Research Article

Received: 5 December 2023 Revised: 1 April 2024 Accepted article published: 16 April 2024 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.8129

Identification, synthesis, and field evaluation of components of the female-produced sex pheromone of *Helopeltis cinchonae* (Hemiptera: Miridae), an emerging pest of tea

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Abstract

Background: *Helopeltis cinchonae* (Hemiptera: Miridae) is a major pest of tea plantations in Asia. Conventional control of pests with pesticides is unsustainable. Therefore, safe and eco-friendly alternatives, such as pheromones, are required to manage the pest.

Results: In gas chromatography–electroantennographic detection (GC–EAD) analysis of whole-body extracts of virgin female *H. cinchonae*, two compounds elicited electroantennogram (EAG) responses from male antennae. These were identified as hexyl (*R*)-3-acetoxybutyrate and (*R*)-1-acetoxy-5-butyroxyhexane using gas chromatography–mass spectrometry (GC–MS) analysis compared to synthetic compounds. This is the first study to report 1-acetoxy-5-butyroxyhexane as an insect pheromone component. The synthetic compounds elicited dose-dependent EAG responses from the antennae of male *H. cinchonae*. In two field trapping experiments, the individual compounds were highly attractive to male *H. cinchonae* when dispensed from polyethylene vials. However, higher catches were obtained with blends of the two compounds in a 1:10 ratio. The blend of racemic compounds was as attractive as the blend of (*R*)-enantiomers.

Conclusions: We reported that 1-acetoxy-5-butyroxyhexane and hexyl 3-acetoxybutyrate are components of the female-produced sex pheromone of *H. cinchonae*, but further work is required on the blend and loading of pheromone and on trap design to provide an optimized system for monitoring and control of this pest. The results may also facilitate the identification of the pheromones of other *Helopeltis* species, which are major pests in many crops.

Supporting information may be found in the online version of this article.

Keywords: 1-acetoxy-5-butyroxyhexane; Brycorinae; electroantennography; *Helopeltis cinchonae*; hexyl 3-acetoxybutyrate; Miridae; pheromone; trapping

1 INTRODUCTION

Helopeltis cinchonae (Mann, 1907) belongs to the Bryocorinae subfamily of mirid bug species (Hemiptera: Miridae),[1] widely distributed across Asia, Africa, Australia, and other geographical regions.[2–4] *Helopeltis* spp. feed on a broad range of host crops, particularly tea, cocoa, and cashew,[3,5,6] and cause significant economic damage.[7] Tea is a major crop of primary economic importance in China.[8] Over the last decade, frequent outbreaks of *H. cinchonae* have constituted a significant threat to Chinese tea plantations.[5] The pest is typically present during the whole growing season in tea plantations, and outbreaks can develop rapidly.[7] *Helopeltis cinchonae* damages the most economically vital part of the tea crop, the fresh, pickable tea shoots. Currently, pesticides

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are widely used for managing *H. cinchonae* in tea plantations, which may lead to pesticide residue problems and a risk to human health.[9] More efficient and eco-friendly control strategies are needed to manage the pest, and pheromones could provide the basis for such strategies.

Female-produced sex pheromones have been identified in various mirid species and are typically saturated and mono-unsaturated, unbranched esters or aldehydes.[10,11] Pheromones for some mirid bugs in the Brycorinae sub-family have been identified, including the cocoa mirids, *Distantiella theobroma* Dist. and *Sahlbergella singularis* Haglund,[12] the aphidophagous mirid, *Macrolophus pygmaeus* Rambur (formerly *Macrolophus caliginosus* Wagner),[13] and the tomato bug, *Nesidiocoris tenuis* (Reuter).[14] All except the latter are esters of 3-hydroxybutyric acid and rather different in structure from those of other mirid species. Synthetic pheromones have been used for monitoring[15–17] and mass trapping[18] of cocoa mirids.

Previous studies reported the attraction of *Helopeltis* male bugs by conspecific females,[19–22] indicating the presence of a sex pheromone. However, there are no reports of successful isolation and identification of pheromone components from these species.[22–24] Sachin *et al*. reported the identification of sex pheromone components of *Helopeltis theivora*. However, no behavioral activity in the field has been reported, and these may have been chemicals from the host plant.[22]

The present study was designed to identify and synthesize components of the female-produced sex pheromone of *H. cinchonae* and to evaluate their attractiveness to male bugs in the field. The results will provide the basis for developing pheromone-based strategies for monitoring and controlling the pest and expand our understanding of chemical communication in *Helopeltis* species.

2 MATERIALS AND METHODS

2.1 Insects

Helopeltis cinchonae nymphs (all stages) were collected from Guangdong province, China (23.38°N, 115.5°E). The pest was identified based on taxonomical characteristics.[1] The *H. cinchonae* bugs were reared on fresh tea shoots in a fine woven mesh (150 cm × 150 cm, and 160 μm aperture) gauze cage (50.5 cm × 50.5 cm × 50.5 cm) throughout the experimental period. The insect-rearing room was maintained at 25 ± 1 °C and 60–70% relative humidity (RH) under a 14 h:10 h light/dark photoperiod. Approximately 800 nymphs were collected, and the fifth nymph instars were separated daily. After adult emergence, individuals were separated by sex and placed in a cage as mentioned earlier. Virgin females and males (3–6-day-old) were used for pheromone collection and other experimental purposes.

2.2 Pheromone collection

To collect pheromone, 3–6-day-old virgin females ($n = 30$), males ($n = 20$), and fifth instar nymphs ($n = 20$) were immersed in dichloromethane (0.5 mL; CNW technologies, ANPEL Laboratories, Shanghai, China) at ambient temperature in 2 mL vials (Agilent Technologies, Santa Clara, CA, USA). After 20 min the supernatant was transferred to a new glass vial, concentrated under a gentle stream of nitrogen to approximately 30 μL, and then transferred to a vial with a 250 μL glass insert (Agilent Technologies), and stored at −20 °C for gas chromatography–electroantennogram detection (GC–EAD) and gas chromatography–mass spectrometry (GC–MS) analyses.

2.3 Chemicals

Full details of the syntheses are given in the Supporting Information Methods S1.

1-Acetoxy-5-butyroxyhexane (**VI**) was synthesized from 1,5-hexanediol (**III**) (Fig. 1). 1,5-Hexanediol is now commercially available but can be synthesized from 5-hexen-1-ol by acetylation, epoxidation, and reduction with lithium aluminum hydride (58% yield). Alternatively, reaction of 5-hydroxypentanal with methyllithium gave 1,5-hexanediol in 85% yield (Fig. 1). Reaction of 1,5-hexanediol (**III**) with 1 equivalent of acetic anhydride and pyridine at 0 °C for 2 h gave 1-acetoxy-5-hydroxyhexane (**V**) with

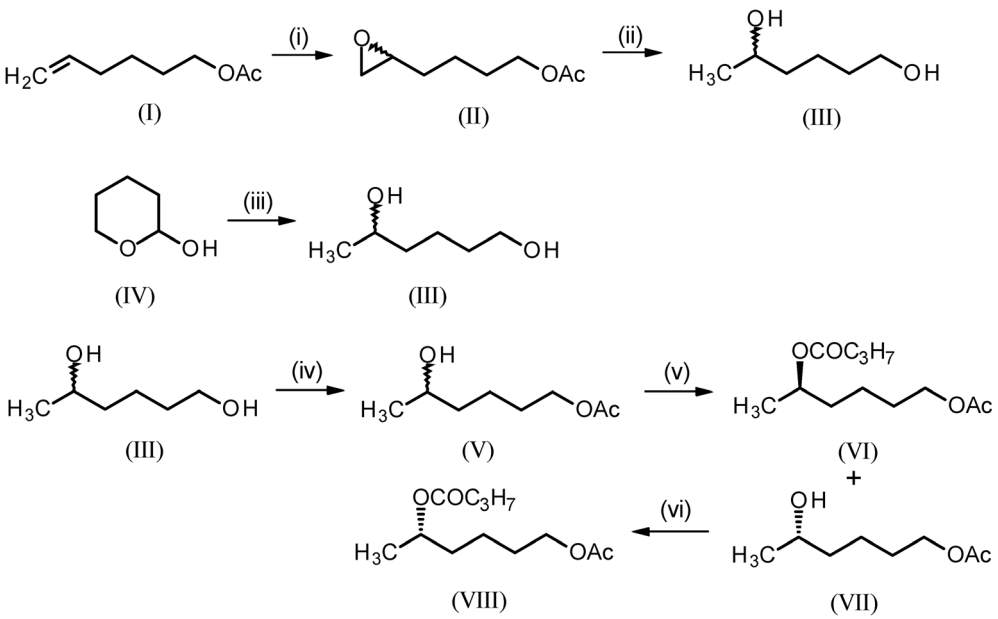

Figure 1. Synthesis of 1-acetoxy-5-butyroxyhexane [reagents: (i) 3-chloroperbenzoic acid/dichloromethane (75%); lithium aluminum hydride/ether (54% overall); (iii) methyllithium in ether/tetrahydrofuran (85%); (iv) acetyl chloride/*N*,*N*-diisopropylethylamine/dichloromethane −78 °C; (v) immobilized lipase from *Candida antarctica*/ether/vinyl butyrate (59% overall); (vi) butyryl chloride/pyridine/dichloromethane 0 °C].

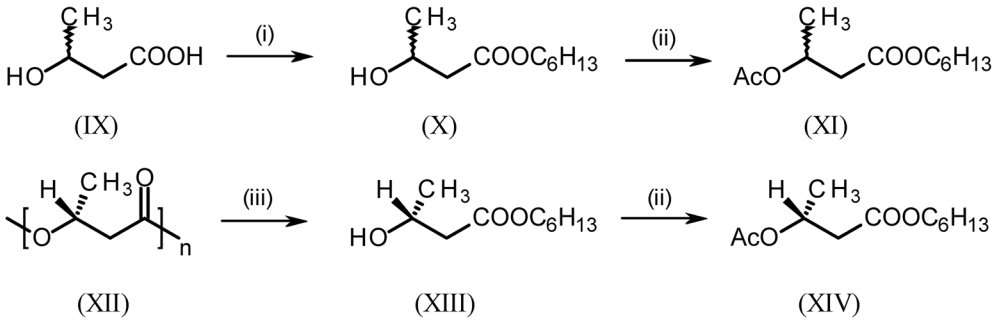

Figure 2. Synthesis of hexyl 3-acetoxybutyrate [reagents (i) trimethylsilyl chloride/hexanol/dichloromethane; (ii) acetic anhydride/pyridine; (iii) hexanol/catalytic sulfuric acid/120 °C].

10–15% 1,5-diacetoxyhexane and 2% of the isomeric 5-acetoxy-1-hexanol. The diacetate could easily be removed by chromatography, but the 5-acetoxy-1-hexanol could not. The preferred synthetic route utilized the selective method for acetylating primary alcohols described previously.[25] Thus reaction of 1,5-hexanediol (**III**) with acetyl chloride and *N*,*N*-diisopropylethylamine in dichloromethane at −78 °C gave 1-acetoxy-5-hydroxyhexane (**V**) containing 1.1% of the diacetate which was removed by chromatography, and < 0.5% of 5-acetoxy-1-hexanol. (Fig. 1).

1-Acetoxy-5-hydroxyhexane (**V**) could be converted to racemic 1-acetoxy-5-butyryloxyhexane with butyryl chloride and pyridine in dichloromethane at 0 °C or to the separate enantiomers by enzymatic kinetic resolution. Thus 1-acetoxy-5-hydroxyhexane (**V**) was mixed with vinyl butyrate in diethyl ether and a catalytic amount of immobilized lipase from *Candida antarctica*. The enzyme selectively converted the (*R*)-enantiomer of **V** to 1-acetoxy-5-butyroxyhexane (**VI**) with 98.6% enantiomeric excess (ee) after 1.25 h. This was separated from unreacted (*S*)-1-acetoxy-5-hydroxyhexane (**VII**; 76.6% ee) by chromatography on silica gel eluted with a gradient of diethyl ether in hexane. The latter could be recycled with vinyl butyrate and enzyme. Reaction for 2 h followed by chromatography gave **VII**, which was reacted with butyryl chloride and pyridine to give (*S*)-1-acetoxy-5-butyroxyhexane (**VIII**) with 99.6% ee.

Hexyl 3-acetoxybutyrate was synthesized as the racemate (**XI**) and the (*R*)-enantiomer (**XIV**) (Fig. 2). Racemic 3-hydroxybutyric acid (**IX**) was converted to the hexyl ester (**X**) with trimethylsilyl chloride and hexanol,[26] and the ester was acetylated with acetic anhydride and pyridine. The (*R*)-enantiomer was conveniently prepared on a large-scale from the readily-available polyhydroxybutyrate (**XII**) by reaction with hexanol and a catalytic amount of concentrated sulfuric acid at 120 °C to give hexyl (*R*)-3-hydroxybutyrate (**XIII**) which was acetylated with acetic anhydride and pyridine to give hexyl (*R*)-3-acetoxybutyrate (**XIV**) with 99% ee.

Racemic 1,5-dibutyroxyhexane (**XV**) was synthesized by reaction of 1,5-hexanediol (**III**) with butyryl chloride and pyridine (Fig. 3). The (*R*)-enantiomer (**XVI**) was synthesized by reaction of 1,5-hexanediol with vinyl butyrate in the presence of immobilized lipase from *Candida antarctica* which rapidly esterified the primary hydroxyl group and stereoselectively esterified the secondary alcohol to the (*R*)-ester with 98% ee (Fig. 3).

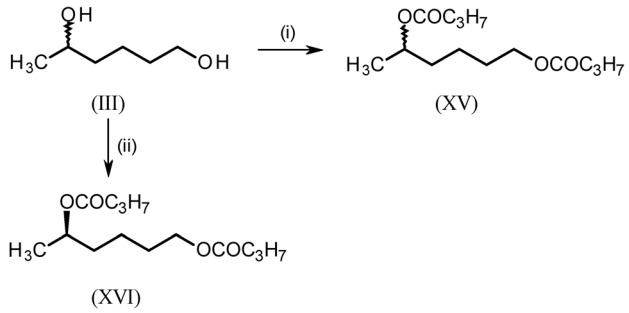

Figure 3. Synthesis of 1,5-dibutyroxyhexane [reagents: (i) butyryl chloride/pyridine/ dichloromethane; (ii) immobilized lipase from *Candida antarctica*/ether/vinyl butyrate].

2.4 Gas chromatography coupled to electroantennographic detection (GC–EAD)

For GC–EAD analysis to detect compounds stimulating receptors on antennae of male *H. cinchonae*, an Agilent 7890 GC (Agilent Technologies, Santa Clara, CA, USA) coupled with an EAD (Syntech, Buchenbach, Germany) was used. The GC was equipped with a non-polar HP-5 column [30 m length × 0.250 mm inner diameter (i.d.) × 0.25 µm film thickness; J&W, Agilent Technologies], and the oven temperature was programmed from 40 °C (0 min hold) to 280 °C at 10 °C min^{-1} and held for 5 min. The injection port was operated in split mode at 200 °C, and helium was used as the carrier gas (1 mL min^{-1}).

For EAD recordings the antenna of a virgin *H. cinchonae* male (3–6-day-old) was excised with fine scissors. A few distal segments of the antenna were cut off with a lancet under a microscope, and then connected with microglass capillaries filled with 0.9% sodium chloride (NaCl) solution. The glass capillaries were prepared by following a previous method.[27] The capillary glass was heated and pulled into the flame of an alcohol lamp. A column cutter was used to cut the capillary column and break it off to make its inner diameter wide enough to insert it into the electrode. Then, the glass capillary was connected with the base segments of the male antenna and mounted between two silver chloride electrodes. The terminal segment of antenna was connected with another glass capillary. The *H. cinchonae* female body extract (1 µL; one female equivalent) was injected into the GC, and the effluent was split (1:3) between the flame ionization detector (FID) and EAD. EAD recordings were done under continuous charcoal-filtered air (400 mL min^{-1}) through a stimulus controller (Syntech) directed to male antennae. The EAD and FID signals were simultaneously recorded using GC–EAD Pro software (Syntech). A compound was considered GC–EAD bio-active if it elicited an EAD response with more than five biological replicates. In total 11 *H. cinchonae* males were used for GC–EAD analysis.

2.5 Gas chromatography coupled to mass spectrometry (GC–MS)

GC–MS analysis of extracts of female *H. cinchonae* (1 µL; one female equivalent) was carried out on an Agilent 7890 GC coupled to an Agilent 5977B mass spectrometer (Agilent Technologies). Analysis was performed on two different columns coated with non-polar HP-5 (30 m length × 0.250 mm i.d. × 0.25 µm film thickness; J&W, Agilent Technologies) or polar DB-23 (30 m length × 0.250 mm i.d. × 0.25 µm film thickness; J&W, Agilent Technologies). The conditions for the HP-5 column were similar to those in GC–EAD analysis. For DB-23 the temperature was programmed from 40 °C for 10 min, then to 210 °C at 3.5 °C min^{-1} and to 230 °C at 30 °C min^{-1} and held for 10 min. Ionization was by electron impact at 70 eV, the ion source temperature was 240 °C, and the scan range was 40–500 *m/z*. Compounds were identified by examination of characteristic fragment ions and retention indices relative to the retention times of *n*-alkanes (Sigma Aldrich, Shanghai, China) and confirmed by comparison of mass spectra and retention indices of standard compounds.

The absolute configuration and enantiomeric excess of natural and synthetic compounds were determined by GC–MS 7890/5977B (Agilent Technologies) analyses using a Cyclodex-B column (30 m length × 0.250 mm i.d. × 0.25 µm film thickness; J&W, Agilent Technologies). The oven temperature was programmed from 50 °C for 2 min, increased at 5 °C min^{-1} to 220 °C, and held for 10 min.

2.6 Electroantennogram (EAG) recordings

Depolarizations of male *H. cinchonae* antennal receptors were recorded in response to synthetic sex pheromone compounds using an IDAC-2 recording system and CS-55 air stimulus controller (Syntech). Solutions of the two sex pheromone compounds **I** and **II**, and their enantiomers were prepared at five doses (2.5, 5, 25, 50, and 100 µg per 20 µL) using dichloromethane as solvent. An aliquot (20 µL) from each solution was applied to a filter paper strip (5 mm × 30 mm) (Whatman No.1), and dichloromethane (20 µL) was used as solvent control. The filter paper applied with a solution of the compound was left inside a fume hood for 2 min to allow the dichloromethane to evaporate. It was then placed into a Pasteur pipette (12.5 cm long) cartridge, which was closed with parafilm for (0.5 h) to vaporize the chemical before starting the recordings. The antennal preparations for EAG recordings were similar to those in GC–EAD analysis, except that the antenna was continuously bathed with a stream of charcoal-filtered humidified air at the flow rate of 800 mL min^{-1}. The tip of the Pasteur pipette cartridge was inserted into a hole (approximately 20 cm upwind from the antenna) in the metal tube, which directed the airstream over the antenna. The test compound was exposed to the antenna by a puff (0.5 s) of air (3 L min^{-1}) into the airstream by pressing the pedal switch. Each stimulus puff was alternated with a solvent control puff, and compounds were tested from low to high dosage. Compounds were tested in randomized order, allowing at least 1 min intervals for the antenna to stabilize. The amplitudes of the antennal depolarizations for each compound were recorded with EAG-pro software (Syntech).

2.7 Measurement of release rates

Low-density polyethylene vials (56 mm × 13 mm × 0.25 mm thickness; Shun Xing Plastic Products Ltd, Guangdong, China), as used in field trapping experiments, were loaded with the *R*-enantiomers of 1-acetoxy-5-butyroxyhexane and hexyl 3-acetoxybutyrate (2 mg each) and maintained under laboratory condition in a wind tunnel at 26 ± 1 °C, and wind speed of 2.3 m s^{-1}. At 2-day intervals, the emitted volatiles from dispensers were collected by placing them in a glass chamber (24 cm × 6 cm diameter) and drawing charcoal-filtered air (200 mL min^{-1}) through the chambers for 3 h. The released volatiles were trapped in a glass column containing Porapak Q (50 mg; 50–80 mesh; Sigma Aldrich), then eluted with dichloromethane (400 µL) and analyzed by GC–MS.

2.8 Field trials

Field trapping experiments were conducted in Jiexi Guangdong, China (23.38°N, 115.5°E). The test compounds were added to a 400 µL volume of dichloromethane and loaded into polyethylene vials (56 mm × 13 mm × 0.25 mm thickness). After evaporation of the solvent, the vials were heat sealed, and compounds diffused through the polyethylene wall. Traps were wing traps (42 cm length × 26.5 cm width × 16 cm height; Pherobio Technology Co. Ltd, Beijing, China) with a white sticky board (42.7 cm length × 28 cm width), and the polyethylene vial pheromone dispenser was placed on the center of the white sticky board. Traps were deployed 10 cm above tea shoots by a plastic pole at 20-m intervals. To minimize the positional effects, the traps were randomly rearranged. Field trapping experiments were done at tea gardens where no insecticides were applied to ensure a resident population of the pest.

Field experiment 1 (28 May–3 July 2023) aimed to evaluate the two EAD-active compounds detected in GC–EAD analysis, 1-acetoxy-5-butyroxyhexane and hexyl 3-acetoxybutyrate (Table 1). Traps baited with dispensers impregnated with 400 µL of dichloromethane only were used as a negative control, and traps baited with ten virgin females (3–6-day-old) from the laboratory-reared colony were used as positive control. The virgin female bugs were caged in muslin-clothed bags (12 cm length × 6 cm width) and fed on fresh tea shoots. Each treatment

Table 1. Loadings and enantiomeric configurations of synthetic compounds evaluated in field trapping experiments

	Dosage (mg)		
Treatment	1-Acetoxy 5-butyroxyhexane	Hexyl 3-acetoxybutyrate	1,5-Dibutyroxyhexane
Field experiment 1			
T1	3.0 (*R*)		
T2		3.0 (*R*)	
T3	2.1 (*S*)	0.9 (*R*)	
T4	2.1 (*R*)	0.9 (*R*)	
T5	1.5 (*R*)	1.5 (*R*)	
T6	0.9 (*R*)	2.1 (*R*)	
T7	Unbaited		
T8	ten virgin females		
Field experiment 2			
T1	2.0 (*R*)		
T2		0.2 (*R*)	
T3	2.0 (*R*)		1.0 (*R*)
T4	2.0 (*R*)	0.2 (*R*)	
T5	2.0 (*R*)	0.2 (*R*)	1.0 (*R*)
T6	4.0 (racemic)	0.4 (racemic)	
T7	Unbaited		

was replicated three times. Trap captures were recorded, and the virgin females, their food, and white sticky boards were changed every day. Lures were not renewed during the experiment.

Field experiment 2 (23 September–30 September 2023) aimed to evaluate further the binary blend of EAD-active compounds and also to investigate the effect of adding a non- EAD-active compound identified in female body extracts of *H. cinchonae*, 1,5-dibutyroxyhexane (Table 1). Each treatment was replicated three times. The trap captures were recorded and white sticky boards were changed daily.

2.9 Statistical analyses

The EAG values of the test compounds were corrected by subtraction of the response to the solvent (dichloromethane), transformed by $\log_{10}(mV + 1)$ to stabilize the variance and were analyzed by one-way analysis of variance (ANOVA). Tukey's *b*-test ($P < 0.05$) was used to determine the significance of differences between the mean EAG values.

For field trial data, trap catches were transformed by $\log_{10}$(trap catch +1) to ensure the homogeneity and normal distribution and were analyzed by one-way ANOVA followed by *post hoc* Tukey's *b*-test ($P < 0.05$). The values of the release rates of both compounds were analyzed by one-way ANOVA followed by *post hoc* Tukey's *b*-test ($P < 0.05$). The statistical analyses were performed with SPSS Statistics 21.0 (IBM, Armonk, NY, USA).

3 RESULTS

3.1 GC–EAD analysis

GC–EAD analysis of whole body extracts of virgin female *H. cinchonae* revealed two consistent bioactive peaks to antennae of male *H. cinchonae* [Fig. 4; compound **I**, retention time (RT) 11.95 min, and compound **II**, RT 12.21 min]. Compound **II** showed relatively higher responses than compound **I**, although compound **I** was present at an extremely low amount in the female extract.

3.2 GC–MS analysis

GC–MS analysis of extract from female *H. cinchonae* on the HP-5 column used in GC–EAD analysis revealed that the peak corresponding to compound **II** was clear in the total ion chromatogram (TIC) at RT 13.33 min (Fig. 5(B)), but compound **I** was barely

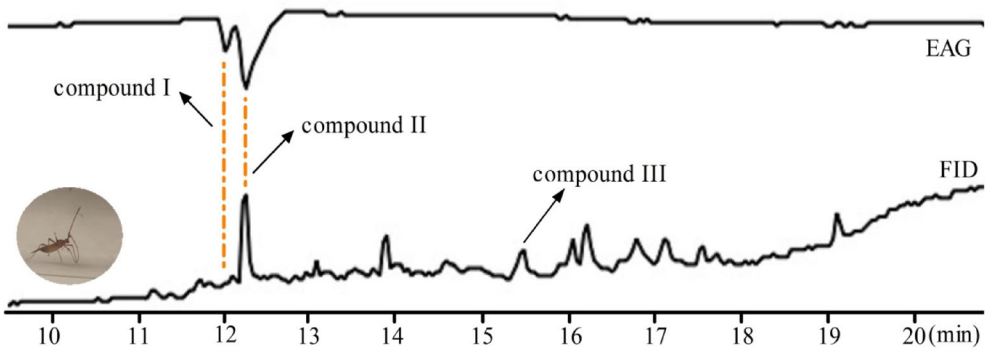

Figure 4. GC–EAD responses of *Helopeltis cinchonae* male antennae to female body extract.

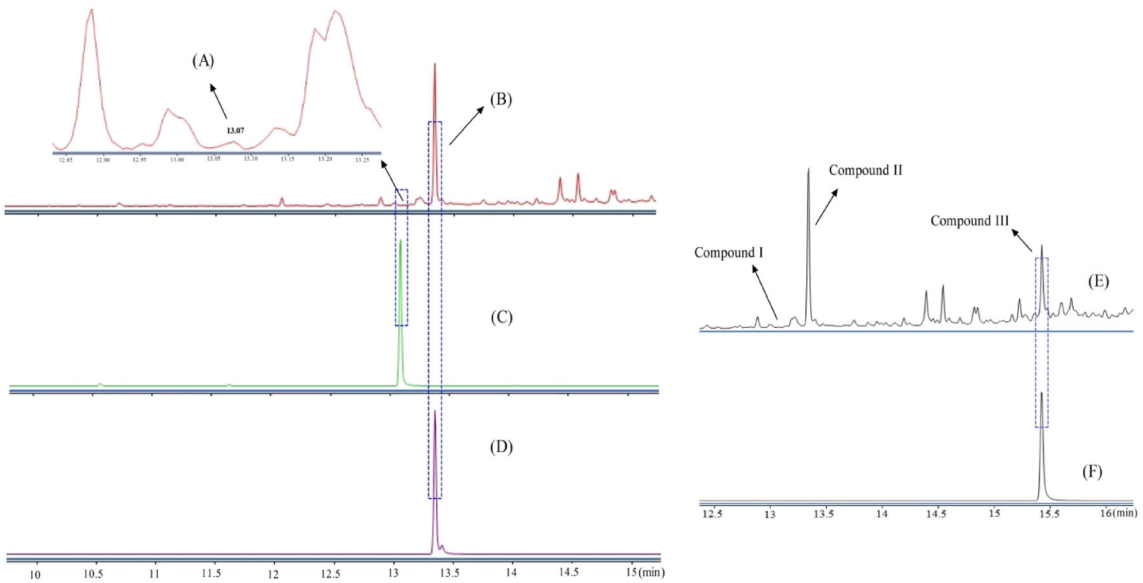

Figure 5. GC–MS analysis of natural and synthetic pheromone components on HP-5 column. (A) Peak corresponding to compound **I** in *Helopeltis cinchonae* female body extract (enlarged), (B) peak corresponding to compound **II**, (C) synthetic hexyl 3-acetoxybutyrate, (D) synthetic 1-acetoxy-5-butyroxyhexane, (E) female body extract, and (F) synthetic 1,5-dibutyroxyhexane.

Table 2. Retention times (RTs) and retention indices (RIs) relative to retention times of *n*-alkanes of candidate pheromone components in gas chromatography–mass spectrometry (GC–MS) analysis of extracts of female *Helopeltis cinchonae* and synthetic compounds on two different GC columns

	HP-5		DB-23	
Compound	RT (min)	RI	RT (min)	RI
Compound **I**	13.07	1479	41.24	1996
Compound **II**	13.33	1500	42.90	2064
Compound **III**	15.42	1661	46.89	2235
Hexyl 3-acetoxybutyrate	13.08	1479	41.24	1996
1-Acetoxy-5-butyroxyhexane	13.34	1500	42.91	2064
1,5-Dibutyroxyhexane	15.42	1661	46.89	2235

detected. However, on injection of a concentrated sample of female extract 1 µL (one female equivalent), a small peak corresponding to compound **I** eluted at 13.07 min (Fig. 5(A)). Neither of the two candidate pheromone compounds was detected in GC–MS analysis of whole-body extracts of virgin male or fifth instar *H. cinchonae* nymphs (Supporting Information Fig. S1).

The two candidate pheromone components were detected in GC–MS analysis on the polar DB 23 column, and retention indices on both columns are shown in Table 2.

The mass spectrum of compound **I** (Fig. 6(A)) shows ions at *m/z* 43 and *m/z* 61, indicating the presence of an acetate group.[28] The ion at *m/z* 103 ($HOCH(CH_3)CH_2COO$) seems to be characteristic of derivatives of 3-hydroxybutyric acid reported as components of the pheromones of related mirid bugs, *D. theobroma* and *S. singularis*[12] and *M. pygmaeus*.[13] The spectrum of compound **I** was identical to that reported for hexyl 3-acetoxybutyrate, a candidate pheromone component of *M. pygmaeus*.[13] The ion at *m/z* 129 confirms the loss of the hexyloxy group ($C_6H_{13}O$) from the molecular ion of *m/z* 230. The mass spectrum and GC retention indices on both columns were confirmed to be identical with those of the synthetic material (Figs 5(C) and S2, and Table 2).

The mass spectrum of compound **II** (Fig. 6(B)) also showed ions at *m/z* 43 and 61, characteristic of an acetate group and ions at *m/z* 71 and *m/z* 89, characteristic of a butyrate ester.[14] A strong ion at *m/z* 82 $(C_6H_{10})^+$ corresponded to a hexadiene fragment ion, suggesting the loss of the two ester groups from a hexane moiety. Synthetic 6-butyroxy-1-acetoxyhexane had mass spectrum and retention indices different from those of compound **II** (data not shown). The ion at *m/z* 115 in the mass spectrum of compound **II** was also observed in the mass spectrum of the pheromone of the saddle gall midge, *Haplodiplosis marginata* (von Roser) (Diptera: Cecidomyiidae), identified as 2-butyroxynonane.[29,30] This probably corresponds to $(C_3H_7C(OH)OCHCH_2)^+$ resulting from a McLafferty-type rearrangement, indicating the butyroxy group is in the 2-position. Synthetic 5-butyroxy-1-acetoxyhexane had mass spectrum and GC retention indices identical to those of compound **II** (Figs 5(D) and S3, and Table 2).

In GC–MS analysis of whole-body extracts of female *H. cinchonae* on the HP-5 column, a third compound (compound **III**) was detected on single ion scanning for butyrate esters at 15.42 min (Fig. 5(E),(F)), although this did not elicit a response in GC–EAD analysis. Compound **III** was not detected in male extracts. The mass spectrum (Fig. S4) had ions at *m/z* 71 and 89, characteristic of butyrate esters, but not *m/z* 43 and 61, characteristic of acetate esters. An ion at *m/z* 82 was consistent with a hexyl dibutyrate, and an ion at *m/z* 115 with at least one of the butyrate esters being at the 2-position. This compound had identical mass spectrum and retention indices on both GC columns to those of synthetic 1,5-dibutyroxyhexane (Fig. S4 and Table 2).

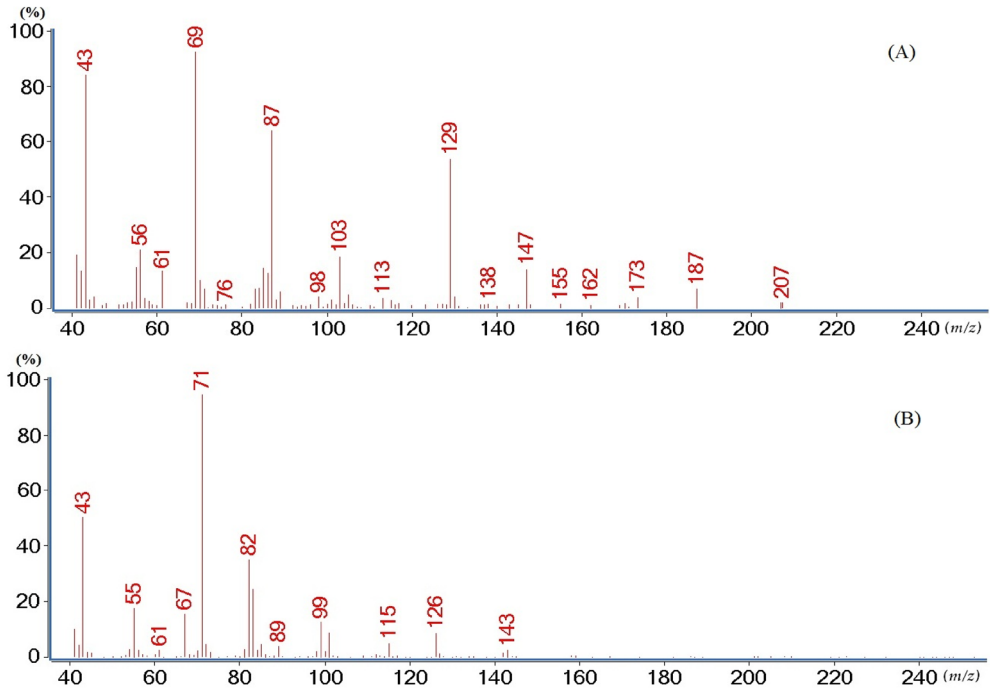

Figure 6. Mass spectra of candidate sex pheromone components in extracts of female *Helopeltis cinchonae*. (A) Compound **I** (hexyl 3-acetoxybutyrate) and (B) compound **II** (1-acetoxy-5-butyroxyhexane).

3.3 Determination of enantiomeric configuration of pheromone components

To determine the enantiomeric configuration of the components of the sex pheromone of *H. cinchonae* female, the female body extract and synthetic compounds were further analyzed by GC–MS using a Cyclodex-B enantioselective column. The (*S*)-enantiomer of hexyl 3-acetoxybutyrate eluted first at 26.19 min and the (*R*)-enantiomer at 26.24 min (Fig. S5(B),(C)). Analysis of female *H. cinchonae* extract showed a peak at 26.24 min (Fig. S5(A)), confirming pheromone **I** as hexyl (*R*)-3-acetoxybutyrate.

In analyses of synthetic 1-acetoxy 5-butyroxyhexane on the Cyclodex-B column, the (*S*)-enantiomer eluted first at 26.79 min, and the (*R*)-enantiomer eluted at 26.87 min (Fig. S6(C),(D)). Analysis of the female *H. cinchonae* extract showed a peak at 26.87 min with the same RT and mass spectrum as the (*R*) enantiomer (Fig. S6(B)). This co-chromatographed with the (*R*)-enantiomer (Fig. S6(A)), confirming the identification of compound **II** as (*R*)-1-acetoxy-5-butyroxyhexane.

Similarly, compound **III** was determined to be (*R*)-1,-5-dibutyroxyhexane by analysis of the Cyclodex-B column (Fig. S7).

3.4 EAG activities of synthetic compounds

In EAG tests of synthetic hexyl 3-acetoxybutyrate and 1-acetoxy-5-butyroxyhexane as racemates and the separate enantiomers, all five compounds elicited EAG responses from male *H. cinchonae* antennae with a significant dose–response (Fig. 7). The strongest EAG response was recorded to the (*R*)-enantiomer of 1-acetoxy-5-butyroxyhexane (compound **II**), followed by the (*R*)-enantiomer of hexyl 3-acetoxybutyrate (compound **I**) and the lowest EAG responses were elicited by the (*S*)-enantiomer of 1-acetoxy-5-butyroxyhexane. The responses elicited by the (*R*)-enantiomers and racemates of both sex pheromone compounds were significantly higher than those to (*S*)-1-acetoxy-5-butyroxyhexane at 100 µg ($F = 17.31$, df = 4,20, $P < 0.001$), 25 µg ($F = 3.90$, df = 4,20, $P < 0.017$), and 5 µg ($F = 5.30$, df = 4,20, $P < 0.004$).

3.5 Release rates of pheromone components

Polyethylene vials loaded with the two pheromone components, hexyl 3-acetoxybutyrate and 1-acetoxy-5-butyroxyhexane, were maintained under constant conditions in the laboratory, and volatiles was collected at intervals of 2 days. Release rates of the two compounds were similar (Fig. 8), However, a significant difference in release rate was observed between the compounds at 6–8 days, 2.85 µg h^{-1} of hexyl 3-acetoxybutyrate ($F = 143.06$, $P < 0.001$), and 3.10 µg h^{-1} of 1-acetoxy-5-butyroxyhexane ($F = 71.53$, $P < 0.001$). The release rates of both compounds then gradually decreased with release rates of 0.60 µg h^{-1} and 0.75 µg h^{-1}, respectively, at day 32.

3.6 Field testing of synthetic compounds

Two field trapping tests were conducted with traps baited with polyethylene vials impregnated with the proposed synthetic pheromone components at various doses and ratios. A negative, unbaited control and positive control of ten virgin females (3–6-day old) were also included. During the whole period, no female *H. cinchonae* were captured in baited or unbaited traps, and only a few non-target insect species, including flies, beetles and moths, were trapped (data not shown).

In field experiment 1, the individual (*R*)-enantiomers of 1-acetoxy-5-butyroxyhexane and hexyl 3-acetoxybutyrate were equally highly attractive to male *H. cinchonae* ($F = 40.40$, df = 7,16, $P < 0.001$) (Fig. 9). Lures containing blends of the (*R*)-enantiomers in 7:3 or 3:7, respectively, were less attractive than

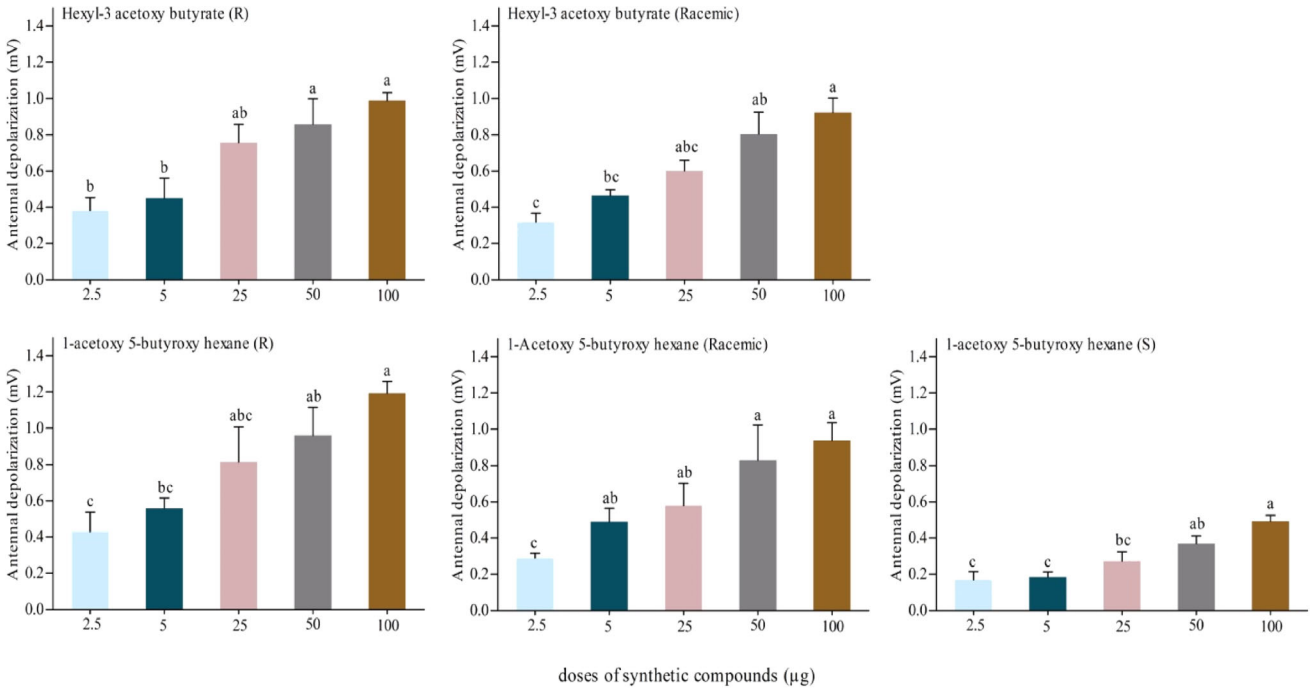

Figure 7. EAG responses (mean ± standard error, mV) of *Helopeltis cinchonae* males ($n = 30$) to synthetic sex pheromone components, hexyl 3-acetoxybutyrate and 1-acetoxy-5-butyroxybutyrate, as racemic mixtures and separate enantiomers at five doses. For each compound, means with different letters are significantly different by Tukey's-*b* test ($P < 0.05$).

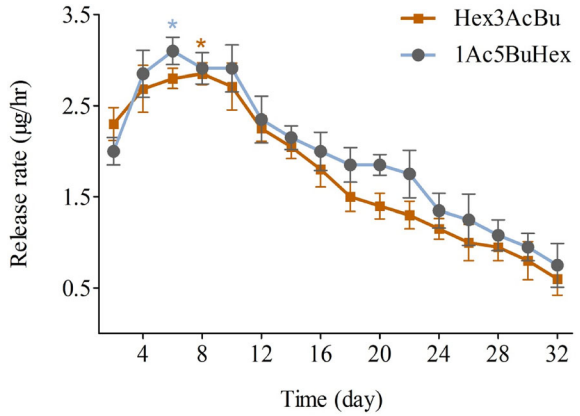

Figure 8. Release rates of hexyl 3-acetoxybutyrate (Hex3AcBu) and 1-acetoxy-5-butyroxyhexane (1Ac5BuHex) from polyethylene vials loaded with 2 mg of each compound and maintained in a laboratory wind tunnel at 26 ± 1 °C, at wind speed of 2.3 m s^{-1}. Results are mean of two replicates.

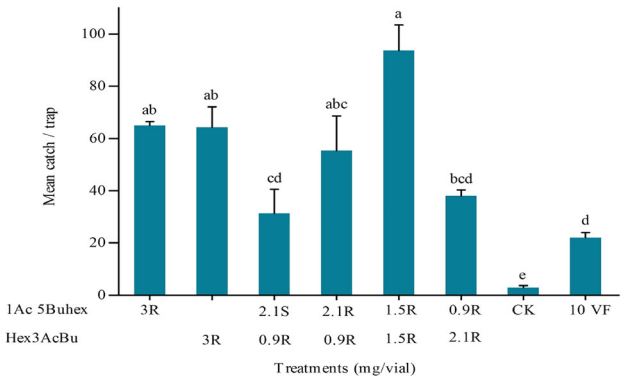

Figure 9. Catches (mean ± standard error) of *Helopeltis cinchonae* males in traps baited with single or binary blends of synthetic sex pheromone components, 1-acetoxy-5-butyroxyhexane (1Ac5BuHex) and hexyl 3-acetoxybutyrate (Hex3AcBu), ten virgin females (10VF) or unbaited (CK) at Jiexi, Guangdong, China. Means with different letters are significantly different (ANOVA $P < 0.05$, followed by *post hoc* Tukey's-*b* test, $P < 0.05$).

those containing the individual compounds, but traps baited with a blend of the (*R*)-enantiomers of both compounds at 1:1 ratio captured the most male *H. cinchonae*, significantly higher than unbaited traps, but not higher than traps baited with the individual compounds. A blend containing the (*S*)-enantiomer of 1-acetoxy-5-butyroxyhexane was less attractive than the corresponding blend with the (*R*)-enantiomer.

Field experiment 2 was aimed to evaluate further binary blends of the EAD-active sex pheromone components and also the effect of adding the non-EAD-active compound identified from extracts of female *H. cinchonae*, 1,5-dibutyroxyhexane. The traps baited with a 10:1 binary blend of the (*R*)-enantiomers of the EAD-active sex pheromone components, 1-acetoxy-5-butyroxyhexane and hexyl 3-acetoxybutyrate, caught significantly more male bugs than the traps baited with the single compounds and unbaited ($F = 43.62$, df = 6,14, $P < 0.001$) (Fig. 10). Traps baited with the blend of equivalent quantities of the racemic compounds caught similar numbers to those in traps baited with the pure enantiomers. Adding 1,5-dibutyroxyhexane did not significantly affect

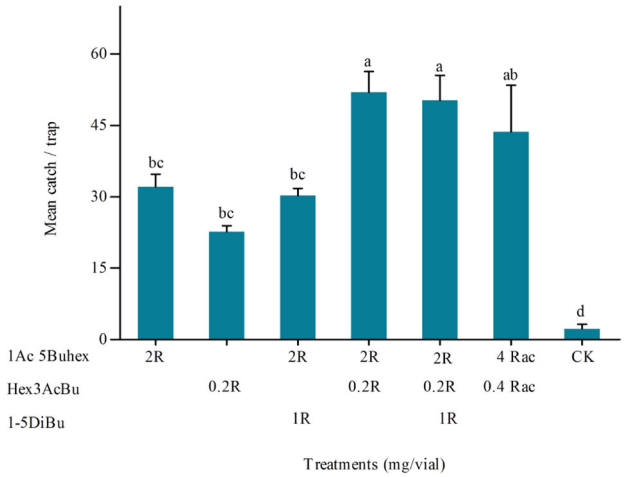

Figure 10. Catches per trap (mean ± standard error) of *Helopeltis cinchonae* males in traps baited with single or binary blend of synthetic sex pheromone compounds 1-acetoxy-5-butyroxyhexane (1Ac5BuHex), and hexyl 3-acetoxybutyrate (Hex3AcBu), 1,5-dibutyroxyhexane (1-5DiBu) or unbaited (CK) at Jiexi, Guangdong, China. Means with different letters are significantly different (ANOVA $P < 0.05$, followed by *post hoc* Tukey's-*b* test, $P < 0.05$).

catches of traps baited with either 1-acetoxy-5-butyroxyhexane alone or as the blend.

4 DISCUSSION

This study aimed to identify components of the female-produced sex pheromone of the tea pest *H. cinchonae*. In the analyses of whole-body extracts of virgin female *H. cinchonae* by GC–EAD, two compounds were detected which elicited EAG responses from antennae of male *H. cinchonae*. These compounds were not observed in analyses of extracts of males or fifth instar nymphs and were thus considered potential pheromone components. The compounds were identified as hexyl (*R*)-3-acetoxybutyrate and (*R*)-1-acetoxy-5-butyroxyhexane, respectively. In field trapping tests, traps baited with either compound singly caught significant numbers of male *H. cinchonae*, but traps baited with binary blends generally caught more bugs. A third compound was detected in GC–MS analysis of the female extract and identified similarly as (*R*)-1,5-dibutyroxyhexane. This compound did not elicit an EAG response in GC–EAD analysis, and adding to the attractive binary blend in field tests did not improve or reduce attractiveness.

Hexyl (*R*)-3-acetoxybutyrate was also identified in whole body washes of females of the mirid bug, *M. pygmaeus*.[13] It was not present in washes of males, but no behavioral responses were observed to this compound.[13] The same 3-hydroxybutyrate motif is found in hexyl (*R*)-3-hydroxybutyrate and hexyl (*R*)-3-[(*E*)-2-butenoyloxy]butyrate, pheromone components of the cocoa mirids, *D. theobroma* and *S. singularis*.[12] These three species belong to the Bryocorinae sub-family, like *H. cinchonae*, suggesting this 3-hydroxybutyrate structure may be characteristic of this sub-family. However, the pheromone of *N. tenuis*, another member of this sub-family, is octyl hexanoate,[14] which is more typical of the pheromones of mirid bugs in other sub-families.

As far as we know, 1-acetoxy-5-butyroxyhexane is a novel structure for an insect pheromone. It is difficult to see any obvious biosynthetic relationship between this compound and hexyl 3-acetoxybutyrate. 3-Hydroxybutyric acid and derivatives are

found in animals, plants, and microbes and probably biosynthesized by condensation of two molecules of acetyl CoA.[31] 1-Acetoxy-5-butyroxyhexane has some resemblance to the pheromones of plant-feeding midges (Diptera: Cecidomyiidae) with a butyrate function in the 2-position and may be biosynthesized similarly.[32] However, the intriguing chemical similarity between these two compounds should be noted. Both are diesters, with one ester grouping of a primary alcohol and the other of a secondary alcohol in the 2-position. Both comprise two-carbon, four-carbon, and six-carbon moieties and have the same molecular formula and hence molecular weight.

Previous studies reported the attraction of male bugs of four *Helopeltis* species by con-specific females,[19–22] which indicated the presence of a sex pheromone. However, it was not possible to isolate and identify the pheromones in these cases.[23,24] Sachin *et al*.[22] reported the identification of components of the sex pheromone of *H. theivora*. Five compounds were identified in solvent extracts of females as (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl butanoate, (*E*)-2-hexenyl pentanoate, 2,4-dimethylpentanal, and (*E*)-2-hexenol. However, these identifications are partially incorrect based on the chromatogram and RTs reported. A binary blend of (*Z*)-3 hexenyl acetate and (*E*)-2-hexenol at a ratio of 1:5 elicited EAG responses and was attractive to male bugs in a wind tunnel bioassay. However, there is no evidence of the field application of those compounds. In our GC–MS analysis of extracts from female *H. cinchonae*, (*Z*)-3-hexenyl acetate, and 2-ethyl-1-hexanol were detected (Fig. S8(A),(B)), although in preliminary behavioral experiments, male *H. cinchonae* bugs did not show a response to either compound (data not shown), and these could well be chemicals from the host plant.

The amount of hexyl (*R*)-3-acetoxybutyrate in whole body extracts of virgin female *H. cinchonae* relative to that of (*R*)-1-acetoxy-5-butyroxyhexane was extremely low (approximately 1%), and its presence was only first detected in GC–EAD analysis, similar with our previous studies.[33] Both compounds were highly EAG-active. In the field experiments, traps baited with the individual compounds caught similar numbers of male *H. cinchonae*. The 7:3, 3:7, and 1:1 blends of the two compounds were not more attractive than the individual compounds, but in the second experiment, a 10:1 blend of (*R*)-1-acetoxy-5-butyroxyhexane and hexyl (*R*)-3-acetoxybutyrate, respectively was more attractive than the individual compounds. Based on laboratory release rate measurements, it may be inferred that the composition of the blend released and loaded will be identical because both compounds were released at similar rates from the polyethylene vials utilized here. Further work is required to optimize the composition and loading of the blend of the two components in the polyethylene vial lures for maximum attractiveness to *H. cinchonae*. In other mirid species, the females often produce well-defined blends of pheromone components,[11,34,35] but males respond to a wider range of blends.[10,36]

The field experiments showed that the racemic 1-acetoxy-5-butyroxyhexane and hexyl 3-acetoxybutyrate are as attractive to male *H. cinchonae* as the (*R*)-enantiomers and also that adding the non-EAD active compound detected in extracts of female *H. cinchonae*, 1,5-dibutyroxyhexane, did not affect attractiveness. However, loading of the pheromone components, that is release rates, may also be important and needs further investigation. Rubber septa loaded with the 1.5 mg:1.5 mg blend of (*R*)-1-acetoxy-5-butyroxyhexane and hexyl (*R*)-3-acetoxybutyrate were not attractive in field tests. The preliminary release rate studies indicated the compounds are released at a much higher rate (data not shown). It is known in other mirid species that the same compounds can be used as pheromones and as defense or alarm compounds depending upon release rates.[34,37–39] In such species, the same compounds can be extracted from males and females,[10,40,41] but this was not the case in *H. cinchonae*.

In the field experiments, the number of non-target species caught in baited and unbaited traps was very low and considered accidental catches or influenced by the color of traps.[42] However, it was observed that most of the male *H. cinchonae* did not land directly on or in the traps but first landed near the trap at a distance of 8–10 cm. Some walked through the white sticky boards towards the lure, but others did not. Thus, further work is required to optimize the trap design to ensure all the bugs attracted are captured.

5 CONCLUSION

Extracts of virgin female *H. cinchonae* contained two compounds that elicited EAG responses from male antennae. These were identified as hexyl (*R*)-3-acetoxybutyrate and (*R*)-1-acetoxy-5-butyroxyhexane. The latter is a novel compound and has not been previously reported as an insect pheromone. These structures may be characteristic of the pheromone components of mirid bugs in the Bryocorinae sub-family. Traps baited with the individual compounds dispensed from polyethylene vials caught large numbers of male *H. cinchonae*. Specific blends of the two compounds were more attractive than the single compounds, and the racemic compounds were as attractive as the (*R*)-enantiomers. Further work is required on the blend of compounds and the loading as well as on trap design to provide an optimized system for monitoring and controlling this pest. The results may also facilitate the identification of the pheromones of other *Helopeltis* species, which are important pests in many crops.

ACKNOWLEDGEMENTS

The authors thank Zou Junhong for his help in field collection of *H. cinchonae*, and field application of sex pheromone compounds in his tea plantations at Jiexi Guangdong, China (23.38°N, 115.5° E). This research was funded by the National Key Research and Development (R&D) Plan (2021YFD1601100; 2022YFD1600803), the Innovation Program of the Chinese Academy of Agricultural Sciences (CAAS-CSCB-202302), A National Key Program for Development of Basic Scientific Research (973 Program) (2012CB114104), National Natural Science Foundation of China (31171862), Modern Agricultural Industry Technology System (CARS-23), and the Division of Science and Technology of Zhejiang Province (2011C22043).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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