pinnelli et al. 2019). In addition, the two PBPs differ in their ligand binding association and dissociation kinetics. Both proteins undergo a two-stage ligand binding process: a rapid association at one or more external site(s), followed by a slow diffusion of the ligand to the interior of the protein. PBP2 binds ligands at its internal binding site very slowly, whereas PBP1 has faster association and dissociation kinetics (Gong et al. 2009, 2010). We have shown that in PBP2, the enantiomers of disparlure differ in the rate at which they bind to the internal binding site (Gong et al. 2009). However, due to their higher rates, the enantioselectivities of the external binding events in both PBPs and of the internalization of ligands in PBP1 have not yet been studied.

Ligand-binding experiments between pheromone binding proteins (PBPs) and hydrophobic ligands (e.g., pheromones) can be performed in two general ways: 1) the ligand and protein are equilibrated in buffer, and the protein-bound ligand is then separated from the free ligand using filtration (Plettner et al. 2000) or 2) the protein is titrated with a fluorescent reporter such as NPN (N-phenyl-1-naphthalamine), and the NPN is then displaced by titration of the PBP-NPN complex with the ligand of interest (Ban et al. 2002; Gong et al. 2010; Gong and Plettner 2011). Disadvantages of equilibrium binding assays include the adsorption of hydrophobic ligands on vial surfaces and the potential loss of bound ligand during the filtration step, which leads to underestimates of binding affinity. Determination of binding constants of the PBP-ligand complex in the second type of experiment depends on the displacement of the fluorescent reporter by a competing ligand with a concomitant decrease in NPN fluorescence. This type of binding experiment does not require any physical separation of bound ligand from unbound ligand (Ban et al. 2002). However, this approach needs the availability of a fluorescent reporter equipped with good binding strength for the PBP under study, whose fluorescence emission is significantly increased when the reporter binds inside the PBP’s binding pocket.

One disadvantage of the use of fluorescent reporters in ligand-binding assays is that various compounds differ in their ability to displace the reporter (due to kinetic factors and incomplete equilibration between aliquot additions during these experiments), rather than reporting the binding strength of the ligand of interest to the PBP. Another disadvantage is the strong fluorescent emission of the reporter in the presence of compounds that are capable of forming micelles (e.g., fatty acids or amphiphilic pheromones). In this

Fig. 1 Structures of disparlure enantiomers and their fluorescent analogues
case, the reporter can occupy the hydrophobic core of the micelle, producing a strong fluorescent peak, identical to that emitted in the binding pocket of the PBP. An example of this problem can be seen in the study by McAffee et al. (2018) when they titrated OBPs 16 and 18 from the honey bee with oleic acid. Given these drawbacks, it would be necessary to conduct binding assays between pheromone and PBPs with fluorescent reporters linked to the ligand of interest.

In this paper, we report the synthesis (Scheme 1) and spectroscopic characterization of fluorophore-tagged disparlure enantiomers 6FAM (+)-disparlure (1a) and 6FAM (−)-disparlure (ent-1a) (Scheme 1), and their binding affinities to two pheromone binding proteins LdisPBP1 and Ldis-PBP2 of gypsy moth. The binding of disparlure to Ldis-PBPs was determined by changes in fluorescence emission intensity of the solution containing LdisPBP1 or 6-FAM-disparlure. The relative changes in fluorescence intensity reflecting binding to LdisPBP1 or LdisPBP2 are quantitated as a function of increasing 6FAM (+)-disparlure (1a) or 6-FAM (−)-disparlure (ent-1a) concentration. To check that the 6-FAM probes bind to the natural binding site, we also displaced each 6-FAM enantiomer with the corresponding non-labelled disparlure.

**Materials and Methods**

**Synthesis of Compounds**

The syntheses of the compounds prepared here are described in the supporting information.

**Determination of Quantum Yield (Φ) and Molar Extinction Coefficient (ε) for 6-FAM (+)-Disparlure (1a)**

To determine the quantum yield (Φ) for the 6-FAM (+)-disparlure (1a), the 6-FAM azide was used as a reference fluorophore as it has the same excitation and emission wavelengths as the 6-FAM (+)-disparlure. The quantum yield of 6-FAM azide is 0.90 at the excitation wavelength of 494 nm. In this experiment, the solutions of the reference and the...
sample (6-FAM (+)-disparlure) with absorbance values from 0.01 to 0.04 were considered to minimize the fluorescence inner-filter effect (IFE). A series of four standard solutions in 50 mM phosphate buffer (pH 8.0) or 1-heptanol were prepared each for 6-FAM (+)-disparlure and 6-FAM azide, with absorbance values between 0.01 to 0.04. The absorption spectra of these solutions were recorded on VWR UV-6300PC double beam spectrophotometer (VWR, Radnor, PA, USA) at an excitation wavelength of 494 nm. The fluorescence emission spectra of both reference and sample solutions were recorded on a fluorescence spectrophotometer (PTI-QuantaMaster, Horiba Instruments Inc., Irvine, CA, USA) over the wavelength range 450–600 nm, at an excitation wavelength of 494 nm. The plots of integrated fluorescence intensity of the emission spectra against the absorbance of both the 6-FAM azide and 6-FAM (+)-disparlure showed a linear relationship (Fig. S7, supporting information). The slope of the linear fit for the 6-FAM azide and 6-FAM (+)-disparlure is used to calculate the quantum yield (Φ) of 6-FAM (+)-disparlure according to the equation below.

\[
\Phi_{FD} = \Phi_{FA} \left( \frac{m_{FD}}{m_{FA}} \right) \left( \frac{\eta_{FD}^2}{\eta_{FA}^2} \right) 
\]

where \( \Phi_{FD} \) is the quantum yield of 6-FAM (+)-disparlure, \( \Phi_{FA} \) is the quantum yield of the 6-FAM azide (standard), \( m_{FD} \) and \( m_{FA} \) are the slopes of the linear fit for the 6-FAM (+)-disparlure and 6-FAM azide, respectively, and \( \eta_{FD} \) and \( \eta_{FA} \) are the refractive indices of the 6-FAM (+)-disparlure and 6-FAM azide solutions, respectively. Since the same buffer was used for both fluorophores, the term \( \eta_{FD}^2/\eta_{FA}^2 = 1 \) and we get:

\[
\Phi_{FD} = \Phi_{FA} \left( \frac{m_{FD}}{m_{FA}} \right) 
\]

To determine the molar extinction coefficient (ε) for 6-FAM (+)-disparlure 1a, a series of four sample solutions was prepared by diluting the 6-FAM (+)-disparlure in phosphate buffer (pH 8) or 1-heptanol. The absorption spectra for these solutions were recorded on a UV/visible spectrophotometer. The plot of concentration of 6-FAM (+)-disparlure vs absorbance at 494nm showed a linear relationship (Fig. S8, supporting information). According to the Beer-Lambert law (A = εbc), where b is the path length of the cuvette, which is 1 cm. Therefore, the slope of the line in the plot of absorbance (A) vs concentration is equal to the molar extinction coefficient, ε (M\(^{-1}\) cm\(^{-1}\)).

**Fluorescence Binding Assay**

To determine the binding affinity of disparlure enantiomers to pheromone binding proteins (Ldis PBP1 and Ldis PBP2), fluorescence binding assays were conducted on a fluorescence spectrophotometer (PTI-QuantaMaster) using 6-FAM tagged-disparlure enantiomers 1a and ent-1a as fluorescent probes. The fluorescence spectra were recorded at room temperature with a silica quartz cuvette (light path = 1 cm). The protein (0.04 μM), LdisPBP1 or LdisPBP2 in 50 mM phosphate buffer (pH 8.0) was titrated with fluorescent probes 1a or ent-1a to final concentrations of 2-60 nM. The complex between the fluorescent probes and LdisPBPs was excited at 494 nm and the fluorescence emission spectra were monitored between 450 to 600 nm.

To measure the dissociation constant (K\(_d\)) of the complexes, the values of the fluorescence intensity of the probes at their emission maximum (after buffer and protein background correction) were plotted against total fluorescent probe concentrations, and the data were fitted into nonlinear regression models using GraphPad Prism 5 (GraphPad Software LLC). To determine the K\(_d\) for the competitor ligands, (+)-disparlure (1) and (−)-disparlure (ent-1) a competitive binding assay was performed. Aliquots of the competitor ligand in ethanol were added to the solution containing 0.04 μM (40 nM) protein and a fixed concentration of fluorescent probe (60 nM). A decrease in the relative fluorescence intensity suggested that the competitor displaced the fluorescent probe from the binding site of the protein. The inhibitory constant (K\(_i\)), was calculated based on the competitor IC\(_{50}\) value using the following equation:

\[
K_i = \frac{[IC_{50}]/(1 + [F]/K_{df})}{(1 + [F]/K_{df})} 
\]

where [F] is the total fluorescent probe concentration and K\(_{df}\) is the binding constant of the fluorescent probe to the LdisPBPs. The dissociation constant of the ligand (K\(_{dl}\), see Eq. 4 below) can then be obtained as follows. The equilibrium between P.F (the protein—fluorescent probe complex), \( L \) (the ligand of interest), and F (the fluorescent reporter) is:

\[
P.F + L \leftrightarrow P.L + F 
\]

\[
K_i = \frac{[P.F][L]/[P.L][F]}{[F]/[P.F]} 
\]

The equilibria between the probe and the protein, as well as the ligand and the protein are:

\[
F + P \leftrightarrow P.FK_{df} = [P][F]/[P.F] 
\]

\[
L + P \leftrightarrow P.LK_{dl} = [P][L]/[P.L] 
\]

Substituting Eq. 5 and 6 into Eq. 4, we get:

\[
K_i \times K_{df} = K_{dl} 
\]
Results

Design of 6-FAM-Tagged Disparlure Enantiomers

The design and convergent synthesis of 6-carboxylfluorescein (6-FAM)-tagged (+)-disparlure analogue (1a) took advantage of the copper (I)-catalyzed Huisgen 1,3-dipolar cycloaddition (also known as Click Reaction) (Huisgen 1963). The target molecule was divided into three parts: (+)-disparlure (1) (pheromone, recognition part for the selective binding to the protein target), and the 6-FAM moiety for detection of binding interactions by changes in fluorescence, which were linked by a diethylene glycol linker to increase the hydrophilicity of the molecule and provide some separation between the recognition part and the fluorophore (Fig. 1).

We used the same approach to prepare (+) and (−)-disparlure enantiomers (Pinnelli et al. 2019) using asymmetric α-chlorination of an aldehyde. This procedure had been used previously in the synthesis of the posticlure enantiomers, which have a trans-epoxide moiety (Kang and Britton 2007). To obtain the cis-epoxide of disparlure we had to invert the configuration of the intermediate anti-chlorohydrin (Pinnelli et al. 2019).

Synthesis of 6-FAM Tagged (+)-Disparlure 1a

To accomplish the synthesis of the 6-FAM (+)-disparlure (1a), two key fragments (i.e., enantiopure cis-epoxy alcohol 2 and ethylene glycol linker 3) were prepared; their synthetic routes are discussed below (Scheme 1). We first prepared enantiopure epoxy alcohol fragment 2 from commercially available 1,12-dodecanediol (4) (Scheme 1).

Three protecting groups (MOM, TBDMS, and benzyl) were tried for monoprotection of diol 4. However, the MOM and TBDMS protecting groups were found to be unstable to the conditions required for the key asymmetric α-chlorination of an aldehyde (Scheme S1, supporting information). Therefore, the benzyl protecting group was utilized for monoprotection of diol 4.

Thus, monobenzylated alcohol 13 was prepared by benzylation of diol 4 with benzylbromide 12 in the presence of NaH/THF at 70 °C (Scheme 1). 1H NMR analysis of the crude reaction mixture indicated that the ratio of mono- and dibenzylated products was ~1:1. Upon purification by flash column chromatography, monobenzylated alcohol 13 was obtained in 44% yield. Alcohol 13 was then oxidized with PCC under standard conditions to its corresponding aldehyde 14, followed by asymmetric α-chlorination with SOMO catalyst 9 (Amatore et al. 2009; Pinnelli et al. 2019) to afford enantiopure α-chloroaldehyde 15 in 53% yield and >98% ee (Fig. S4). Previous studies have shown that the addition of a nucleophile to a chiral α-chloroaldehyde produces predominantly the 1,2-anti-chlorohydrin (Kang and Britton 2007; Shibuya et al. 2008; Pinnelli et al. 2019). Therefore, 5-methyl-1-hexyne (16) was treated with n-butyllithium followed by slow addition of α-chloroaldehyde 15 to afford 1,2-anti-chlorohydrin 17. 1H NMR data of the crude reaction mixture revealed a diastereomeric ratio (dr) of ~20:1 (anti:syn). The crude reaction mixture was purified by column chromatography yielding the 1,2-anti-chlorohydrin 17 as a pale-yellow oil in 68% yield.

To prepare epoxy alcohol 2, the stereochemistry at the C-8 carbon of 1,2-anti-chlorohydrin 17 was inverted under Mitsunobu conditions as reported previously (Pinnelli et al. 2019). Thus, compound 17 was treated with benzoic acid (18), triphenylphosphine (TPP), and disisopropyl azadiacarbomylxylate (DIAD) in dry THF under inert atmosphere, producing the completely inverted product 19 in 74% yield. Then, a one-pot deprotection of the benzyl group and alkyne reduction of the 1,2-syn-chloroester 19 (10% Pd/C in MeOH, H2) gave alcohol 20. Basic hydrolysis of the benzoate function of 1,2-syn chloroester 20 then yielded the key enantiopure cis-epoxy alcohol 2 in 82% yield (Scheme 1). The required ethylene glycol linker 3 for esterification of 2 then was prepared as shown in Scheme S2.

The key fragments 2 and 3 were coupled under Steglich conditions, using DIC (N,N′-disopropylcarbodiimide) as coupling reagent and DMAP (N,N′-dimethylaminopyridine) as a catalyst, leading to alkyne ester 24, completing the alkyne intermediate for a Click Reaction employing 6-FAM azide as the coupling partner. Alkyne 24 and 6-FAM azide 25 were subjected to a copper-catalyzed azide-alkyne cycloaddition using THPTA (tris(benzyltriazolylmethyl)amine), sodium ascorbate, and copper (II) sulphate at room temperature, producing the desired (+)-disparlure fluorescent probe 1a in 57% yield (Scheme 1). 6-FAM (−)-disparlure was then prepared in analogous fashion, using SOMO catalyst ent-9 in the asymmetric α-chlorination stage (Schemes S3 & S4).

Determination of Enantiomeric Excess (ee) of Epoxy Alkenes 24 and ent-24

To determine the ee for the target molecules 6-FAM (+)-disparlure 1a and 6-FAM (−)-disparlure ent-1a, a scalemic sample of epoxy alkyne (Fig. 2) was prepared from nearly equal amounts of enantiopure epoxy alkenes 24 and ent-24 (Scheme 1 and S4). This scalemic epoxy alkyne was then subjected to the Click Reaction using 4-bromophenylazide (26) as coupling partner to give the corresponding scalemic triazole 27. HPLC analyses showed baseline separation of the scalemic triazole (Fig. 2), allowing us to determine the ee of alkenes 24 and ent-24. Enantiopure epoxy alkenes 24 and ent-24 then were transformed to their corresponding triazoles using the same conditions. HPLC analysis of the
resulting derivatives determined that both probes had >98% ee (Fig. S3 & Table 2).

Characterization of 6-FAM (+)-Disparlure 1a

The absorbance and emission spectra of 6-FAM (+)-disparlure 1a were recorded in phosphate buffer (pH 8) and 1-heptanol. A shift of 9 nm was noticed when comparing the excitation maximum of 1a in 1-heptanol (λ_max 485) with that in phosphate buffer (λ_max 494) (see supporting information). The molar absorptivity (ε) of 1a was determined in 1-heptanol (ε_{485 nm} = 3021 M^{-1} cm^{-1}) and in phosphate buffer (ε_{497 nm} = 16,823 M^{-1} cm^{-1}) from absorption spectra recorded at different concentrations of 1a. Compound 1a shows maximal emission at 515 nm with a Stokes shift and quantum efficiency of (Φ) 0.62 in 1-heptanol, whereas it exhibits maximum emission at 520 nm with a Stokes shift of 26 nm and quantum efficiency of (Φ) 0.86 in phosphate buffer (Table 1).

Fluorescence Emission of 6-FAM Tagged Disparlure Enantiomers upon Binding to LdisPBPs

We examined the binding affinity of 6-FAM tagged disparlure enantiomers 1a and ent-1a to LdisPBP1 and LdisPBP2 by fluorescence binding assays. When excited at 494 nm, fluorescent probes 6-FAM (+)-disparlure 1a and 6-FAM (+)-disparlure ent-1a in phosphate buffer (pH 8) show fluorescence emission with maxima at 520 nm. For example, Fig. 3A shows the emission spectra of fluorescent probe 1a in phosphate buffer and the presence of LdisPBP1, with

![Figure 2](image)

**Fig. 2** Determination of enantiomeric excess (ee) of epoxy alkynes 24 and ent-24 by forming phenyltriazoles. A Reaction used to create the phenyltriazoles of scalemic disparlure with a linker. The major isomer that forms in the reaction is shown. B HPLC chromatogram of the resulting product.
increasing fluorescence emission intensity with fluorescent probe 1a concentration.

The increase in fluorescence intensity is a probable consequence of restricted rotation of the fluorophore upon binding to PBP. It has been reported that fluorescent probes show weak fluorescence in buffer due to fast vibrational relaxation of the singlet excited state through internal bond rotations (Haidekker and Theodorakis 2007; Yu et al. 2015). When a fluorescent probe binds to a protein, a large fluorescence increase can occur due to restricted bond rotation of the fluorophore and, consequently, less thermal relaxation. The increase in fluorescence intensity allows measurements of binding sites of restricted rotation of the fluorophore upon binding to a protein, a large fluorescence increase can occur due to restricted bond rotation of the fluorophore and, consequently, less thermal relaxation. The increase in fluorescence intensity allows measurements of the interaction between probes and proteins. Therefore, we titrated LdisPBP1 and LdisPBP2 with 6-FAM (+)-disparlure 1a and 6-FAM (−)-disparlure ent-1a to determine the dissociation constants (Ki) as a measure of the strength of binding. The smaller the Ki, the stronger the interaction. Figure 4 shows the isotherms for the LdisPBP1/fluorescent disparlure enantiomer pair, for both of which we could detect significant binding affinities. The concentration dependence of fluorescent disparlure enantiomers binding to LdisPBP can be described by a hyperbolic curve, as expected for a one-site binding model (Fig. 4), for which dissociation constants (Ki) could be calculated. The novel fluorescent disparlure analogues appeared to be much stronger ligands for the LdisPBP1 and LdisPBP2, with dissociation constants in the nanomolar range (Fig. 4, Table 2), than the fluorescent probe NPN (which had Kd values 1.3 ± 0.3 µM for LdisPBP1 and 8.6 ± 0.6 µM for LdisPBP2 (Gong et al. 2010).

Displacement of 6-FAM Tagged Disparlure by Disparlure

The fluorescence of bound fluorescent probes 6-FAM (+)-disparlure 1a and 6-FAM (−)-disparlure ent-1a was followed as disparlure (same enantiomer as the probe) was titrated, to measure the binding affinities of disparate enantiomers to LdisPBP1 and LdisPBP2 and to check whether fluorescent probes 1a and ent-1a bind within the same binding pocket of LdisPBPs as the fluorescent probe. The fluorescence emission spectra of the mixture decreased in intensity upon addition of disparlure (Fig. 5A), suggesting that disparlure was displacing the fluorescent disparlure analogue from the binding pocket of LdisPBPs as disparlure was titrated into the mixture. This decrease can be taken as a measure of binding affinity of disparlure for LdisPBPs at the same binding site as that occupied by the fluorescent probe.

The displacement constant (Ki) values determined for the disparate enantiomers in competition with fluorescent probes 1a and ent-1a binding to LdisPBPs ranged between 132 and 211 nM. For LdisPBP1, the (+)-disparlure (I) and (−)-disparlure (ent-1) exhibited different fluorescent probe displacement properties with Ki values of 165 and 132 nM, respectively. In the case of LdisPBP2, the best fluorescent probe competitor was found to be (+)-disparlure (Ki = 144 nM) (Table 3). These Ki values show that the disperslure enantiomers can effectively displace the corresponding enantiomer of the fluorescent probe from the binding pocket. Furthermore, an estimate of the Ki values for the free disperslure enantiomers from the displacement.

Table 1 Absorption and fluorescence properties of 6-FAM (+)-disparlure 1a in phosphate buffer (pH 8).

| ωex [nm] | ωem [nm] | SS [nm] | εm [M⁻¹ cm⁻¹] | Φa |
|----------|----------|---------|---------------|-----|
| 494      | 520      | 26      | (16.8 ± 0.3)×10³ | 0.86 ± 0.02 |

SS Stokes shift; ε molar extinction coefficient; Φ quantum yield

*Values are reported ± standard error (SE) of 3 replicates.

Fig. 3 A 6-FAM (+)-disparlure 1a emission spectra. 1a bound to LdisPBP1 was excited at 494 nm and its emission spectrum (blue trace) was recorded. The red trace shows the emission spectrum of 1a in buffer and the green trace shows the emission background obtained with only PBP in buffer. B Fluorescence emission spectra were recorded with increasing doses of 6-FAM (+)-disparlure 1a, titrated into LdisPBP1. The fluorescence emission from 6-FAM (+)-disparlure 1a/LdisPBP1 complex determined by subtracting the bottom trace (red) from the upper trace (blue) in part A.
data (see derivation above) gave $K_d$ values (Table 3) within the range observed previously by other methods (e.g. Plettner et al. 2000). The enantiomer selectivity is also consistent with previous studies.

**Discussion**

We covalently linked 6-carboxyfluorescein (6-FAM) to the gypsy moth sex pheromone (+)-disparlure (1) and its enantiomer (−)-disparlure (ent-1) (Fig. 1) by adding a linker with a terminal alkyne moiety to disparlure and then performing a copper-catalyzed Click Reaction between the alkyne and 6-carboxyfluorescein azide (6-FAM azide). We chose 6-carboxyfluorescein (6-FAM) as a fluorescent reporter because of its high fluorescence quantum yield (0.93) and good water solubility, and because of its derivatives such as 6-FAM azide are commercially available. In addition to the high quantum yield, it has excellent absorption and emission properties (Sjöback et al. 1995; Zhang et al. 2014). To our knowledge, this is the first report describing the synthesis of a fluorophore-tagged insect pheromone. We expect that such fluorophore-tagged pheromones will provide researchers with a viable alternative to radiolabeled pheromones and fluorescent probes, such as NPN, that have been used in PBP-pheromone binding assays. To date, the use of fluorophore-tagged pheromones in the study of pheromone binding protein interactions has not been reported.

The results of the binding assays showed that the 6-FAM tagged disparlure enantiomers 1a and ent-1a bound to both LdisPBP1 and LdisPBP2 with nanomolar dissociation constants. When binding affinities of the LdisPBP for the 6-FAM tagged disparlure enantiomers were compared, LdisPBP1 had a significantly higher affinity towards 6-FAM (-)-disparlure whereas LdisPBP2 bound the 6-FAM (+)-disparlure more strongly (Table 2). This is consistent with the body of previous equilibrium dissociation studies on LdisPBP1 and LdisPBP2 with (+)-disparlure (1) and (−)-disparlure (ent-1). LdisPBP1 binds preferentially to (−)-disparlure (ent-1) whereas LdisPBP2 binds (+)-disparlure (1) more strongly (Plettner et al. 2000; Yu and Plettner 2013). Similarly, multiple studies with the NPN displacement assay have shown that PBPs bind pheromone components selectively. For example, PBP2 and PBP3 from the Chinese silk oak moth, Antheraea pernyi, and the giant silk moth, Antheraea polyphemus, preferentially bind to one pheromone component, e.g. the aldehyde [(E,Z)-6,11-hexadecadienyal] for PBP2, whereas PBP3 has a higher affinity for the other pheromone component [(E,Z)-6,11-hexadecadienyl acetate] (Maida et al. 2003). A difference in pheromone binding affinity also has been observed for PBP3 from Ostrinia furnacalis which binds strongly to the sex
pheromone components (E)-12-tetradecenyl acetate and (Z)-12-tetradecenyl acetate, whereas PBP4 and PBP5 bind selectively to (E)-11-tetradecenyl acetate and (Z)-11-tetradecenyl acetate, respectively (Zhang et al. 2017). Similarly, selective sex pheromone binding by PBPs has been observed for PBP1 and PBP2 of the tea geometrid moth Ectropis obliqua (Sun et al. 2019; Yan et al. 2020) and PBP2 of the tobacco budworm H. virescens (Große-Wilde et al. 2007).

The approach outlined here could be applied to other insect pheromones, provided the main functional groups of those pheromones that interact with the pheromone binding sites are not altered by the addition of the linker and the probe. For example, the pheromone components of A. polyphemus, (E6,Z11)-6,11-hexadecadienyl acetate and (E6,Z11)-6,11-hexadecadienal and (E6,Z11)-6,11-hexadecadienyl acetate would need to have the alkene and aldehyde or acetate functional groups intact. An additional hydroxyl group could be installed on C-16, then linked to the alkyne linker and coupled to the fluorophore azide, as described here.

Our finding of the same enantioselectivity of the two PBPs from the gypsy moth and Kd values in the same order as previously determined shows that the probes we have prepared here can be used for more detailed studies of PBP-ligand kinetics than were possible before. In a previous study, Gong et al. (2009) followed the kinetics of (+)-disparlure and its enantiomer binding to LdisPBP2 by adding a fluorescent tag (dansyl) to the PBP. The fluorescence of this tag decreased when pheromone was bound to the protein. The limitations in that study were: (1) only PBP2 could be studied in this way because the fluorescence of dansylated PBP1 drifted and (2) only the slow binding to the internal binding site could be monitored. In another study, Gong et al. (2010) followed the association and dissociation of NPN with both gypsy moth PBPs. However, the structure of NPN differs

Table 2 Binding of 6-FAM disparlure enantiomers 1a and ent-1a to gypsy moth pheromone binding proteins LdisPBP1 and LdisPBP2

| Protein  | Ligand           | Kd (nM) | Enantiomeric excess of the probe |
|----------|------------------|---------|---------------------------------|
| LdisPBP1 | 6-FAM (+)-disparlure 1a | 27.3 ± 2.2 | 98.4%                           |
|          | 6-FAM (−)-disparlure ent-1a | 18.6 ± 2.0 | 98.8%                           |
| LdisPBP2 | 6-FAM (+)-disparlure 1a | 14.1 ± 1.7 | 98.4%                           |
|          | 6-FAM (−)-disparlure ent-1a | 47.3 ± 3.5 | 98.8%                           |

*Values represent the mean ± S.E of 3 replicates

Fig. 5 A Example of the decrease of 6-FAM (+)-disparlure 1a/LdisPBP1 fluorescence emission intensity at maximum (520 nm) at increasing concentrations of competitor (+)-disparlure (1). B Competition of 6-FAM (+)-disparlure 1a binding to LdisPBP1 (see Fig. S12 (supporting information) for competition binding curves of 6-FAM (−)-disparlure ent-1a with LdisPBP1 and 6-FAM disparlure enantiomers with LdisPBP2). Data shown are the mean of 3 independent measurements. Points represent means ± S. E of 3 replicates

Table 3 Inhibition of 6-FAM disparlure binding to LdisPBP2 by disparlure enantiomers

| Protein  | Competitor | IC50 (nM) | Ki (nM) | Kd (µM) |
|----------|------------|-----------|---------|---------|
| LdisPBP1 | (+)-disparlure | 410 ± 4.6 | 165 ± 1.9 | 4.5 ± 0.4 |
|          | (−)-disparlure | 414 ± 9.8 | 132 ± 2.3 | 2.5 ± 0.3 |
| LdisPBP2 | (+)-disparlure | 556 ± 10  | 144 ± 2.6 | 2.0 ± 0.3 |
|          | (−)-disparlure | 210 ± 4.8 | 211 ± 4.9 | 10.0 ± 6.4 |

Competitor concentrations causing a decay of fluorescence emission to half maximal intensity were determined as IC50 values from curves resulting from competition binding assays as shown in Fig. 5 and Fig. S12 (supporting information). Ki values were calculated according to $K_i = IC_{50}/([LdisPBP] 	imes [6-FAM dis] = IC_{50}/([LdisPBP] 	imes [6-FAM dis])$. [6-FAM dis]$ = free 6-FAM dis concentration; $K_{d} (6-FAM-dis) \times K_i = K_d (disparlure)$

*Values are the ± S. E of 3 replicates

*Calculated from the IC50 and dissociation constants of the ligand (see above)

*Calculated from the $K_{d} (6-FAM-dis) \times K_i = K_d (disparlure)$
greatly from that of the pheromone and the authors could only monitor rapid external binding steps with that method. The slow internalization step did not lead to a change in NPN fluorescence and was, therefore, silent in those experiments. With the probes described in this paper, we can monitor both the rapid external binding and the slow internalization steps of the probes with both LdisPBP1s. These results will be published in due course. This, in turn, will help us understand the mechanism by which these pheromones are bound to their cognate PBPs in a multi-step process. These multi-step binding mechanisms of pheromones to PBPs are likely functionally significant.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s10886-021-01318-2.

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