Identification of the Sex Pheromone of the Common Forest Looper *Pseudocoremia suavis*

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June 2004

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Frontispiece: An adult male common forest looper *Pseudocoremia suavis*

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Date: 28th June 2004   Date: 28th June 2004

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This report summarises work undertaken in 2002-2004, for the New Zealand Forest Health Research Collaborative, to identify the sex pheromone of the common forest looper, *Pseudocoremia suavis* (Lepidoptera: Geometridae). *Pseudocoremia suavis* is a polyphagous native moth whose larvae also eat the leaves of *Pinus radiata*. In the past *P. suavis* has been responsible for major deforestation in *P. radiata* plantations notably at Eyrewell Forest, North Canterbury and in Kaingaroa Forest, Bay of Plenty.

During 2002-2003 we identified four electrophysiologically active compounds in sex pheromone gland extracts of *P. suavis*, using coupled gas chromatography-electroantennogram detection (GC-EAD). Among these four antennally active compounds, two compounds consistently elicited a strong antennal response. However, preliminary analysis in 2002-2003, by coupled gas chromatography-mass spectrometry (GC-MS) to determine their chemical structure, indicated that the active compounds were present in relatively small amounts and therefore large numbers of moths would be required to obtain useful quantities, for any further qualitative analysis.

In December of 2003, when enough moths became available from a colony established at Forest Research, we analysed the pheromone gland extract by GC-MS. From mass spectral data obtained for three of the four active compounds, we established that the active compounds were most likely to be the unsaturated epoxides, (*Z*)-6-9,10-epoxynonadecene (active 1), (*Z*,*Z*)-3,6-9,10-epoxynonadecadiene (active 2) and (*Z*,*Z*)-3,6-9,10-epoxyheneicosadiene (active 4). Epoxides are known to be pheromones in the geometrid family and within the subfamily Ennominae, to which *P. suavis* belongs, nineteen carbon compounds are the most frequently occurring.

In 2004 the racemic form of (*Z*)-6-9,10-epoxynonadecene was synthesied by HortResearch chemists and a subsequent comparison of mass spectral data, has confirmed this compound as active 1. Similarly comparison of the mass spectra of active 4 with a racemic standard has established this active to be (*Z*,*Z*)-3,6-9,10-epoxyheneicosadiene. It is likely but not guaranteed that a synthetic blend of the two compounds eliciting the strongest antennal response (actives 1 and 2), will be enough for attraction of *P. suavis* males in the field. However, these types of compounds (epoxides) have chiral centres and therefore will have enantiomeric isomers, which may or may not be important to achieve an attractive blend capable of trapping male *P. suavis* on sticky bases, in delta pheromone traps within *P. radiata* plantations.

It is expected that completion of this project will realistically require another 1-2 years and so further work will be undertaken as part of our FRST funded activity in the Biosecurity of New Zealand Forests programme.

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INTRODUCTION

The common forest looper, *Pseudocoremia suavis* (Lepidoptera: Geometridae), is a polyphagous native moth whose larvae eat the leaves of a wide variety of native and exotic trees and shrubs, including southern beech, podocarps and conifers (Dugdale, 1958). In the 1950's and again in the 60's *P. suavis* was responsible for major defoliation in *Pinus radiata* plantations at Eyrewell Forest in North Canterbury (White, 1974), and caused considerable damage in Douglas-fir stands in Kaingaroa Forest, Bay of Plenty in the 1970's (Alma, 1977). It was considered useful therefore to identify its sex pheromone as a tool to study its ecology, long-term phenology including detection and delimitation surveys, if another outbreak was to occur. A sex pheromone may also be useful for controlling any future outbreak, for example, using mating disruption, lure and kill and/or mass trapping technologies.

To date, the sex pheromones of more than 120 geometrids have been identified (El-Sayed, 2004), although none from New Zealand, but typically this family of moths have sex pheromones consisting of single or multi-component blends of compounds such as hydrocarbons and/or epoxides. One species is known to use an unsaturated ketone as a component of its sex pheromone (Buser et al., 1985). There are over 30 species of *Pseudocoremia* in New Zealand (Dugdale, 1988).

MATERIALS AND METHODS

Insect Rearing

A lab colony of *P. suavis* was started by collecting live female moths attracted to a UV light over 12 nights, from November 2002 to January 2003 in Eyrewell Forest. For egg laying, captured female moths were placed individually in 400 ml clear plastic jars with fine mesh lids. A dental wick soaked in 10% honey solution and a tissue strip (an egg laying substrate made of four layers of 2 ply facial tissue, stapled and cut into 10 mm by 20 mm rectangles) was added to each jar. Additional eggs were obtained from wild *P. suavis* reared from larvae collected in Eyrewell Forest. These larvae were reared in the laboratory on *Pinus radiata*, and adults were added to mating cages (described below) along with F1 adults from the colony.

Tissue strips with eggs were placed in small pottles until eggs hatched. Neonate larvae and remaining unhatched eggs were then transferred to a 1 L rearing jar. All larvae were reared on new growth *P. radiata* needles obtained from roadside hedges in Halswell, Christchurch. Rearing was carried out at room temperature (approximate daytime temperatures: mean 23°C, min 12°C, max 30°C) under fluorescent lights with a 12L:12D photoperiod. First and second instar larvae were reared in 1 L jars. Fresh pine branch tips, checked for insects and spiders, were provided as food, with the cut end of each branch tip wrapped in parafilm to reduce drying out. Neonate larvae are highly phototactic, so to ensure larvae remain on the pine, rather than dying on the inside of the jar, two branch tips were curled around the base of the jar, with two more filling the rest of the jar. Jars were laid on their side with the base facing the light.

The pine was replaced every third day for first and second instar larvae. To do this, all the old branch tips were gently removed. Two new tips were placed in the base of the jar as described above, then two of the old tips with the most larvae on them were placed in the middle of the jar, and the mesh top put back on. Larvae moved to the light, off the old pine onto the new material.

After the second instar, once larvae were less phototactic, they were moved to clear plastic boxes (320 x 310 x 80 mm, with mesh-covered ventilation holes in the lid), and pine branches were changed every fifth day. Larvae from old branches were brushed off onto new branches. A light mist of water was applied to the boxes every few days if needed, but care was taken not to let rearing boxes become so moist that condensation formed. Frass and cut pine needles from the growing larvae were left in the boxes to provide a substrate for pupation. Pupae were removed from each box once the majority of larvae had pupated.

Moth Mating

Pupae of both sexes were placed together in open Petri dishes on the floor of a large mesh cage (600 x 600 x 600 mm), with some shredded paper to simulate leaf litter. Honey water on cotton wool was also provided in the cage. Female moths, newly emerged and mated, were removed from the cage each morning and placed in plastic pottles, along with a honey water soaked dental wick and a tissue strip, for egg laying, as described above.

Pheromone gland extracts

The pheromone glands of 24 – 48 hr old female *P. suavis* were excised and placed into 20 – 30 µl of n-hexane, contained within a liquid nitrogen cooled 0.5-ml V-vial (Wheaton, Millville, New Jersey, USA). Gland extracts were taken from females 2 – 3 hr into the scotophase, when female moths were observed to be actively calling. After all glands had been removed, the vial and its contents were brought to room temperature (ca. 5 min) and the liquid phase transferred to a 1.1 ml tapered-bottom clear glass vial (Alltech, Illinois, USA). The volume of extract was then reduced to ca. 10 µl using a stream of argon before being stored in a freezer at -18°C.

Gas chromatography and electroantennographic detection (GC-EAD)

Pheromone gland extracts of *P. suavis* females were analyzed by GC-EAD using a Varian 3800 gas chromatograph equipped with both polar and non-polar columns, coupled to an EAD Recording Unit (Syntech Research and Equipment, Hilversum, Netherlands). Extracts were first run on a DB-5 (Agilent Technologies, California, USA) capillary column (30 m x 0.25 mm ID x 0.5 µm film thickness) and then a polar DB-Wax capillary column (Agilent Technologies, California, USA) (30 m x 0.25 mm ID x 0.5 µm film thickness). Both capillary columns had 1:1 split outlets. Helium was used as the carrier gas at a flow rate of 1 ml/min and injections were in splitless mode. The injector temperature was set at 220°C and the GC oven temperature programmed from 80°C (1 min hold) to 240°C at 10°C/min and held for 30 min. An excised male *P. suavis* antenna was positioned between two glass electrodes, containing BE Ringer's solution with 10% polyvinylpyrrolidone (Molecular Weight 360,000) (Sigma Chemical Co., N.S.W., Australia). Each glass electrode held a length of 1 mm silver wire that electrically connected the preparation to the recording unit's preamp. The EAD exit port temperature was maintained at 200°C and the antennal preparation placed in a charcoal filtered and humidified 400 ml/min airstream. Kováts indexes (Kováts, 1965; Marques et al., 2000) were calculated for the antennally active compounds

Gas chromatography – mass spectrometry (GC-MS)

In 2002 initial GC-MS analysis conducted at Forest Research (Rotorua) of *P. suavis* pheromone gland extracts used a Fisons 8060 gas chromatograph coupled to a Fisons Platform-II mass spectrometer (VG-MicroMass, Manchester, UK) with an ionization voltage of 70 eV and a mass range of 41 *m/z* to 400 *m/z*. Separations were on a 30 m x 0.25 mm i.d. x 0.25 µm phase thickness, Supelcowax™-10 capillary column (Supelco, USA). The temperature of the MS ion source was 280°C, the transfer line from the GC to the ion source

was 250°C., and the GC injection port was at 1μl samples were injected in splitless mode into an injection port maintained at 180°C. The GC oven temperature was held at 80°C for 1 min, raised to 230°C at a rate of 20°C/min, and held for 20 min. The head pressure (helium) was held at 14 psi.

In August of 2003 a GC-MS analysis of a 25 FE (female equivalent) gland extract of *P. suavis* was conducted at HortResearch (Palmerton North) on a VG-70S Magnetic Sector Mass Spectrometer (VG-MicroMass Manchester, UK), using an ionization voltage of 70 eV and a mass range of 30 *m/z* to 500 *m/z*. Separations were on a 30 m x 0.25 mm i.d. x 0.25 μm phase thickness, ZB5 capillary column (Phenomenex, California, USA). The temperature of the MS ion source and the transfer line from the GC to the ion source was set at 240°C. A 3 μl aliquot was injected in splitless mode into the injection port maintained at 220°C. The oven temperature was kept at 80°C for 1 min, raised to 240°C at a rate of 10°C/min, and held for 30 min. The head pressure (helium) was held at 10 psi.

In December of 2003, a 58 FE gland extract of *P. suavis* was analysed at HortResearch, Lincoln by GC-MS on a VF-5ms capillary column (30 m x 0.25 mm ID x 0.25 *μ*m film thickness) (Varian Inc., California, USA), using a Varian 3800 gas chromatograph in splitless injection mode coupled to a Varian 2200 MS. Helium was used as the carrier gas at a flow rate of 1 ml/min and the trap temperature maintained at 200°C. The injector temperature was set at 220°C and the GC oven temperature programmed from 80°C (1 min hold) to 240°C at 10°C/min and held for 30 min. Prior to analysis of a 1 μl aliquot, the gland extract had been reduced to a volume of ca. 10 μl under a stream of argon.

Synthesis of (*Z*)-6-9,10-epoxynonadecene
(*Z*)-6-9,10-epoxynonadecene (7) was synthesized in 8 steps (Zhang et al., 1999) from commercially available THP protected propargyl alcohol (1) (Figure 1). The first step involved alkylation of the protected alcohol by treatment with *n*-butyl lithium followed by the addition of bromononane and tetrabutyl ammonium iodide. Overnight reaction of the crude product with *p*-toluene sulphonic acid in methanol gave the desired deprotected hydroxy alkyne (2). The hydroxy alkyne (2) was readily reduced in refluxing THF by lithium aluminium hydride to give hydroxy alkene (3), which in turn was converted to triol (4) using a Sharpless dihydroxylation (Sharpless et al., 1992). Triol (4) was converted to epoxy tosylate (5) by treatment with sodium hydride and subsequent reaction with tosyl imidazole. The 1,2-epoxide was opened by addition to heptyne treated with *n*-butyl lithium and boron trifluoride THF complex to give a hydroxy tosylate, which was readily converted to 9,10-epoxynonadec-6-yne (6) by potassium carbonate in methanol at room temperature. Epoxynonadec-6-yne (6) was then reduced using Lindlar's catalyst under an atmosphere of hydrogen at 0 ºC. Chromatography gave (*Z*)-6-9,10-epoxynonadecene (7) as a colourless oil.
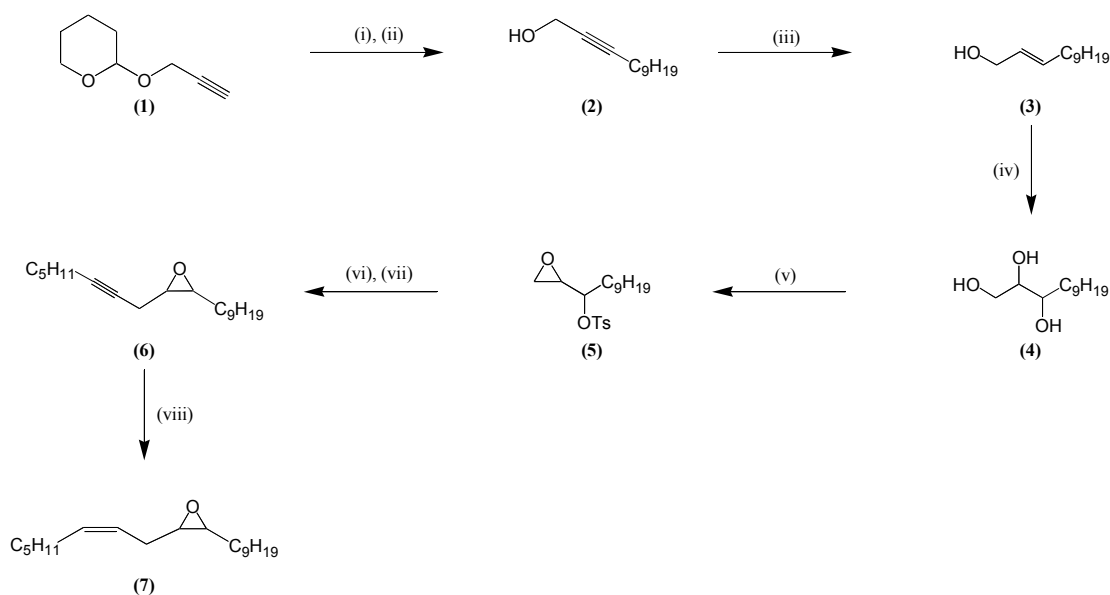
Figure 1: Synthesis of (*Z*)-6-9,10-epoxynonadecene. Reagents and conditions: (i) *n*-BuLi, bromononane, TBAI, THF, -78 °C→r.t, 26 hrs. (ii) *p*-TsOH, MeOH, r.t, 18 hrs, 34% 2 steps. (iii) LiAlH$_4$, THF, reflux, 1.5 hrs, 74%. (iv) AD mix, MeSO$_2$NH$_2$, *t*-BuOH, H$_2$O, 5 °C, 4 hrs, 70%. (v) NaH, *p*-TsIm, THF, 0 °C→r.t, 18 hrs, 24%. (vi) *n*-BuLi, heptyne, BF$_3$.THF complex, THF, -78 °C→r.t, 3 hrs. (vii) K$_2$CO$_3$, MeOH, r.t, 1 hr, 59% 2 steps. (viii) Lindlar's catalyst, MeOH, 0 °C, 79%.

Experimental

2-Dodecyn-1-ol (2).

A solution of tetrahydro-2- (2-propynloxy)-2H-pyran (**1**) (10 mL, 0.071 mol) in tetrahydrofuran (160 mL) was cooled to −78 °C, *n*-butyl lithium (1.6 M, 45 mL, 0.072 mol) was added slowly and the reaction mixture was allowed to warm to room temperature. After 30 minutes bromononane (12.5 ml, 0.065 mol) was added followed by tetrabutylammonium iodide (2.4 g, 0.0065 mol), the reaction mixture was then heated to reflux for 26 hours. After cooling to room temperature ammonium chloride (100 mL, sat. aq.) was added. The resulting layers were separated and the aqueous layer extracted twice with diethyl ether (75 mL), the combined organic fractions were dried over magnesium sulphate, filtered and concentrated *in vacuo*. The resulting brown oil was then taken up in methanol (100 mL) and *p*-toluene sulphonic acid (900 mg) was added in one portion. The reaction mixture was allowed to stir for 18 hours before the removal of methanol *in vacuo*. The crude product was purified by column chromatography on silica, eluting with 10% ethyl acetate/90% petroleum spirit to give the title compound as pale yellow crystals (4 g, 0.022 mol, 34% 2 steps).

2E-Dodecenol (3).

Alcohol (**2**) (4 g, 0.022 mol) was taken up in tetrahydrofuran (75 mL) and lithium aluminium hydride (950 mg, 0.025 mol) was added in one portion. The reaction mixture was stirred at reflux for 1.5 hours before the addition of ammonium chloride (50 mL, sat. aq.), the resulting layers were separated and the aqueous layer extracted twice with diethyl ether (50 mL), the combined organic fractions were dried over magnesium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography on silica, eluting with 10% ethyl acetate/90% petroleum spirit to give the title compound as colourless oil (3.04 g, 0.016 mol, 74%).

1,2,3-Dodecan-triol (4).

A solution of alcohol (**3**) (3.03 g, 0.016 mol) in *t*-butanol (60 mL) and water (60 mL) was cooled to 5 ºC before the addition of AD mix α (12 g), AD mix β (12 g) and methanesulfonamide (1.5 g, 0.015 mol). After stirring for four hours sodium sulfite (20 g) was added and the reaction mixture was stirred for a further hour before the addition of ethyl acetate (50 mL). The layers were separated and the aqueous phase extracted twice with ethyl acetate (50 mL), the combined organic fractions were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Recrystallization from ethyl acetate/petroleum spirit gave the title compound as a white powder (2.5 g, 0.012 mol, 70%).

3-Tosyl 1,2-epoxydodecane (5).

Triol (**4**) (2.5 g, 11.5 mol) in tetrahydrofuran (75 mL) was added to a stirred suspension of sodium hydride (3 equiv. 758 mg, 34.5 mmol) in tetrahydrofuran (75 mL) at 0 ºC. The reaction mixture was allowed to warm to room temperature and after 30 minutes *p*-tosyl imidazole (2 equiv. 5.2 g, 23.0 mmol) was added. After 18 hours ammonium chloride (100 mL, sat. aq.) was added the layers were separated and the aqueous phase extracted twice with diethyl ether (50 mL), the combined organic fractions were washed with water (50 mL), dried over magnesium sulphate, filtered and the solvent removed *in vacuo*. Column chromatography on silica eluting with 15% diethyl ether/85% petroleum spirit followed by recrystallization from petroleum spirit gave the title compound as a white solid (0.95 g, 2.8 mmol, 24%).

9,10-epoxynonadec-6-yne (6).

n-Butyl lithium (3.5 equiv. 1.6 M, 6.5 mL, 10.33 mmol) was slowly added to a solution of heptyne (4 equiv. 1.13 g, 11.8 mmol) in tetrahydrofuran (30 mL) at −78 ºC. The reaction mixture was stirred for 20 minutes before the addition of boron trifluoride tetrahydrofuran complex, after a further 20 minutes of stirring epoxide (**5**) was added in tetrahydrofuran (50 mL). The reaction mixture was allowed to warm to room temperature and after 3 hours ammonium chloride (50 mL) was added, the layers were separated and the aqueous phase extracted twice with diethyl ether (50 mL), the combined organic fractions were dried over magnesium sulphate, filtered and concentrated *in vacuo*. The crude material was taken up in diethyl ether and filtered through celite the solvent was removed *in vacuo*. The resulting yellow oil was taken up in methanol (35 mL), to this solution was added potassium carbonate (1.9 g, 13.7 mmol). After stirring for one hour the reaction mixture was diluted with diethyl ether (50 mL), filtered through celite and concentrated *in vacuo*. Purification by column chromatography on silica eluting with 6% diethyl ether/94% petroleum spirit gave the title compound as pale yellow oil (460 mg, 1.65 mmol, 59% 2 steps).

(Z)-6-9,10-epoxynonadecene (7).

Lindlar's catalyst (Pd/C poisoned with Pb, 9% w/w, 14 mg) was added to a solution of epoxide (**6**) (150 mg, 0.54 mmol) in methanol (7 mL), the reaction vessel was then flushed with hydrogen and stirred under hydrogen for three minutes before flushing with nitrogen. A small aliquot was taken for analysis by GC-MS, this process was repeated until GC-MS revealed other side products starting to form at which point the reaction mixture was filtered through celite with diethyl ether washings (50 mL) and concentrated *in vacuo*. Purification by column chromatography on silica eluting with 5% diethyl ether/95% petroleum spirit gave the title compound as colourless oil (120 mg, 0.54 mmol, 79%).

RESULTS

Progress in 2001/02

Preliminary GC-EAD and GC-MS analysis

Preliminary analysis of the pheromone glands extracts of *P. suavis* by GC-EAD during 2001/2002 revealed two major antennally active compounds present in very small amounts. GC-EAD's also suggested that additional active compounds were present in the female gland extracts. However, analysis of gland extracts at Forest Research in Rotorua using GC-MS to identify these two major active compounds, was unsuccessful in obtaining useful spectra. This was due to the very small amounts of material produced by females and the small amount of material available to work with.

Progress in 2002/03

GC-EAD

When sufficient moths became available in January of 2003, GC-EAD's were again repeated (Figure 1) using both polar (DB-Wax) and non-polar (DB-5) columns. This work confirmed the presence of the two major antennally active compounds (actives 1 and 2) and in addition the presence of two minor antennally active compounds (actives 3 and 4).

While actives 1 and 2 were visible in the FID trace, both actives 3 and 4 were not visible, even though male antennae consistently responded at the appropriate retention times. Table 1 below summarises the relative retention times (Kovat's indexes) of the two major antennally active compounds for both polar and non-polar columns.

Table 1:   Relative retention times (Kovat's indexes) of the two major antennally active compounds present in female pheromone gland extracts of *P. suavis*, eluting from non polar and polar gas chromatograph capillary columns

Compound	Kovat's Indexes[a]	
	ZB-5 column	**ZB-Wax column**
Active 1	2052	2341
Active 2	2058	2405

[a] GC conditions: Varian 3800 gas chromatograph; splitless injections of a 4 μl aliquot of gland extract in hexane; 80°C (1min) to 240°C at a rate of 10°C/min and held for 30 min; Helium as carrier gas at a rate of 1ml/min. Injector 220°C and Detector 300°C.

Male antennae were also tested to a range of synthetic standards of unsaturated hydrocarbons, epoxides and ketones and responded well to these types of compounds. Of the synthetic standards tested with male *P. suavis* antennae, only one compound ((*Z,Z*)-3,6-9,10-epoxyheneicosadiene) had the same retention time as that of active 4 on both polar and non-polar columns.
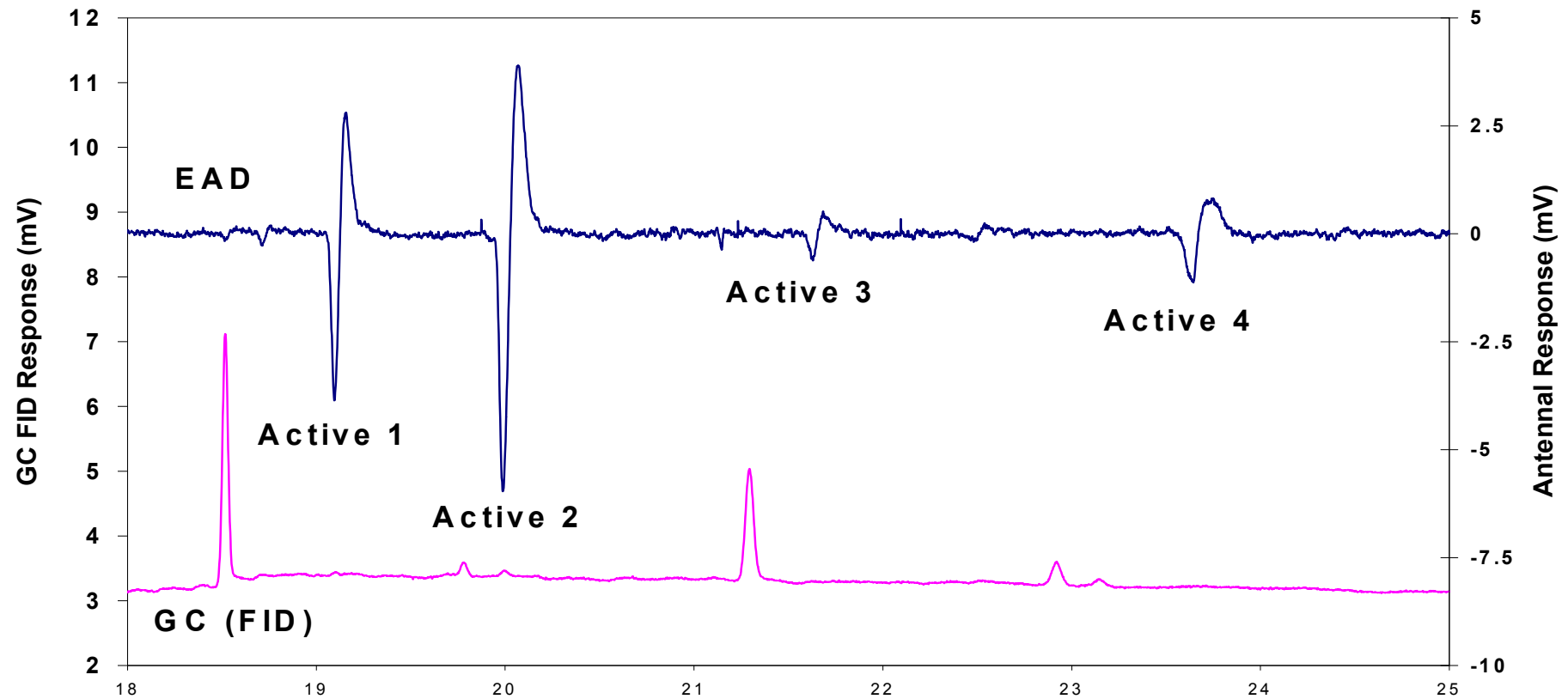
Figure 1: Coupled gas chromatograph-electroantennogram (GC-EAD) of a *P. suavis* male antenna responding to the active compounds in pheromone gland extracts, eluting from a polar (DB-Wax) gas chromatograph column (FID=Flame ionisation detector of gas chromatograph).

Progress in 2003/04

GC-MS – Active compound identifications

When larger numbers of female *P. suavis* became available in December 2003 GC-MS analysis at HortResearch, Lincoln, produced good spectra for actives 1, 2 and 4. Their retention times on the GC-MS column were compared with the retention times of male antennae responding to actives in the same extract using GC-EAD analysis. It was then possible to tentatively identify actives 1 and 4 and confirm the tentative identification of active 2 (achieved earlier by GC-MS work completed at HortResearch in Palmerston North in August 2003) by obtaining good diagnostic spectra on our mass spectrometer at the HortResearch, Lincoln site. As the compounds tentatively identified are not easily synthesised we were limited as to where and how we could obtain standards, in order to confirm the pheromone components of *P. suavis*. Expertise in synthesis of these specific compounds lies with only a few chemical ecology teams, most notably in Europe and Japan.

Just prior to Xmas 2003-04, a number of extracts were sent by courier to the laboratory of Professor Wittko Francke, of the Institute of Organic Chemistry at Hamburg University in Germany, for further analysis and to obtain standards of the actives identified. Unfortunately, due to the ill health of Professor Wittko Francke the extracts have not been analysed. We have also contacted the laboratory of Professor Tetsu Ando from Tokyo University of Agriculture and Technology, Japan, from whom we had previously obtained standards of (*Z*,*Z*)-3,6-9,10-epoxyheneicosadiene. However, to date we have not been able to obtain standards from him for pheromone component confirmation.

Fortuitously, our chemists at HortResearch, Palmerston North have had considerable experience with synthesis of mono-epoxides for the Ministry of Agriculture and Forestry, as part of the pheromone identification of the Painted Apple Moth, *Teia anartoides*. When it was clear that our attempts to source standards from other laboratories was likely to be unsuccessful, we asked them to synthesise the racemic forms of the two major active components of the sex pheromone of *P. suavis*. To date they have synthesised active 1 and currently are working on the synthesis of active 2.

Identification of active 1 as (*Z*)-6-9,10-epoxynonadecene

Synthesis of (*Z*)-6-9,10-epoxynonadecene by HortResearch chemists at Palmerston North in April-May of 2004 has made it possible to compare the spectra of active 1 with the spectra of a known synthetic standard, using the same mass spectrometer and identical analytical conditions. While the intensity of the spectral data differed slightly from that of published data (Ando et al., 1995), the spectra and retention time of active 1 was identical to that of the synthetic standard (*Z*)-6-9,10-epoxynonadecene using our Varian 2200 mass spectrometer. Because the synthetic (*Z*)-6-9,10-epoxynonadecene was only ca. 90% isomerically pure (i.e. it contained ca. 10% of (*E*)-6-9,10-epoxynonadecene) it was also possible to confirm the configuration of the double bond at carbon 6 as taking the *Z* form rather than the *E* form, because the two forms were easily separated on the VF-5ms capillary column (*Z* form elutes before the *E* form).

Both the synthetic standard and active 1 from the gland extract had a base peak of *m/z* 67, a molecular ion (M^+) of *m/z* 280 and diagnostic ions (relative intensities) at *m/z* 79 (35) and M-125 (9). Any differences in intensity of other diagnostic ions was minimal and could be attributed to the difference in amount of material available for analysis in the gland extract, when compared to that of the standard (Table 3). Spectral comparisons of C20 and C21 mono-epoxide standards to published data are also shown in Table 3 and serve to illustrate

and reinforce that differences in mass spectra occur for the same compound when using different machines.

Identification of active 4 as (*Z,Z*)-3,6-9,10-epoxyheneicosadiene

Comparison of the mass spectra of active 4 with that of a synthetic racemic standard on the Varian 2200 mass spectrometer has confirmed the identification of this minor component as (*Z,Z*)-3,6-9,10-epoxyheneicosadiene. While the intensity of the spectral data differed slightly from that of published data (Ando et al., 1993), the spectra and retention time of active 4 was identical to that of the synthetic standard (*Z,Z*)-3,6-9,10-epoxyheneicosadiene using our Varian 2200 mass spectrometer. Both synthetic standard and active 4 from the gland extract had a molecular ion (M^+) of m/z 306 and diagnostic ion (relative intensities) at m/z 108 (26 and 25 respectively). Published data and the spectra of the synthetic standard and active 4 both had a base peak of m/z 79. Any differences in intensity of other diagnostic ions was minimal and could be attributed to the very small amount of material available for analysis in gland extract, when compared to that of the standard (Table 2).

Putative identification of active 2 as (*Z,Z*)-3,6-9,10-epoxynonadecadiene

Analysis of female *P. suavis* pheromone gland extract (fresh 25 FE) by GC-MS at HortResearch in Palmerston North in August of 2003 tentatively identified active 2. The spectra obtained had a molecular ion with an m/z value of 278, suggesting a 19 carbon epoxydiene. By comparison of mass spectral library data (NIST and Wiley mass spectrometer libraries) it was determined that active 2 was most likely to be (*Z,Z*)-3,6-9,10-epoxynonadecadiene. When larger numbers of *P. suavis* females became available in December 2003, fresh extract was analysed by GC-MS at HortResearch Lincoln. Comparison of the mass spectra of active 2, with published relative intensities of the molecular and fragmentation ions of epoxydienes (Ando et al., 1993), provided corroboration that this major component of the sex pheromone of *P. suavis* was indeed the epoxydiene (*Z,Z*)-3,6-9,10-epoxynonadecadiene.

Both the published data and active 2 have a base peak of m/z 79 and a molecular ion (M^+) of m/z 278 (see Table 2). The difference in intensity of the m/z 108 ion between the published data and data from the Varian 2200 mass spectrometer can be explained by differences in spectra produced on different machines. An intensity of 27% for the m/z 108 ion for active 2 is in line with values obtained on our machine for the epoxydiene homologue (*Z,Z*)-3,6-9,10-epoxyheneicosadiene. The epoxydiene (*Z,Z*)-3,6-9,10-epoxynonadecadiene is a known pheromone in the geometrid family (El-Sayed, 2004) and within the subfamily Ennominae, to which *P. suavis* belongs, nineteen carbon dienic compounds are the most frequently occurring.

Table 2: Comparison of relative intensities (%) of the molecular ion (M[+]) and some diagnostic fragment ions in the mass spectra of actives 2 and 4, from the gland extract of *Pseudocoremia suavis*, with published epoxydiene data (actives 2 and 4) and standard (active 4). (After Ando et al., 1993)

	M[+] (*m/z*)	79[c]	108[c]	111	M-109[c]	M-72	M-69[c]	M-29[c]	M-18	M[+]
					Relative intensity of ions, *m/z* (%)					
(Z,Z)-3,6-9,10-epoxynonadecadiene[a]	278	100	66	3	5	2	2	2	9	4
Active 2[b] from pheromone gland extract	278	100	27	5	0.48	0.88	2.72	10	3	8
Z3,Z6-9,10-epoxyheneicosadiene[a]	306	100	81	5	4	1	2	2	8	3
Z3,Z6-9,10-epoxyheneicosadiene[b] (standard)	306	100	26	7	0.06	1	2	9	5	8
Active 4[b] from pheromone gland extract	306	100	25	7	1.56	0	0.78	3	0.78	0.78

[a]Ando et al., 1993
[b]Spectra measured with a Varian 2200 mass spectrometer.
[c] Characteristic ions assumed to be produced by fragmentation of 9,10-epoxydienes.

Table 3: Comparison of relative intensities (%) of some diagnostic fragment ions in the mass spectra of active 1 from the gland extract of *Pseudocoremia suavis*, with published data for mono-epoxides of 6,9-dienes and standards. (After Ando et al., 1995)

	Base ion (*m/z*)	M[+] (*m/z*)	71	79[c]	99[c]	110	113	124[c]	127	153	M-153	M-127	M-125[c]	M-114[c]	M-111[c]	M-100[c]	M-97	M-71
								Relative intensity of fragment ions, *m/z* (%)										
(Z)-6-9,10-epoxynonadecene[a]	81	280	38	49	9	67	24	26	3	18	3	18	38	3	29	2	6	12
(Z)-6-9,10-epoxynonadecene[b] (standard)	67	280	21	35	5	20	9	10	1	9	1	9	9	1	3	1	2	6
Active 1[b] from pheromone gland extract	67	280	17	35	3	17	4	9	1	7	1	7	9	1	2	1	1	4
(Z)-6-9,10-epoxyeicosene[a]	81	294	28	43	11	44	19	21	2	13	1	4	16	2	16	1	2	10
(Z)-6-9,10-epoxyeicosene[b] (standard)	95	294	31	32	8	22	6	6	2	12	1	4	20	1	3	0	4	12
(Z)-6-9,10-epoxyheneicosene[a]	81	308	33	50	17	71	32	31	3	20	2	8	24	5	27	2	5	13
(Z)-6-9,10-epoxyheneicosene[b] (standard)	67	308	30	29	5	24	8	9	1	11	1	10	11	1	2	1	3	12

[a]Ando et al., 1995
[b]Spectra measured with a Varian 2200 mass spectrometer.
[c] Characteristic ions assumed to be produced by fragmentation of 9,10-epoxides.

DISCUSSION

The identification of the sex pheromones of Lepidoptera can sometimes be relatively straightforward and quick. However, it is not unusual for the identification of a moth's sex pheromone to be particularly challenging and take a considerable period of time to complete. While some Lepidoptera require only a single compound for complete behavioural activity (i.e. comprising initial male activation, followed by flight and eventual arrival at a pheromone source), many moths' sex pheromones are complex multi-component blends. According to the GC-EAD data obtained in this study, it is likely that the sex pheromone of *P. suavis* is a multi-component blend rather than a simple single component blend.

We have identified four active components in gland extracts that consistently elicit a response in male moth antennae using GC-EAD analysis. We have also been able to confirm the chemical structure of two of these actives as (*Z*)-6-9,10-epoxynonadecene (active 1) and (*Z,Z*)-3,6-9,10-epoxyheneicosadiene (active 4) and have putatively identified active 2 as the epoxydiene (*Z,Z*)-3,6-9,10-epoxynonadecadiene. To date our chemists at HortResearch, Palmerston North, have successfully synthesised a racemic mixture of active 1 and currently are synthesising a racemic mixture of the second major active, (*Z,Z*)-3,6-9,10-epoxynonadecadiene. We have also attempted to source standards of all actives from other chemical ecology workers both in Europe and Japan who also have considerable expertise with epoxide pheromones.

While it has not been possible to obtain enough material to produce a chromatograph for active 3, due to the small amount of this compound present in gland extracts, it is likely that this compound is either an epoxyeicosene or epoxyeicosadiene. It is also likely but not guaranteed, that a synthetic blend of the two major active compounds 1 and 2, will be enough for attraction of *P. suavis* males in the field. Addition of the minor actives to the blend may or may not significantly enhance trap catch of males. In some lepidopteran the addition of minor components to a lure significantly enhances trap catch (e.g. *Colotois pennaria*, Szöcs et al., 1993), while for others the addition of a minor component does not enhance trap catch (e.g. *Oraesia* excavata; Ohmasa et al., 1991).

These types of compounds (epoxides) have chiral centres and therefore will have enantiomeric isomers, which may or may not be important to achieve an attractive blend. For example in some moths the use of the opposite enantiomer can inhibit male attraction (Miller et al., 1991), while for others a racemic mix is necessary for attraction (Gries et al., 1999). The ratio of enantiomers in the blend may also be important for attraction of male moths to a synthetic lure placed in a pheromone trap (Gries et al., 1999). Trap catch to a synthetic blend can be compared to trap catch using caged virgin females to determine whether a synthetic blend is as attractive as the natural pheromone source.

Because there are over thirty known species of geometrids in the genus *Pseudocoremia* present in New Zealand, it is likely that an attractive blend will require specific enantiomers and/or ratios of the epoxide actives. This is mainly because of the sexual selection for a narrow communication channel, due to the inefficiency of hybridising with related species. If enantiomeric specificity is the case, then realistically the completion of this project may take another 1-2 years. Our chemists at HortResearch in Palmerston North are capable of synthesising enantiomers of the actives but any synthesis is a complicated and drawn-out procedure. For example, each enantiomer of active 1, the epoxide (*Z*)-6-9,10-epoxynonadecene can take 6-8 weeks to produce.

CONCLUSIONS AND FUTURE WORK

Funding for the work to date has been provided by the New Zealand Forest Health Research Collaborative, but this is only for a two year period and will finish in June 2004. Therefore, any future work to complete this project will be carried out as part of the FRST programme Biosecurity of New Zealand Forests and will focus on the following:

1) The sourcing or synthesis of the epoxydiene (*Z,Z*)-3,6-9,10-epoxynonadecadiene, tentatively identified as active 2 and confirming its identity.

2) When the identity of active 2 is confirmed as the epoxydiene (*Z,Z*)-3,6-9,10-epoxynonadecadiene by comparison with synthetic standard, then trapping trials will be undertaken to evaluate the attractiveness of a lure comprising racemic forms of the two major components, actives 1 and 2.

3) Further refinement of the lure may be required to achieve male moth trap catch. This could include specificity of an enantiomeric blend and comparison with caged virgin females.

ACKNOWLEDGEMENTS

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