

Sex Pheromone of the Carpenterworm, *Holcocerus insularis* (Lepidoptera, Cossidae)

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Sex Pheromone, (Z)-3-Tetradecenyl Acetate, *Holcocerus insularis*

By means of thin-layer chromatography (TLC), electroantennogram (EAG), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) and field tests, (Z)-3-tetradecenyl acetate(Z3-14:Ac), (E)-3-tetradecen-1-ol(E3-14:OH), and (Z)-3-tetradecen-1-ol(Z3-14:OH) at a ratio of 51:39:10 were identified from the female sex pheromone gland extracts of the carpenterworm, *Holcocerus insularis* Staudinger (Lepidoptera, Cossidae). The average amounts of Z3-14:Ac, E3-14:OH and Z3-14:OH in a single sex pheromone gland of calling moth were 7.29 ± 2.72 ng, 5.72 ± 2.43 ng and 1.44 ± 0.56 ng, respectively. This is the first time that Z3-14:Ac was identified as a component of lepidopteran sex pheromone. Traps baited with rubber septa impregnated with Z3-14:Ac (500 µg / septum) were more effective than the traps baited with virgin female. The addition of the E3-14:OH and Z3-14:OH to rubber septa baited with Z3-14:Ac did not modify *H. insularis* male attraction, but E3-14:Ac slightly enhanced trap catch.

Introduction

The carpenterworm moth, *Holcocerus insularis* Staudinger (Lepidoptera, Cossidae) is a destructive forest pest widely distributed throughout China and ex-USSR. In China, the larvae inflict serious damage to broad-leaved trees and fruit trees by tunneling into their trunks. The trunks of damaged tree are full of wounds and holes that eventually can cause the death of entire tree (Wang and Zhang, 1993).

In the Cossidae moths, the sex pheromones of three species, *Prionoxystus robiniae* (Solomon *et al.*, 1972), *Cossus cossus* (Capizzi *et al.*, 1983), *Cossus mongolicus* (Qi *et al.*, 1990), and the sex attractants of two species, *Acosinus centeresis* (Doolittle *et al.*, 1976a,b) and *Prionoxystus piger* (Landolt *et al.*, 1985), have been reported. In this paper, we report the isolation and identification of *H. insularis* sex pheromone, as well as the results of field trapping tests.

Methods and Materials

Insects

Insects were collected from ash trees in Beijing, China. Some blocks (ca. 80 cm length) of ash trees

were cut off, which were damaged by *H. insularis* in the summer before emergence, and were maintained in a screen cage (2×2×2 m) in the shade out of door to allow natural eclosion. Virgin male and female moths were sexed. Virgin male moths were removed out immediately after emergence. The female ones remained in the original cage for observation and extraction.

Collection of sex pheromone from calling females

24 hr observation of 1~2-day-old female moths revealed that the moths began calling and mating ~5 hr after sunset. Sex pheromone glands were extruded out immediately by applying gentle pressure to the female's abdominal tips to force eversion of the ovipositor and were excised with small scissors and immersed in re-distilled hexane (ca. 30 µl / tip) for 1 hr at 0° C. The hexane extracts were transferred and pooled to a clean conical glass vial and kept in a freezer at -10° C. The extracts were concentrated carefully as needed by N₂ prior to analysis.

Thin-layer chromatography of sex pheromone

The active extracts were subjected to TLC on the plate with silica gel G60 developed with petro-



leum ether : diethyl ether (1:1 v/v). A mixture of Z3-14:OH and Z3-14:Ac was run as a standard on another same plate. Horizontal strips were scraped off from the plate and extracted with re-distilled hexane.

Electroantennogram

Electroantennogram (EAG) assays were conducted as previously described (Roelofs *et al.*, 1971; Zhang and Meng, 2000). Electrophysiological responses of male antennae to a series of C14 unsaturated alcohol and acetates and TLC extracts were determined.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS)

The GC of sex pheromone gland extracts and standard compounds were performed on a HP 5890 gas chromatograph fitted with a flame ionization detector (FID) and a splitless injector. Two fused silica capillary columns, A (HP-1, 50 m, id. 0.22 mm, film thickness 0.33 μ m; Paloalto California) and B (BP10-0.5, 50 m, id. 0.42 mm, film thickness 0.33 μ m; SGE, Australia) were used with a temperature program of 120° C for 1 min, then 4° C / min to 220° C, isothermal 20 min for the column A and 120° C for 1 min, then 4° C / min to 200° C, isothermal 25 min for the column B. GC-MS analyses were performed on a Trace 2000 gas chromatograph (GC) interfaced with a Voyager mass spectrometer (MS). Two fused silica capillary columns, C (DB-5, 60 m, id. 0.25 mm, film thickness 0.25 μ m) and D (RTX-5, 15 m, id. 0.33 mm,

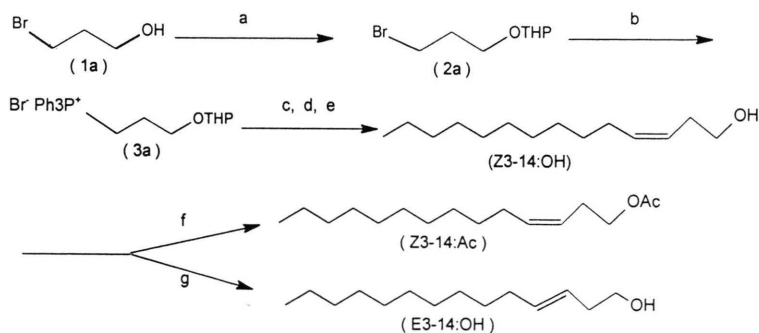
film thickness 0.25 μ m), were used with a temperature program of 50° C for 1 min, then 10° C / min to 270° C, isothermal 20 min. Mass spectral data and retention times of selected peaks on both columns were compared to corresponding data from reference standards.

Synthesis of Z3-14:Ac, E3-14:OH and Z3-14:OH

The title compounds were synthesized (Scheme 1 or 2) via Wittig reaction routs (Horiike *et al.*, 1980) and acetylene routs (Henrick, 1977):

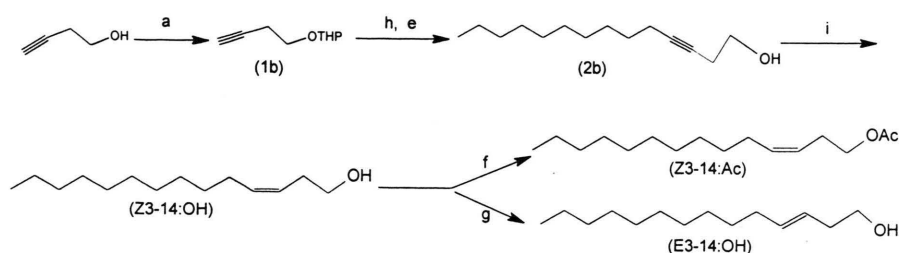
In Scheme 1, commercially available 3-brom-1-propanol (**1a**) was protected at the hydroxyl group to form tetrahydropyranyl derivation **2a** which was further converted into triphenyl phosphonium salt (**3a**). Wittig reaction of **3a** with undecylic aldehyde (key step), deprotection of the reaction product, produced (Z)-3-tetradecen-1-ol. The alcohol was transformed to (Z)-3-tetradecenyl acetate or isomerized to (E)-3-tetradecen-1-ol.

In Scheme 2, the tetrahydropyranyl derivation **1b** of 3-butyn-1-ol was elongated with 1-bromodecane. The deprotection of the reaction product gave tetradecen-3-yn-1-ol (**2b**), which was transformed directly to the (Z)-3-tetradecen-1-ol by hydrogenation over P-2Ni catalyst. The alcohol was further converted into (Z)-3-tetradecenyl acetate. The syntheses of other C14 acetates were carried out via the same way. All products were purified by silica gel column. The purity of compounds was confirmed to be more than 96% by gas chromatography.



Scheme 1

a: DHP, HCl; b: $\text{Ph}_3\text{P} / \text{C}_6\text{H}_6$; c: $\text{n-BuLi} / \text{DMSO}$; d: $\text{CH}_3(\text{CH}_2)_9\text{CHO}$; e: CH_3OH , P-TsOH; f: CH_3COCl , $\text{C}_5\text{H}_5\text{N}$; g: $\text{NaNO}_2 / \text{HNO}_3$.



Scheme 2

h: $n\text{-BuLi}$ / THF, $\text{CH}_3(\text{CH}_2)_9\text{Br}$ / HMPA; i: H_2 / P-2Ni.

Field tests

Field trapping tests were done during the *H. insularis* flight season (June 1 to July 20 2000) in Beijing (China) where the larvae were collected. Test chemicals in hexane were loaded on green rubber septa (sleeve type). White Delta sticky traps were hung on ash trees at ca. 2 m height, 10~20 m interval, and insects trapped were checked every day. 1~2-day-old virgin females were put into a small screen cage in the middle inside the trap as bait for comparison. Hexane was used as control. For each formulation, 3 replicates were deployed in a randomized block. The experiments were designed to examine attraction of different chemical composition and the optimum dosage of each active component.

Results

Analysis of sex pheromone gland extracts

20 female equivalent (FE) sex pheromone gland extracts were subjected to TLC, and separated into three bands, R_{f1} 0.07 (TLC₁), R_{f2} 0.35 (TLC₂), and R_{f3} 0.69 (TLC₃) on the plate. The TLC₂ and TLC₃ showed R_f as synthetic compounds E3-14:OH (or Z3-14:OH) and Z3-14:Ac, respectively. The bands were scraped off from the plate and extracted with re-distilled hexane. The extracts (1FE / 20 μl) were assayed for male antenna responses by EAG. The extract of TLC₃ caused high EAG (4.3 mV), TLC₂ moderate (1.9 mV), and TLC₁ hardly any response.

The GC of female sex pheromone gland extracts and a series of *Z* and *E* isomers of monounsaturated C₁₄ standards were made on either of the capillary column A and B under different temper-

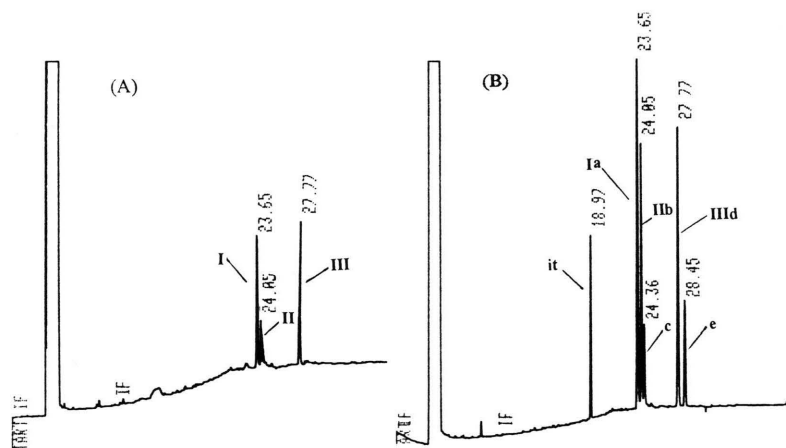


Fig. 1. (A) GC (BP10-0.5) of the pheromone gland extracts of *H. insularis* and (B) a mixture of 0.5 μl crude extract and 0.5 μl blend of equal 2,6-di-tert-butyl-*p*-cresol (it: internal standard 10 ng), E3-14:OH(a), Z3-14:OH(b), 14:OH(c), Z3-14:Ac(d) and 14:Ac(e).

ature condition. In comparison of the retention times (Rt), peaks I, II and III (Fig. 1A) consistently co-chromatographed with synthetic standard compounds E3-14:OH, Z3-14:OH, and Z3-14:Ac. The extract (TLC₂) gave two well-separated peaks (I and II) and the extract (TLC₃) gave one peak (III). When 0.5 μ l extract and 0.5 μ l blend of equal 2,6-di-tert-butyl-*p*-cresol (it: internal standard 10 ng), E3-14:OH(a), Z3-14:OH(b), 14:OH(c), Z3-14:Ac(d) and 14:Ac(e) were injected simultaneously into the column B, the peaks corresponding to compounds a, b and d

were heightened (Fig. 1B). The results, together with those already mentioned, strongly suggest that E3-14:OH, Z3-14:OH and Z3-14:Ac are present in the sex pheromone of *H. insularis*. Moreover, quantitative analysis by GC showed that titer (ratio) of them varied around a mean of 5.72 ± 2.43 ng (39), 1.44 ± 0.56 ng (10) and 7.29 ± 2.72 ng (51), respectively, in a single calling female sex pheromone gland.

TIC showed the presence of three peaks (Fig. 2A, peak I–III) in the extracts, which were identical with those of synthetic standard com-

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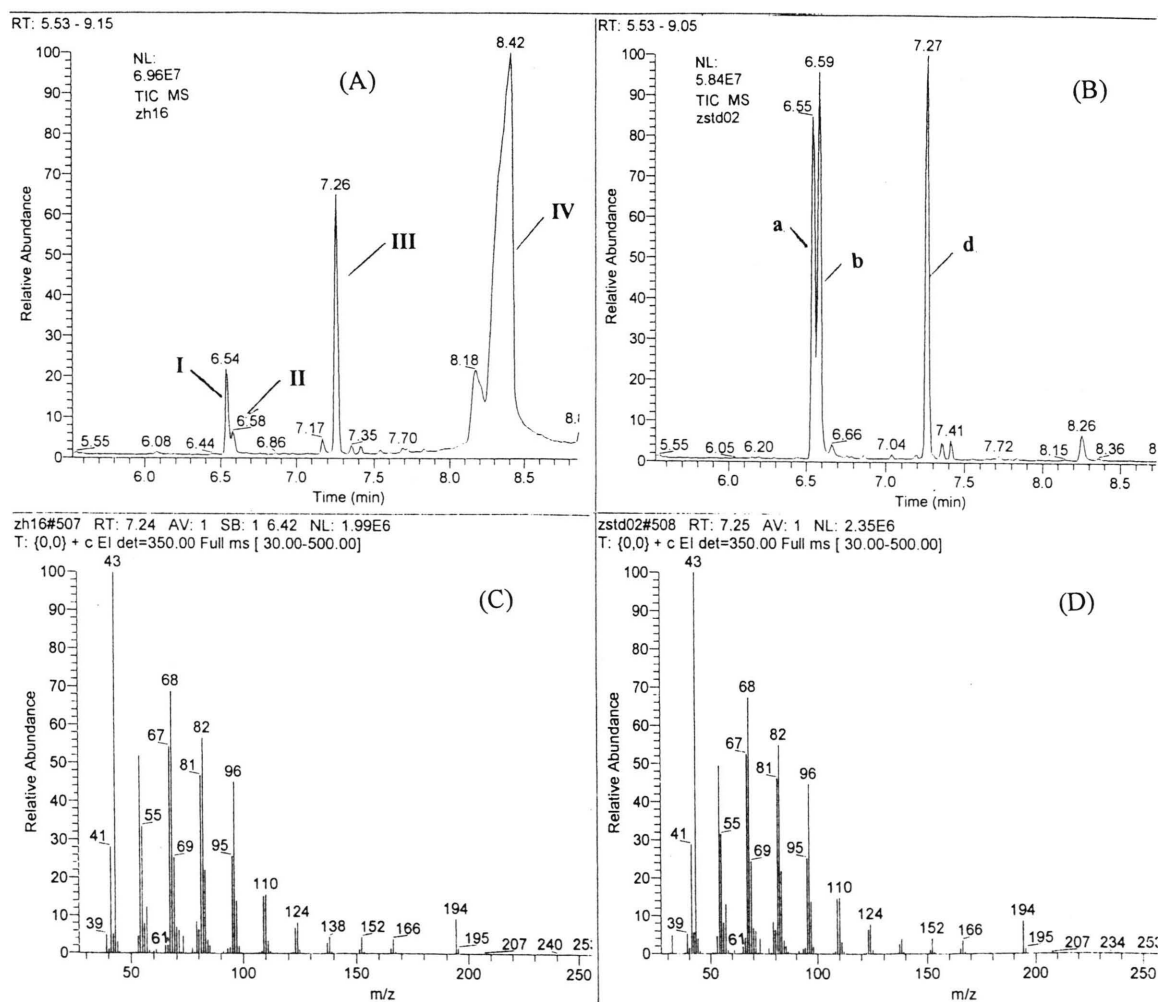


Fig. 2. Mass spectra with EI (70eV) showing the TIC of sex pheromone gland extracts of *H. insularis* (A) and synthetic standard compounds E3-14:OH (a), Z3-14:OH (b) and Z3-14:Ac (d) (B); MS data for peak III (C) and Z3-14:Ac(d) (D) on the RXT-5 column.

pounds (Fig. 2B, a, b and d). All mass data were listed in Table I. Although peaks I–III produced a common ion fragment of m/z 194, which indicated that they had similar straight-chain structure, peak I, II had characteristic ion fragments of m/z 31 (CH_2OH^+) and 71 ($\text{C}_4\text{H}_6\text{OH}^+$) of alcohol (Silverstein *et al.*, 1981), peak III had m/z 61 ($\text{CH}_3\text{COOH}_2^+$) and 43 ($\text{O}=\text{C}^+\text{CH}_3$) of acetate (Brown *et al.*, 1988). This confirmed that peak I, II was alcohol and peak III acetate. CI-MS further indicated the peak I (II) and peak III to have molecular weight 212 and 254, respectively. By the relative intensities of five diagnostic pairs of the predominant ions – $[(m_1/z) / (m_2/z)]: 54/55, 67/68, 81/82, 81/95$ and $95/96$, the similarity indices between compound I–III and each of the double-bond positional isomers of C14 alcohol and acetates were calculated according to the fussy reasoning method (Horiike *et al.*, 1990, 1991). The comparison of these indices indicated that peak I (II) and III were 3-tetradecen-1-ol and 3-tetradecenyl acetate. Peak IV (Fig. 2A) is a saturated 16-carbon fatty acid (MW 256), which was not detected in GC owing to lower column temperature. Finally, the comparison of the R_f (TLC), R_t (GC)

and spectral data (GC-MS) with those of authentic synthetic compounds enabled us to conclude that peak I–III were E3-14:OH, Z3-14:OH and Z3-14:Ac, respectively.

The EAG responses of male antennae to compounds identified from female sex pheromone glands and analogues differing in double-bond position and number are summarized in Fig. 3. Male antennae produced the strongest responses to Z3-14:Ac (5.9 mV), and moderate to E3-14:OH (2.0 mV) and Z3-14:OH (2.6 mV). It should be noted that Z3E5-14:Ac, E3-14:Ac and Z9-14:Ac exhibited strong EAG response comparable to Z3-14:Ac, but Z5-14:Ac, the sex pheromone component of *Cossus cossus*, weaker. All results show that distinct responses were caused only by tetradecenyl acetates and to a lesser extent by the corresponding alcohol, and *cis*-isomers elicited much stronger responses than *trans*-isomers. These suggest that the double-bond position of tetradecenyl acetate be in 3-position and *cis*-isomer.

Field tests

Preliminary field tests were carried out around Beijing and the results are shown in Table II. The

Table I. Mass data for components of sex pheromone gland extract and standard compounds.

Component	Standard	m/z (relative intensity of major ions) [assignment]
I	E3-14:OH	212 (< 0.5)[M^+], 194 (6)[$\text{M}^+ - \text{H}_2\text{O}$], 166(5)[$\text{M}^+ - \text{C}_2\text{H}_5\text{OH}$], 109(11), 96(42), 95(34), 82(76), 81(52), 68(100), 67(63), 55(99), 54(36), 31(13)[CH_2OH^+]
II	Z3-14:OH	194 (2)[$\text{M}^+ - \text{H}_2\text{O}$], 166(2)[$\text{M}^+ - \text{C}_2\text{H}_5\text{OH}$], 109(12), 96(34), 95(31), 82(63), 81(82), 68(100), 67(83), 55(72), 54(31), 31(10)[CH_2OH^+]
III	Z3-14:Ac	194(8) [$\text{M}^+ - 60$], 152(5)[$\text{C}_{11}\text{H}_{20}^+$], 110(16)[$\text{C}_8\text{H}_{14}^+$], 96(44), 95(26), 82(56), 81(46), 68(67), 67(54), 61(1)[$\text{CH}_3\text{COOH}_2^+$], 55(32), 54(52), 43(100)[$\text{O}=\text{C}^+\text{CH}_3$]

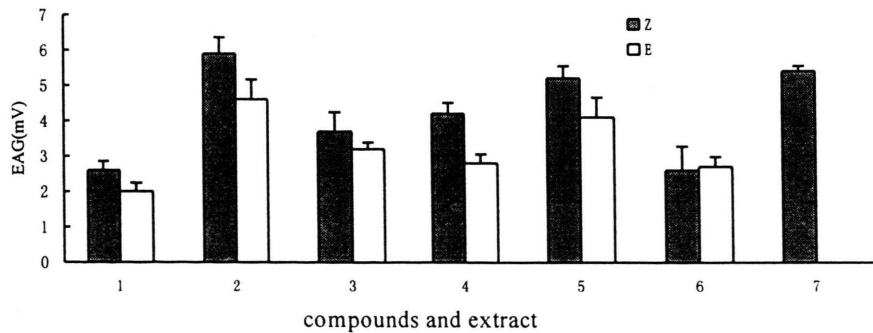


Fig. 3. EAG responses of male *H. insularis* to compounds at a dose of 10 μg und sex pheromone extract; 6 replicates. Bars indicate mean \pm SE; 1:Z3-14:OH,E3-14:OH; 2:Z3-14:Ac,E3-14:Ac; 3:Z5-14:Ac,E5-14:Ac; 4:Z9-14:Ac,E9-14:Ac; 5:Z3E5-14:Ac,E3E5-14:Ac; 6:Z12-14:Ac,E12-14:Ac; 7: 10FE extract.

compounds identified in sex pheromone extract and some analogues on the basis of their high EAG activities were included in these tests. The results indicated that Z3-14:Ac was essential for the selective attraction of *H. insularis* male moths in the field, which, at a dose of 500 µg, showed more attractive to males than virgin females. On the contrary, E3-14:OH and Z3-14:OH had hardly attraction to males, and addition of them to Z3-14:Ac did not show any antagonistic or synergistic effect. Even though E3-14:Ac was not positively identified in sex pheromone extracts, the results showed it had weak attractiveness. When it was added to Z3-14:Ac at the ratio tested, the trap catches of male moths were slightly increased. This showed E3-14:Ac to have a possible synergistic effect. Although Z3E5-14:Ac and Z9-14:Ac presented strong EAG activity, neither had attractiveness to *H. insularis* male moths.

Table II. Field attraction of *H. insularis* males to various chemical^a.

Trap lure composition	Dose µg/septum	Males caught/trap mean ± SE ^b
moths	6 virgin females	20.8 ± 3.4c
Z3-14:Ac	500	48.0 ± 2.8ab
E3-14:Ac	500	6.4 ± 1.7d
Z3-14:Ac+E3-14:Ac	500+300	51.8 ± 4.6a
Z3-14:Ac+E3-14:OH	500+300	45.2 ± 3.7ab
Z3-14:Ac+Z3-14:OH	500+300	41.3 ± 2.6b
Z3-14:OH	500	1.2 ± 0.5d
E3-14:OH	500	0.8 ± 0.4d
Z3E5-14:Ac	500	0.00d
Z9-14:Ac	500	0.00d
Hexane (control)	1000 µl	0.00d

^a Caught in Beijing June 17~ July 1, 2000.

^b Six replicates, means followed by the same letter are not significantly different by Duncan's multiple range test ($p = 0.05$).

Discussion

The analytical data, electrophysiology and field tests indicated that the extracts from the sex pheromone glands of *H. insularis* contained three compounds, E3-14:OH, Z3-14:OH and Z3-14:Ac. The main active component of the extracts was characterized as Z3-14:Ac, which had not been identified

as Lepidoptera sex pheromone before, although it was reported as sex attractants in a few of Gelechiidae species (Arn *et al.*, 1992, 2000). *H. insularis* belongs to the Cossidea, in which the common structures of sex pheromones identified consist of monounsaturated docenyl acetates or tetradecenyl acetates with the double-bond in position 5 or diunsaturated tetradecadienyl acetates with the conjugated double-bond in position 3,5.

Although the E3-14:OH and Z3-14:OH were detected significant amount in the extracts from the sex pheromone gland, and caused a moderate EAG response to the male moths, they could hardly catch the male moths. Since we did not observe any significant difference in the catches between Z3-14:Ac and the mixture of Z3-14:Ac and E3-14:OH (or Z3-14:OH), we intended to obtain more information about the biological role of the compounds in further field assays and investigating the release rate of sex pheromone components by female *H. insularis*.

The E3-14:Ac, the isomer of Z3-14:Ac, although not detected in the extracts, is a potential synergistic component of the Z3-14:Ac. This result is similar to the sex attractant for carpenterworm, *Prioxystus robiniae* (Doolittle and Solomon, 1986).

EAG results showed that Z3E5-14:Ac and Z9-14:Ac exhibited strong EAG response comparable to Z3-14:Ac, however, none of which had any attraction to male *H. insularis*.

Synthetic Z3-14:Ac, at a dose of 500 µg, is an effective attractant, which is currently used in experimental programs for monitoring the flight period and abundance of *H. insularis*, in order to integrate management of the insect in all parts of the country.

Acknowledgments

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