Comparative Pheromone Analyses of Three *Bactrocera* Fruit Fly Species of Economic Importance

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Table of Contents

Declaration of Originality

I, Danielle Holgate, declare that this work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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List of Abbreviations

AcOH	Acetic acid
ADI	Acceptable daily intake
AIBN	Azobisisobutyronitrile
APVMA	Australian Pesticide and Veterinary Medicines Authority
ARfD	Acute reference dose
BnBr	Benzyl bromide
Bu_3SnH	Tributyltin hydride
t-BuOH	tert-Butanol
TBDMSCl	tert-Butyldimethylsilyl chloride
n-BuLi	*n*-Butyllithium
m-CPBA	meta-Chloroperoxybenzoic acid
δ	NMR chemical shifts in parts per million
d	Doublet
dd	Doublet of doublets
DCM	Dichloromethane
DMA	Dimethylacetamide
DMF	Dimethylforamide
EAG	Electroantennography
EI	Electron ionisation
equiv.	Equivalent
Et	Ethyl
EtOAc	Ethyl acetate

EtOH	Ethanol
GC	Gas chromatography
GC-IR	Gas chromatography-infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
Hz	Hertz
*i*Pr	Isopropyl
J	Coupling constant
LDA	Lithium diisopropylamide
m	Multiplet
m/z	Mass to charge
MAT	Male annihilation technique
MD	Mating disruption
MeCN	Acetonitrile
MeOH	Methanol
MHz	Megahertz
min	Minutes
MS	Mass spectrometry
MT	Mass trapping
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
ppm	Parts per million
Pr	Propyl
q	Quartet

rt	Room temperature
s	Singlet
SIT	Sterile insect technique
Et_3N	Triethylamine
t	Triplet
TBAI	Tetrabutylammonium iodide
TBAF	Tetrabutylammonium fluoride
THF	Tetrahydrofuran
TIC	Total ion count
TLC	Thin layer chromatography
TsOH	*p*-Toluenesulfonic acid

Abstract

Fruit flies are significant horticultural pests in many countries, including Australia. Organophosphate insecticides have been typically used to control fruit fly infestation, however these compounds have been banned in recent years, hence the development of alternative control methods is of paramount importance. In this study, the chemical profiles for males and females of three pest species in Australia, *Bactrocera frauenfeldi*, *B. kraussi* and *B. musae*, were investigated. Compounds were identified from rectal gland extracts and headspace collections, and confirmed by comparison of GC retention times and MS fragmentation patterns to authentic samples. In total, 27 compounds were preliminarily identified in the chemical profiles, and 17 were confirmed by comparison to authentic samples. Across all species, and both sexes, three main chemical classes were present: esters, spiroacetals, and acetamides. Also, a larger number of compounds were identified in rectal gland extracts than headspace collections. Females typically produced a wider range of compounds, including acetamides, spiroacetals and esters, with the latter being the major components. Males typically produced one major component, usually a spiroacetal, and had additional minor components. The only exception was males of *B. musae,* who were found to produce a range of compounds in their chemical profile, predominantly esters.

Chapter 1: Introduction

1.1 Preamble

Fruit flies are significant horticultural pests, with an estimated 46 endemic and exotic species considered economic threats to Australian horticulture.[1] Between 2006 and 2009, the average annual value of fruit fly susceptible produce in Australia was an estimated 5.3 billion AUD.[2] The total value of Australian horticulture production was valued at over 9 billion AUD in 2012-13.[3] In Australia, the most destructive fruit fly pest species include the Queensland fruit fly, *Bactrocera tryoni* (Froggatt), and the Lesser Queensland fruit fly, *B. neohumeralis* (Hardy), which are known to infest approximately 90 commercial and wild hosts. Moderate pest species include the Mango fruit fly, *B. frauenfeldi* (Schiner), the Banana fruit fly, *B. musae* (Tryon) and *B. kraussi* (Hardy). These species are well established in the Torres Strait Islands and Northern Queensland, and are found as far south as Townsville. Each of these species infests a range of both wild and commercial hosts, with eight major commercial hosts between them.[4,5]

Fruit flies have been controlled in Australia by synthetic insecticides including dimethoate and fenthion. These insecticides have been recently restricted[6,7] and there is an urgent need for alternative methods for the control of fruit fly populations. Current alternative control methods include the use of a male-specific chemical lure and/or food baits (*e.g.* hydrolysed protein) to attract fruit flies to traps or killing devices. Combining the lure with an insecticide serves to kill the trapped flies and protect the commercial crop.[8,9]

Pheromones are compounds produced by animals, including fruit flies, to affect the behaviour of other members of the same species,[10] and as such may be employable in fruit fly population monitoring and control. It is therefore of interest to investigate the chemical profiles for economically important *Bactrocera* pest species in order to identify chemicals to serve as lead compounds in lure development. The aim of this project was to qualitatively analyse rectal gland extracts and headspace collections from *B. frauenfeldi*, *B. kraussi* and *B. musae* to determine chemical profiles and confirm compound identifications with samples obtained commercially or *via* synthesis.

1.2 The Fruit Fly Life Cycle and Economic Costs

Fruit flies cause extensive damage to fruit and vegetable crops as part of their life cycle. The fruit fly life cycle begins when gravid females oviposit eggs into the host fruit or vegetable. The eggs hatch into larvae that feed on the internal structure of the host, damaging the fruit or vegetable and inducing decay and premature fruit drop.[1,8,11-13] The site of oviposition can also become infected and blemish the exterior of the produce.[1,4] An example of the type of damage caused by fruit fly larvae is shown

in Figure 1.1. After several days, when the larvae have grown into maturity, they pupate, either directly in the fruit or vegetable, or in the ground, from which adult flies emerge to complete the life cycle.[8,11,14] This can be completed in 3-4 weeks under favourable conditions,[1,14] and this short life cycle makes it possible for an uncontrolled population to increase rapidly.[11]

Figure 1.1. Left: Female *B. frauenfeldi.*[15] Right: Fruit fly larvae feedings tracks in ripe Cavendish banana.[16]

Fruit flies are major economic pests for a variety of reasons, including their short life cycle, capacity to infest a large number of hosts, and their ability to disperse.[1,17] The cost of fruit fly management and research projects in Australia (2003-2008) has been estimated as 128 million AUD.[1,8] In Victoria, approximately 3.2 million AUD was spent on management programs in 2007-08, which included operational costs, research projects and community awareness.[18] Over 75% of Australia's fruit and vegetable exports, valued at approximately 640 million AUD in 2012-13, are susceptible to fruit fly damage.[3] Fruit fly infestation in production areas can impede domestic and international trade, as trading partners consider fruit flies as major quarantine pests.[3] Quarantine procedures designed to avoid cross-border incursions can seriously impede domestic and international trade, limiting market access and thus reducing profitability.[10] For example, susceptible produce transported into South Australia and Tasmania, and exported from Australia to countries such as the United States and Japan, are subject to strict phytosanitary requirements.[18]

In Australia, the most destructive fruit fly species' are *B. tryoni* and *B. neohumeralis.*[8,18] In 2000, the estimated cost of *B. tryoni* induced damage was 28.5 million AUD.[19] Whilst these species pose a significant threat, an estimated 46 endemic and exotic species are considered to be economic threats to Australian horticulture.[1] These endemic species are mostly concentrated in the Queensland region, and include the three species that are the subject of the study reported in this thesis: *B. frauenfeldi, B. musae* and *B. kraussi*. *Bactrocera frauenfeldi* is an invasive polyphagous pest species, with 22 commercial and wild hosts in Northern Queensland.[20] The species is established in the Torres Strait Islands and Northern Queensland, and is also widely distributed in Papua New Guinea.[4,5] *Bactrocera*

musae is a minor polyphagous pest species, and predominantly infests banana crops by laying eggs in immature green bananas.[4,21,22] It is distributed in the Torres Strait Islands and north-east Queensland, where bananas are an important commercial crop.[4,5,22] *Bactrocera kraussi* is a moderate polyphagous pest species, and is distributed in the Torres Strait Islands and north-east Queensland. These species are considered economically important pest species, and infest six commercial hosts in Australia, including grapefruit, mandarin, orange, mango, banana and guava.[4,5]

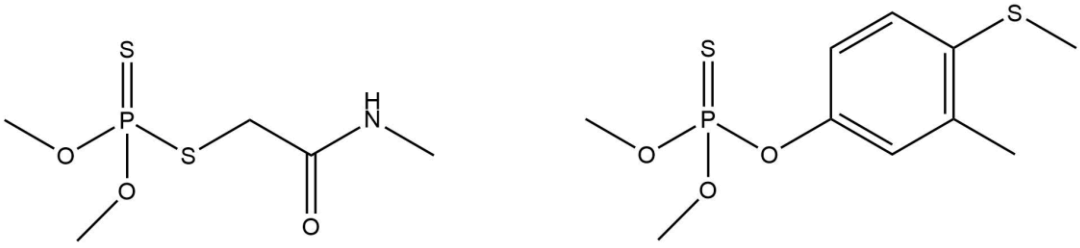
1.3 Fruit Fly Control Methods

1.3.1 Organophosphate Insecticides

Insecticides have been the most successful and widely used fruit fly control method. These are typically contact insecticides, which require only contact rather than ingestion to be effective. These insecticides can be applied by cover spraying, where the fruit/vegetable or entire tree is covered, or through under-tree spraying, where the insecticide is only applied to the ground. With cover spraying, the insecticide kills adult fruit flies prior to oviposition. With under-tree spraying, the insecticide kills larvae after leaving the host, or fruit flies after pupating.[23]

Contact insecticides are typically organophosphates, including dimethoate and fenthion (Figure 1.2). These chemicals act as acetyl cholinesterase inhibitors, thereby affecting the function of the insect's central nervous system.[23] These insecticides have been used extensively to control destructive fruit fly species in Australia, including *B. tryoni* and the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann).[23,24] Organophosphates are also human acetyl cholinesterase inhibitors and are therefore hazardous to human health.[8,23] Exposure to excessive amounts of organophosphate insecticides can have adverse health effects, including severe hypotension,[25] severe neurotoxic effects,[26] and death.[26] The levels of organophosphates to which consumers are exposed to are typically much lower than those that cause these adverse health effects; however, those working in the horticulture industry may be at an increased risk. Additionally, organophosphate insecticides are not environmentally benign and are particularly hazardous to birds and fish.[27,28] Due to these concerns, the Australia Pesticide and Veterinary Medicines Authority (APVMA) conducted reviews into dimethoate and fenthion, including examination of both dietary intake and toxicology. The review into dimethoate resulted in a lowering of the acceptable daily intake (ADI) of dimethoate to 0.001 mg kg^{-1} day^{-1}, with an associated acute reference dose (ARfD) of 0.02 mg kg^{-1} body weight.[6] Similarly, the review into fenthion resulted in a lowering of the ADI to 0.002 mg kg^{-1} day^{-1}, with an associated ARfD of 0.007 mg kg^{-1} body weight.[7] Given these new values, the APVMA determined that many common use patterns of dimethoate and fenthion would exceed the ADI and/or ARfD. As a result, the use of dimethoate has been suspended on many crops in Australia, whilst fenthion use

has ceased.[6,7] With the phasing out of organophosphate insecticide use in Australia, the development of alternative fruit fly control methods is of paramount importance.



Dimethoate
O,O-Dimethyl-*S*-[2-(methylamino)-2-oxoethyl] dithiophosphate

Fenthion
O,O-Dimethyl-*O*-[3-methyl-4-(methylsulfanyl)phenyl] phosphorothioate

Figure 1.2. Structures of dimethoate and fenthion.

1.3.2 Mass Trapping and Male Annihilation Technique

Two well established alternative fruit fly control methods include mass trapping (MT) and male annihilation technique (MAT). These methods use a chemical lure to attract adult fruit flies to a trap or killing device, thus allowing for population monitoring, as well as removing fruit flies from the population and decreasing population growth.[29] The lures used are typically food baits (*e.g.* hydrolysed protein), or male-specific chemical lures. The traps/killing devices also typically contain an insecticide, allowing the crops to be protected without applying the insecticide to the crop itself.

In MT, a variety of lures are used to attract and trap fruit flies, although male-specific chemical lures are typically the most potent and most widely used. Current commercial male lures include raspberry ketone and methyl eugenol, which are natural products, and cuelure and melolure, analogues of raspberry ketone found to have higher potency than raspberry ketone[23,30] (Figure 1.3). In contrast, lures for attracting females are commonly food baits, such as orange-ammonia solution. Hydrolysed protein baits can be used to attract both sexes, but will typically capture a higher proportion of females.[9,30,31] MT is a commonly used method for fruit fly control and eradication. One study used a hydrolysed protein bait mixed with orange juice, brown sugar and ammonium acetate to remove approximately 8900 male and female fruit flies from the *B. minax* (Enderlein) population over a 12 week period in the Hubei Province in China.[9]

MAT uses a male-specific lure combined with an insecticide to attract and kill male fruit flies.[8,23] Whilst these lures are only specific for one sex, the advantage of MAT over MT is that capture rates are much higher, and fewer MAT devices are needed to cover an area.[29,30] A MAT trap will typically use an absorbent material to contain both the lure and the insecticide. Absorbent materials that have been used include compressed particle blocks, cotton wicks, bucket traps, fibreboard discs,[32] Min-U-Gel, and moulded paper fibre.[8,12] MAT has proven to be a successful technique, and has been used to

eradicate *B. tryoni* from Easter Island[8] and *B. papayae* (Drew & Hancock) from Northern Queensland.[33] MAT is effective in controlling and decreasing fruit fly populations by removing males from the population, thus decreasing the number of mated females and therefore the number of ovipositions and larvae that cause crop damage.[29]

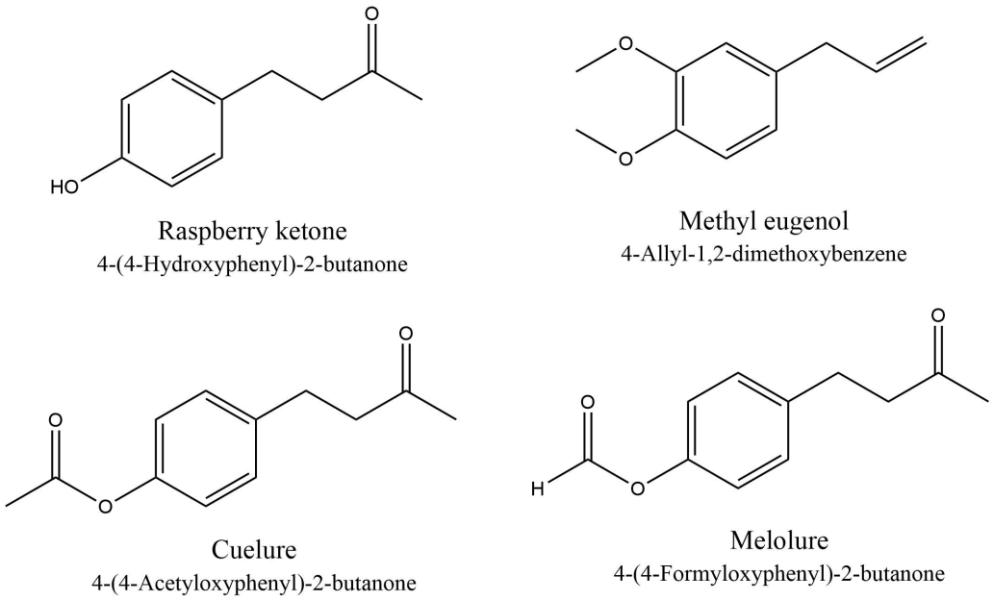


Figure 1.3. Structure of raspberry ketone, methyl eugenol, cuelure and melolure.

Although there are cases where chemical lures have been used effectively to monitor, decrease or eradicate fruit fly populations, 50% of the identified *Bactrocera* species do not respond to any commercial lure or have a limited response.[34] For example *B. cucumis* (French) does not respond to any male lures, although this species can be caught using a 'cucumber volatile blend' or orange-ammonia solution.[35] Additionally, there are no known effective female lures for commercial use. Given the phasing out of organophosphate insecticides, and that females cause direct damage to fruits and vegetables through ovipositions, the development of female targeted lures is of particular interest. Such lures would aid in controlling, monitoring and decreasing fruit fly populations, and therefore more effectively protect horticultural produce.

1.3.3 Sterile Insect Technique

Sterile Insect Technique (SIT) involves rearing large numbers of males of the target pest species, exposing them to irradiation to induce reproductive sterility, and releasing the sterile males into the target pest population.[17] The aim is for the sterile males to compete with the wild males in mating with wild females, thus preventing the females from reproducing. Over time, this reduces the fruit fly population substantially.[36] Another application of SIT involves the use of a chemosterilant, which is a sterilising agent, combined with a lure. This avoids the need to rear the target pest species by inducing reproductive sterility in wild males, which mate with wild females and thus prevent reproduction.[17]

SIT has been used to strongly suppress *B. tryoni* in areas in New South Wales, Victoria and South Australia, and has been used to eradicate the species from Perth, Western Australia.[36] SIT may also be combined with other control methods, such as MT and MAT, to further reduce pest populations. Typical approaches include using MAT before releasing sterile males into the environment. In doing so, this aims to remove as many wild males from the population as possible before introducing sterile males, so that the sterile males have a higher chance of mating with wild females.

1.4 Fruit Fly Pheromones

Pheromones are chemical compounds produced and secreted by an animal that affect the behaviour of another member of the same species. Many types of animals, including fruit flies, are known to produce and respond to pheromones.[10] Possibly the most widely studied, and of most commercial interest, are sex pheromones, which are released by members of one sex to attract the opposite sex.[10] Male fruit flies secrete sex pheromones and store in a reservoir in the rectal glands[11,37,38] (Figure 1.4). During courting and mating, males converge on a site at a specific time of day to perform competitive courting rituals to attract, court and mate with females. This aggregation is known as a lek.[12,39,40] The males initiate sexual behaviour by releasing sex pheromones to attract females.[12,41,42] Attracted females then choose a mate based on the courtship display performed, which includes wing vibration, olfactory and tactile cues.[12,42] Males that join mating aggregations typically have a greater probability of securing a mate; therefore males can also be attracted to the signals of other males.[40,43] It has been classically considered that males are the major sex pheromone producers, but it is now known that females also produce and release sex pheromones. In some species, such as *B. oleae* and *Zeugodacus cucurbitae* (Coquillett), the roles are reversed and females perform competitive courting and release sex pheromones to attract males.[12] As a result, the study of pheromone profiles of both males and females is of interest, particularly for lure development.

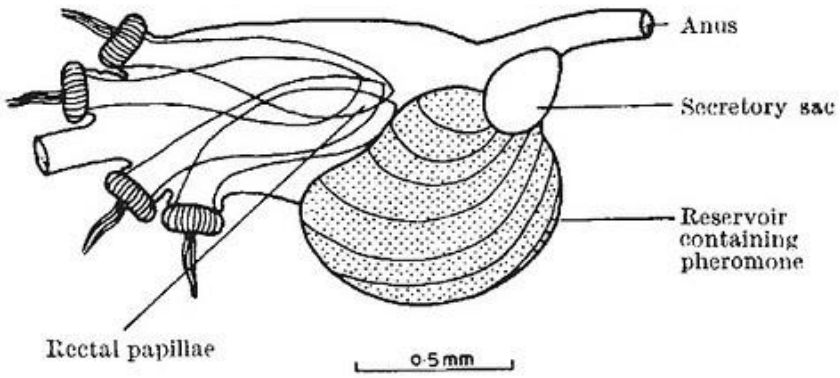


Figure 1.4. The rectum of a sexually mature male *B. tryoni* showing the rectal gland (reservoir containing pheromone).[44]

1.4.1 Pheromones for Fruit Fly Control

In addition to already established food and male lures, sex pheromones may also be used as potential chemical lures, given their inherent ability to attract the opposite sex during mating. The use of pheromones is of interest as they are generally species-specific, are effective in small amounts, and are capable of eliciting long-distance responses.[45,46] Pheromones are particularly advantageous because of their high specificity, and partial pheromone blends are typically sufficient for attraction.[45] Pheromone programs are most effective with low to moderate population densities, or when employed in small, isolated areas.[47,48] Pheromones can be employed with MT, MAT and/or MD methods.[45,48,49] Given that there are currently no effective commercial female lures, the sex pheromones of males may serve as a starting point in identifying and developing female specific lures.

MT and MAT involve similar approaches, in that a high density of pheromone-baited traps and/or killing devices are placed in strategic positions within the crop. These typically include a sex pheromone combined with another lure. Other lures used include visual stimuli, such as yellow boards, as well as food baits.[47,50,51] Large numbers of males and females are trapped and/or killed, impacting the mating pattern of the pest.[52] MT and MAT have successfully been used to control *B. oleae* populations in Europe. In MT studies undertaken in Tanagra Voeotias, Greece, traps containing the major pheromone component of *B. oleae*, 1,7-dioxaspiro[5.5]undecane, as well as ammonium bicarbonate (food attractant) and deltamethrin (insecticide), were compared to bait sprays over a four year period. The results indicated that lower fly populations and infestation levels were obtained in the MT orchard.[47] In MAT studies undertaken in Markopoulo, Greece, killing devices containing 1,7-dioxaspiro[5.5]undecane, deltamethrin, sugar solution and glycerol were compared to bait sprays. These devices were found to be sufficient in maintaining a low fruit fly population and kept fruit infestation at a level similar to that obtained in the control crops treated three times with insecticide.[50] MT and MAT are not sufficient alone in controlling fruit fly populations in large areas, however when used in conjunction with timely insecticide treatments with fruit phenology, effective management can be achieved whilst reducing the number of required insecticide treatments.[50,53] Compared to bait sprays, MT and MAT utilising a pheromone lure are approximately 30% more expensive.[47] As MT and MAT approaches are increasingly adopted worldwide, considerable cost reductions are expected.

Mating disruption (MD) involves disrupting insect mating communication systems with minute amounts of pheromonal chemicals, thus creating confusion by saturating the atmosphere with excessive amounts of sex pheromones[49,52] (see Section 1.4). As a result, fewer females are able to mate in order to lay viable eggs within host crops, therefore reducing the pest population.[48,49] Most research and implementation of MD has been focused on lepidopteran pests, an order of insects that

includes moths, skippers and butterflies.[45,54] In North America, a major cotton pest, the pink bollworm (*Pectinophora gossypiella*), is controlled by a commercial pheromone product with the tradename Glossyplure H.F.[49,55] The product contains a synthetic pheromone mixture dissolved in *n*-hexane and contained in hollow fibres, which are sealed at one end.[49] In 1989, an area-wide program using Glossyplure H.F. was implemented in Parker Valley, Arizona. In a five year period, crop damage decreased by 99%, and control costs decreased by 33%.[55] MD is also employed to protect apples and pears from a number of lepidopteran pests in Australia. Registered MD pheromone products include Isomate CTT© pheromone, specific for the codling moth (*Cydia pomonella*), Disrupt OFM©, specific for the oriental fruit moth (*Grapholita molesta*), and Isomate LBAM Plus© pheromone, specific for the light brown apple moth (*Epiphyas postvittana*).[56] MD has not been employed commercially with *Bactrocera,* and studies into its application have been limited to *B. oleae.* In preliminary MD studies researchers placed 176 pheromone 'sachets' in a one hectare site in Spain and sachets were replaced every 4 weeks. Over a 2 month period, trap catches decreased by approximately 98%, compared to a control site.[54] Given the success of pheromone programs employed for lepidopteran pests, it is feasible that MD methods can also be employed for controlling and monitoring *Bactrocera* species. As there is a current need for alternative fruit fly control methods, the determination of the pheromone profiles of different *Bactrocera* pest species, and the identification of the most attractive components, is of interest in the development of new and effective fruit fly lures.

1.4.2. Previous Work on *Bactrocera* Chemical Profiles

Given that sex pheromones are produced in the rectal glands of fruit flies and emitted as volatile components, studies on rectal gland extracts and headspace (volatile) collections of fruit flies serve as a valuable starting point in pheromone identification. The first investigation into the chemical profile of the rectal gland extracts of two *Bactrocera* fruit fly species was conducted in 1979 by Bellas and Fletcher[57] on *B. tryoni* and *B. neohumeralis*. Since then, the chemical profiles of many different fruit fly species have been published, including *B. jarvisi* (Tryon), *B. cucumis, B. kraussi,* and *B. oleae*.[38] These studies have mainly focused on profiling the chemical components of male rectal gland extracts, however recent studies also look at examining headspace collections. This is due to the fact that pheromone compounds are released into the atmosphere during sexual calling, and therefore examining headspace collections can serve to identify the compounds most likely acting as pheromones.[12,38,45,58,59] The study of female chemical profiles, through both rectal gland extracts and headspace collections, has also been increasingly reported within the literature in recent years.[58,60,61] Subsequent behavioural studies, such as cage trials, can determine which compounds identified in the chemical profiles act as pheromones.[51,57,62,63]

It is common practice to use gas chromatography-mass spectrometry (GC-MS) for the identification of fruit fly volatile compounds.[11,57-60,62,64,65] This technique separates complex chemical mixtures (*e.g.* rectal gland extracts or headspace collections) into individual components *via* GC, allowing sequential analysis of the components by MS. The MS fragments a compound, and matching the fragmentation pattern to a mass spectral library allows for the preliminary identification of individual compounds.[66,67] In cases where the compound is not listed in a spectral library, detailed analysis of the fragmentation pattern can assist compound identification.[67] Ultimately, comparison of retention times and mass spectra to authentic samples must be made to definitively identify compounds by the GC-MS technique. Given increases in the sensitivity of detection techniques, there is value in looking again at previously studied species, as new compounds may be detected and identified.

1.4.2.1 *B. tryoni and B. neohumeralis*

Bellas and Fletcher collected and examined the rectal gland extract of sexually mature males of *B. tryoni* and *B. neohumeralis.*[57] Six amides (Figure 1.5) were identified *via* GC-MS in both *B. tryoni* and *B. neohumeralis*: *N*-3-methylbutylpropanamide (major component), *N*-3-methylbutylacetamide, *N*-(3-methylbutyl)-2-methylpropanamide, *N*-2-methylbutylpropanamide, *N*-2-methylbutylacetamide, and *N*-(2-methylbutyl)-2-methylpropanamide (in decreasing order of abundance). These amides were present in similar ratios in both laboratory-reared and wild male flies, eliminating the possibility of dietary artefacts that can arise from multiple generations of laboratory-rearing.[11,38,57] A subsequent study by Lewis[68] on the rectal gland extract of *B. tryoni* males confirmed the findings of Bellas and Fletcher,[57] and also identified 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane-3-ol as a minor component (Figure 1.5).

Behavioural tests of *B. tryoni* and *B. neohumeralis* with the six amides identified indicated that a mixture of the amides could function as a short-range stimulant. This short-range stimulant was found to increase the sexual excitement of the female once she had arrived in the vicinity of the males.[11,38,57] There was no evidence of long-range attractive properties. As both *B. tryoni* and *B. neohumeralis* contain the same compounds in similar ratios, it appeared unlikely that these amides acted to sexually isolate the species.[11] Subsequent studies of the headspace collections of male *B. tryoni* identified *N*-3-methylbutylpropanamide, *N*-3-methylbutylacetamide, *N*-(3-methylbutyl)-2-methylpropanamide and *N*-2-methylbutylpropanamide in a similar proportion to the glandular composition, and the acids 2-methylpropanoic acid and 2-methylbutanoic acid. The rectal gland extracts were also found to contain 2-methylpropanoic acid and 2-methylbutanoic acid, as well as the six amides previously reported. The presence of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane-3-ol was not detected.[38] All compounds identified from the gland extracts are shown in Figure 1.5.

Booth *et al*.[58] examined the headspace collections and abdominal extracts of female *B. tryoni.* GC-MS of the headspace collections identified *N*-3-methylbutylpropanamide and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane as major components. *N*-3-Methylbutylacetamide, (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane were identified as minor components. GC-MS analysis of the abdominal extracts identified eight spiroacetal compounds (Figure 1.6), with (*2S,6R,8S*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane as the major component. Nonanal and decanal were identified as minor components (Figure 1.7).

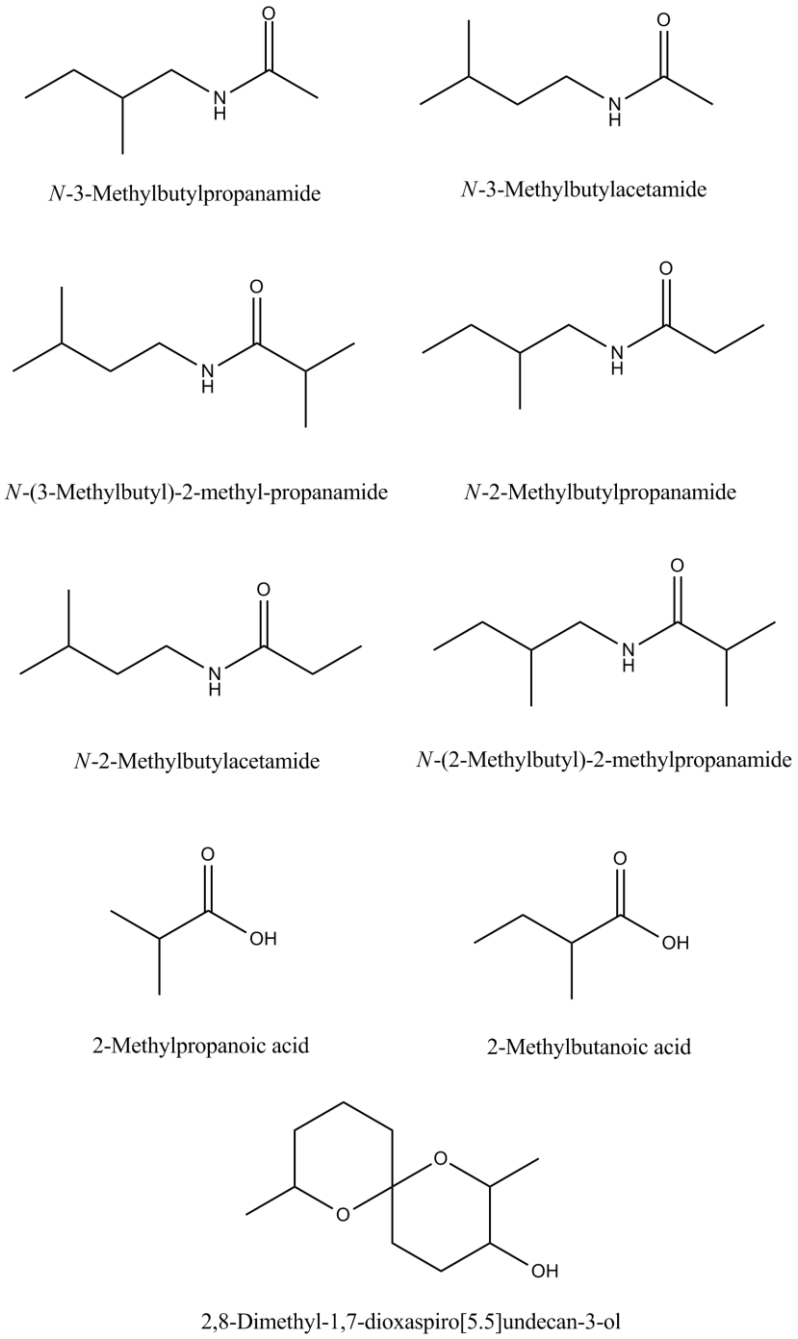


Figure 1.5. Compounds identified in *B. tryoni* and *B. neohumeralis* male rectal gland chemical profiles.

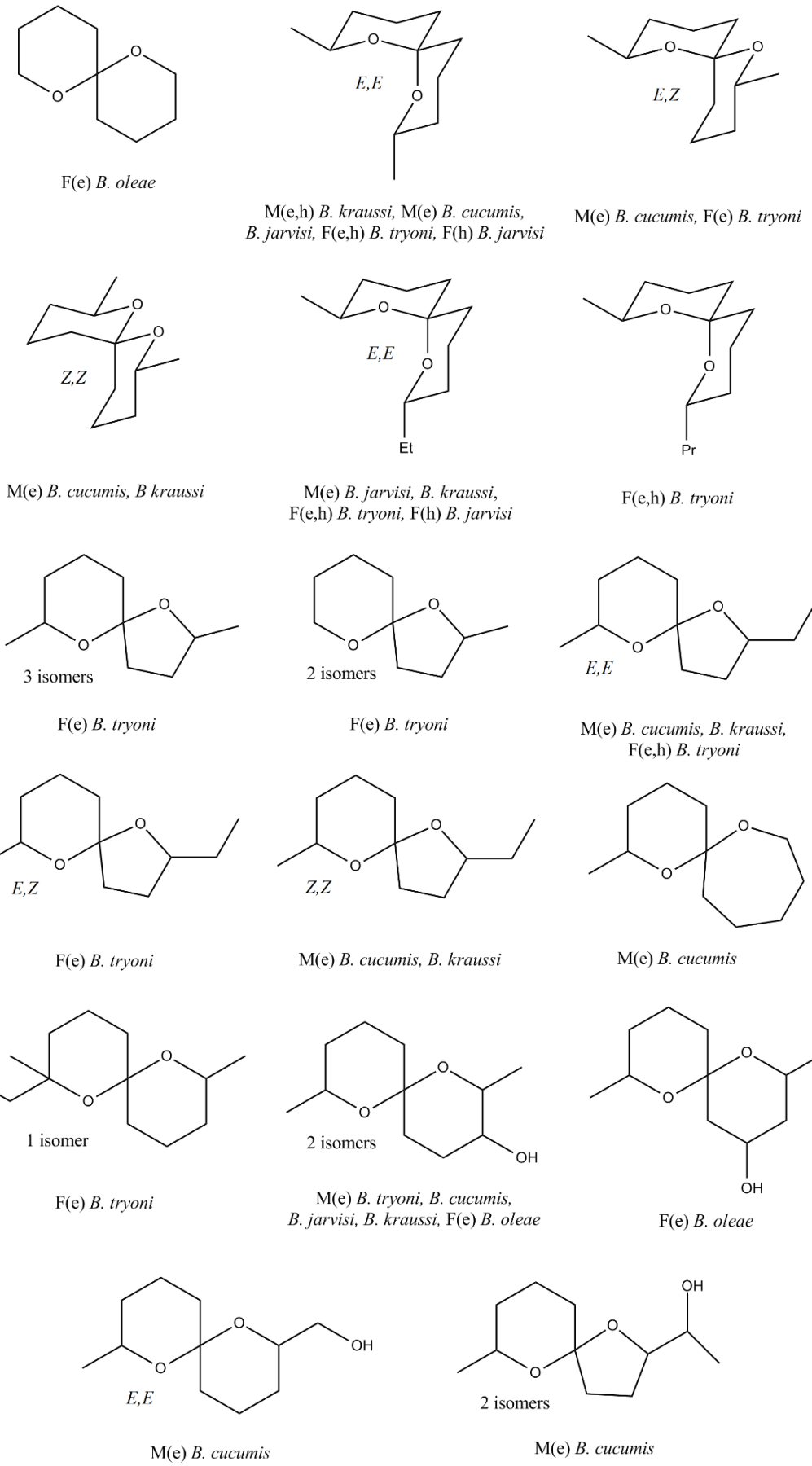


Figure 1.6. Spiroacetal components identified in *Bactrocera* chemical profiles. M = Male, F = Female, e = Gland extract, h = Headspace collections.

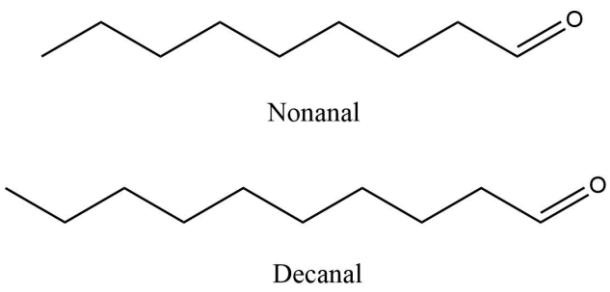


Figure 1.7. Aldehydes identified in *B. tryoni* female headspace collections.

1.4.2.2 *B. oleae*

Laboratory and field tests by Haniotakis *et al.*[69] found that female olive fruit flies released pheromones that acted as male sex attractants. Studies undertaken by Baker *et al*.[70] identified that the major component of the female rectal gland is 1,7-dioxaspiro[5.5]undecane (Figure 1.6), with additional significant components as α-pinene, nonanal and ethyl laurate[63] (Figure 1.8). The spiroacetal was found to be the most attractive compound, however a mixture of the four major compounds attracted more males than the spiroacetal alone.[63] Minor components included 1,7-dioxaspiro[5.5]undecane-3- and 4-ol and sixteen long-chain esters.[71,72]

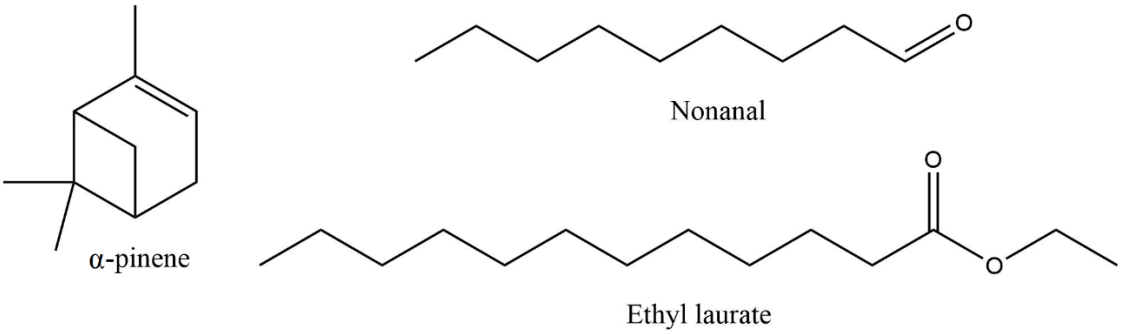
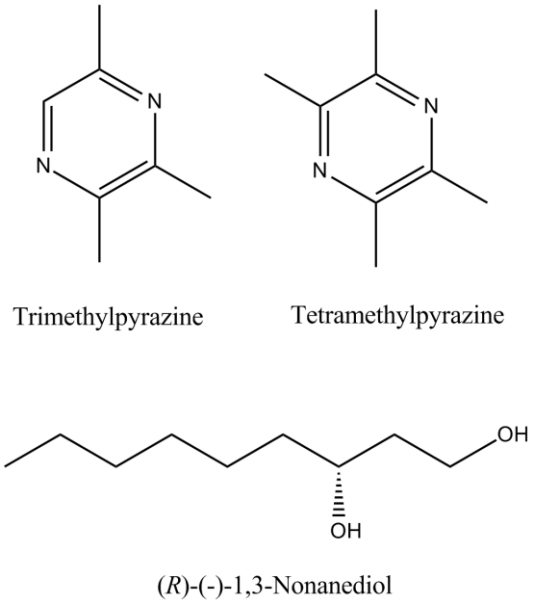


Figure 1.8. Major compounds identified in *B. oleae* female rectal gland extracts.

1.4.2.3 *B. cucumis*

Kitching *et al*.[37] examined the rectal gland extracts of male *B. cucumis.* GC-MS analysis identified (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane as the major component. The (*E*,*Z*) and (*Z*,*Z*) isomers were also identified as minor components. A total of twelve compounds were identified, including nine spiroacetals (Figure 1.6) and three other compounds (Figure 1.9).



Trimethylpyrazine Tetramethylpyrazine

(*R*)-(-)-1,3-Nonanediol

Figure 1.9. Compounds identified in *B. cucumis* male rectal gland extracts.

1.4.2.4 *B. jarvisi*

Fletcher and Kitching[38] reported that the rectal gland extracts of male *B. jarvisi* contain a large number of components (> 50). These compounds were present at a low level, with significant inconsistency between extracts, and possible contamination. Chemical species present included ethyl esters, terpenoids, substituted pyrazines, spiroacetals, and both saturated and unsaturated long chain acids and alcohols. The structures of the reported spiroacetals are shown in Figure 1.6 and the structures of the other compounds are shown in Figure 1.10. Headspace collections of female *B. jarvisi* contained ethyl dodecanoate and (*2S,6R,8S*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane as major components, and ethyl tetradecanoate and (*E,E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane as significant components. The structures of the spiroacetals identified are shown in Figure 1.6.

1.4.2.5 *B. kraussi*

Fletcher *et al.*[59] examined the rectal gland extracts and headspace collections of male *B. kraussi.* In the rectal gland extracts, one major, six significant, and five minor components were identified. In the headspace collections, four components were identified. The major component of both the gland extracts and the headspace collections was (*2S,6R,8S*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. The spiroacetals identified are shown in Figure 1.6 and other identified components are shown in Figure 1.11.

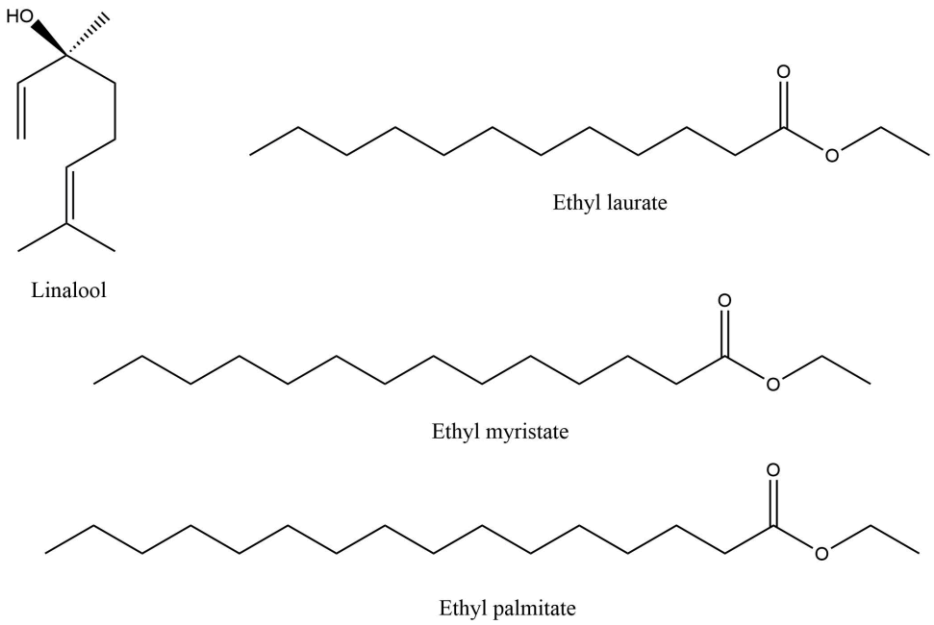


Figure 1.10. Reported compounds identified in *B. jarvisi* male rectal gland extracts.

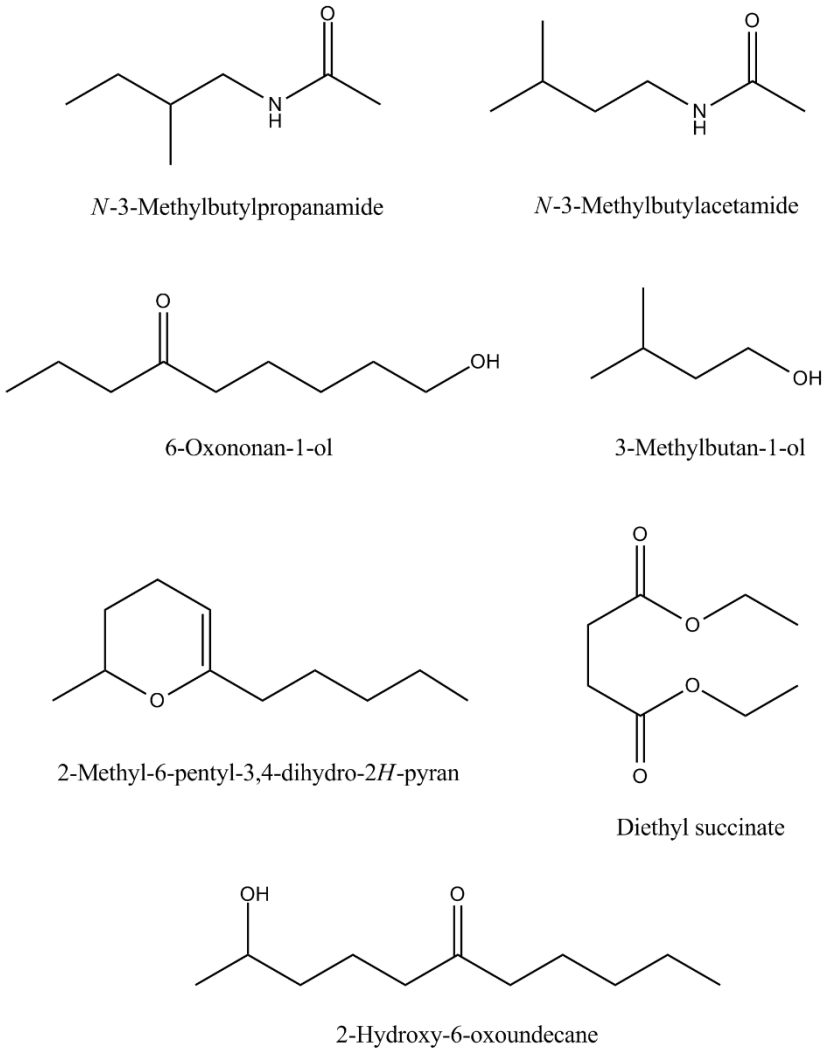


Figure 1.11. Compounds identified in *B. kraussi* male rectal gland extracts.

1.5 Aims

Given the restrictions placed on the use of insecticides for fruit fly control methods in recent years, there is a strong need for research into and development of alternative fruit fly control methods. The development of potent lures, for both males and females, is of particular interest. The overall aim of the project was to compare and determine the chemical pheromone profiles of three economically important pest species, *B. frauenfeldi, B. musae* and *B. kraussi.* This was achieved by:

i) Examining the chemical profiles of rectal gland extracts and headspace collections of males and females of the species studied using GC-MS.

ii) Identifying the major components of the chemical profiles through comparison of the GC-MS profiles to authentic samples obtained commercially or *via* synthesis and obtaining sufficient samples for assessing their attraction to fruit flies.

Chapter 2: Chemical Profiles of *B. frauenfeldi, B. musae* and *B. kraussi*

This chapter presents studies on the collection and analysis of rectal gland extracts and headspace collections of *B. frauenfeldi, B. musae* and *B. kraussi*.

2.1 Results and Discussion

2.1.1 Collections

Rectal gland extracts and headspace collections were collected by the candidate (see Section 5.1). Flies for rectal gland extracts and headspace collections were obtained in May 2016 in Cairns from the Queensland Government Department of Agriculture, Fisheries and Forestry. Rectal gland extractions and headspace collections in May 2016 were performed by the candidate at the Daintree Rainforest Observatory (James Cook University) in Cape Tribulation, Queensland. *n*-Hexane and ethanol extracts were obtained using laboratory-reared *B. frauenfeldi*, *B. kraussi* and *B. musae* (13-25 days old). For rectal gland extracts, three replicates per sex per species per solvent were collected (except for *B. frauenfeldi*, where two replicates per sex for *n*-hexane extracts were collected). For headspace collections, five replicates per sex per species per solvent were collected (except for *B. frauenfeldi*, where four replicates per sex per species per solvent were collected). Additional *n*-hexane extracts[37] of the rectal glands for laboratory-reared males and females of *B. frauenfeldi*, *B. kraussi* and *B. musae* (10-16 days old) were collected in Cairns in September 2014 by Dr Jeanneth Pérez from the Behavioural Biology Research Group (within Macquarie University's Department of Biological Sciences).

Rectal gland extracts were paired with headspace collections to both confirm the presence of major components identified in the rectal gland extracts, and to determine which compounds were being released into the gas phase. Headspace collections were collected during sexual calling time periods. Sex pheromones act through release into the gas phase during calling, dispersal throughout the atmosphere, and detection by the opposite sex.[12,45] Therefore, identifying the compounds released into the gas phase during calling acts to identify the components most likely to be detected by the fruit flies, and therefore the components most likely to act as sex pheromones. These compounds serve as strong lead compounds for biological testing, such as electroantennography (EAG), cage and field studies to confirm their role as sex pheromones.

n-Hexane was primarily used as the extraction solvent as literature procedures for fruit fly volatile analyses typically use a hydrocarbon solvent (*e.g.* pentane) for extraction of volatile compounds, which are often non-polar.[37,65,68] Ethanol extracts were also collected and analysed by GC-MS, to

determine if any polar compounds were present in the rectal glands that were not extracted into the *n*-hexane solvent. The ethanol extracts were very low in concentration, and were not investigated further in this project. Comparison of the GC-MS traces from these solvent systems showed that there was typically no difference in major (60-100% in abundance) and moderate (30-60% in abundance) components, however additional minor peaks were present. These are likely to be polar compounds (*e.g*. alcohols and acids) that were not extracted into the non-polar hydrocarbon solvent. Further investigation of the ethanol extracts is needed to identify minor components and to determine the complete chemical profiles.

Some rectal gland extracts and headspace collections were collected by the candidate in May 2016 on days of heavy rain. Some fruit fly species (laboratory-reared) are known to respond strongly to weather changes,[73] even when housed indoors. For fruit flies, light intensity and humidity affect mating behaviour, and so during heavy rain, calling activity and the release of pheromones may decrease.[73,74] It is ideal not to collect samples during heavy rain, however given time constraints we could not wait for the weather to change, and the samples were collected. This may have affected the quantities of compounds released, and therefore the chemical profiles obtained. Additional rectal gland extracts and headspace collections would need to be taken in future work during favourable conditions to confirm the results reported here.

2.1.2 GC-MS Analysis

Chemical profiles were determined separately for the different collection times (2014 and 2016) and for males and females of *B. frauenfeldi, B. musae* and *B. kraussi.* These results are summarised in Table 2.1. Structures for the compounds listed in Table 2.1 are shown in Figure 2.1. Representative GC-MS traces for rectal gland extracts and headspace collections are shown in Appendices A-C.

Compound	Species	Sex	Abundance			Retention Time (min)	Similarity Index (%)
			Rectal gland extracts		Headspace		
			2014	2016			
Ethyl caprate (1)	*B. frauenfeldi*	F	+	-	-	15.401	87
	B. musae	F	+	-	-	15.406	93
Ethyl tridecanaote (**2**)	*B. frauenfeldi*	F	+	-	-	18.981	77
	B. kraussi	F	+	+	+	18.983	86
	B. musae	F	+	-	-	18.987	77
Methyl laurate (**3**)	*B. frauenfeldi*	F	+	+	+	17.067	97
	B. kraussi	M	+	-	-	17.073	96
		F	+	+	+	17.072	96
	B. musae	M	+++	-	-	17.069	97
		F	+	+	+	17.074	96
Ethyl laurate (**4**)	*B. frauenfeldi*	F	+++	+++	+++	17.877	97
	B. kraussi	M	+	-	-	17.847	94
		F	+++	+++	+++	17.879	97
	B. musae	M	+++	-	-	17.851	95
		F	+++	+++	+++	17.882	96
Propyl laurate (**5**)	*B. kraussi*	F	+	+	+	18.930	94
	B. musae	F	+	+	+	19.937	95
Isoamyl laurate (**6**)	*B. kraussi*	F	+	+	+	20.544	91
Methyl myristate (**7**)	*B. frauenfeldi*	F	+	+	+	19.369	95
	B. kraussi	F	-	+	+	19.366	93
	B. musae	F	+	+	+	19.377	94
Ethyl myristate (**8**)	*B. frauenfeldi*	F	+++	++	++	20.077	95
	B. kraussi	M	+	-	-	20.057	88
		F	+++	+++	++	20.078	96
	B. musae	M	+++	-	-	20.064	95
		F	+++	+++	++	20.077	95

Ethyl palmitate (**9**)	*B. frauenfeldi*	F	**+++**	**+++**	**+**	22.085	94
	B. kraussi	M	**+**	-	**-**	22.064	85
		F	**++**	**++**	**+**	22.084	92
	B. musae	M	**++**	**-**	**-**	22.066	91
		F	**++**	**++**	**+**	22.088	92
Ethyl palmitoleate (**10**)	*B. frauenfeldi*	F	**+++**	**+++**	**+**	22.109	94
	B. kraussi	M	**+**	-	**-**	22.073	89
		F	**++**	**++**	**+**	22.094	94
	B. musae	M	**++**	**-**	**-**	22.082	88
		F	**++**	**++**	**+**	22.108	93
Ethyl elaidate (**11**)	*B. frauenfeldi*	F	**+**	**+**	**-**	23.934	93
	B. kraussi	F	**+**	**+**	**-**	23.935	93
	B. musae	F	**+**	**+**	**-**	23.935	93
Ethyl oleate (**12**)	*B. frauenfeldi*	F	**+**	**+**	**-**	23.872	94
	B. kraussi	F	**+**	**+**	**-**	23.881	95
	B. musae	F	**+**	**+**	**-**	23.881	93
2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**13**)	*B. frauenfeldi*	M	**+++**	**+**	**-**	11.630	-
		F	**++**	**+**	**-**	11.638	-
	B. kraussi	M	**+++**	**+++**	**++**	11.652	-
		F	**+**	**+**	**+**	11.649	-
	B. musae	F	**+**	**+**	**+**	11.651	-
2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**14**)	*B. kraussi*	M	**+**	**+**	**-**	13.027	-
		F	**+**	**+**	**-**	13.028	-
	B. musae	F	**+**	**+**	**-**	13.033	-
2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**15**)	*B. frauenfeldi*	M	**+++**	**+++**	**+++**	13.320	-
		F	**+**	**+**	**-**	13.269	-
	B. kraussi	M	**+**	**+**	**-**	13.340	-

N-3-Methylbutylacetamide (**16**)	*B. kraussi*	M	+	+	+++	12.880	96
		F	+	+	+	12.884	94
	B. musae	M	++	-	-	12.882	96
		F	+	+	+	12.886	94
Diethyl succinate (**17**)	*B. kraussi*	M	+	+	-	13.310	91
6-Oxononan-1-ol (**18**)	*B. kraussi*	M	+	+	-	15.896	-
(*R*)-(+)-Citronellal (**19**)	*B. frauenfeldi*	M	+	-	-	12.239	93
		F	+	-	-	12.258	92
	B. kraussi	M	+	-	-	12.245	91
		F	+	-	-	12.249	91
	B. musae	M	+	-	-	12.243	92
		F	+	-	-	12.246	93
Ethyl 4-ethoxybenzoate (**20**)	*B. frauenfeldi*	M	+	-	-	18.363	72
		F	+	-	-	18.364	78
	B. musae	M	+	-	-	18.364	68
		F	+	-	-	18.375	73

Table 2.1. Compounds tentatively identified in chemical profiles for *Bactrocera* species studied. +++ = major component, ++ = moderate component, + = minor component, - = not detected.

Compounds were considered major components if they fell between 60-100% in abundance, moderate if between 30-60%, and minor if below 30%, when compared to the peak with the highest total ion count (TIC), which was given a relative abundance of 100%. For rectal gland extracts of *B. kraussi* and *B. musae*, peaks were considered genuine if present in at least four of the six replicates collected (across 2014 and 2016). For rectal gland extracts of *B. frauenfeldi*, peaks were considered genuine if present in at least three of the five replicates collected (across 2014 and 2016). If the compound was present in rectal gland extracts from one year only, peaks were considered genuine if present in at least two of the three replicates taken. For headspace collections of *B. kraussi* and *B. musae*, peaks were considered genuine if they were present in at least three of the five replicates collected (or three of the four replicates collected for *B. frauenfeldi*) (from May 2016), unless peaks were contaminants identified through comparison to blanks. Compounds were identified by matching to library mass spectral data or literature mass spectra.[60,75-79]

GC-MS was chosen for analysis of the volatiles in both the rectal gland extracts and headspace collections. Given the complexity of the samples, their low concentration (fruit flies typically produce and release chemicals on the micro to nanomole scale[11,49]), and their volatility, GC-MS was the most suitable technique. Using GC-MS, complex mixtures can be separated into individual compounds in the GC component, allowing percentage composition analysis, and components can be sequentially introduced into the MS to allow structural elucidation. In the MS, ionisation causes fragmentation, generating a mass spectrum specific to the compound. This mass spectrum can be searched through spectral databases, leading to preliminary compound identification.[66,67]

GC-MS and subsequent searching through spectral databases NIST21 and NIST107 lead to the tentative identification of 27 compounds. Four compounds were unable to be tentatively assigned through libraries. The three spiroacetals (**13**, **14** and **15**) and **18** were tentatively assigned based on matches to literature mass spectra. These compounds, shown in Table 2.1, were subject to confirmation as described in Chapter 3. Seven esters were found not to be present in the GC-MS traces, and are not included in Table 2.1 or Figure 2.2, or described further here (see Section 3.2.1). Compounds were ignored if they were identified as contaminants, which predominantly included siloxane compounds. Hydrocarbon peaks were also ignored, given the similarity of mass spectra, ambiguity surrounding parent ion peaks, and their presence as minor components.

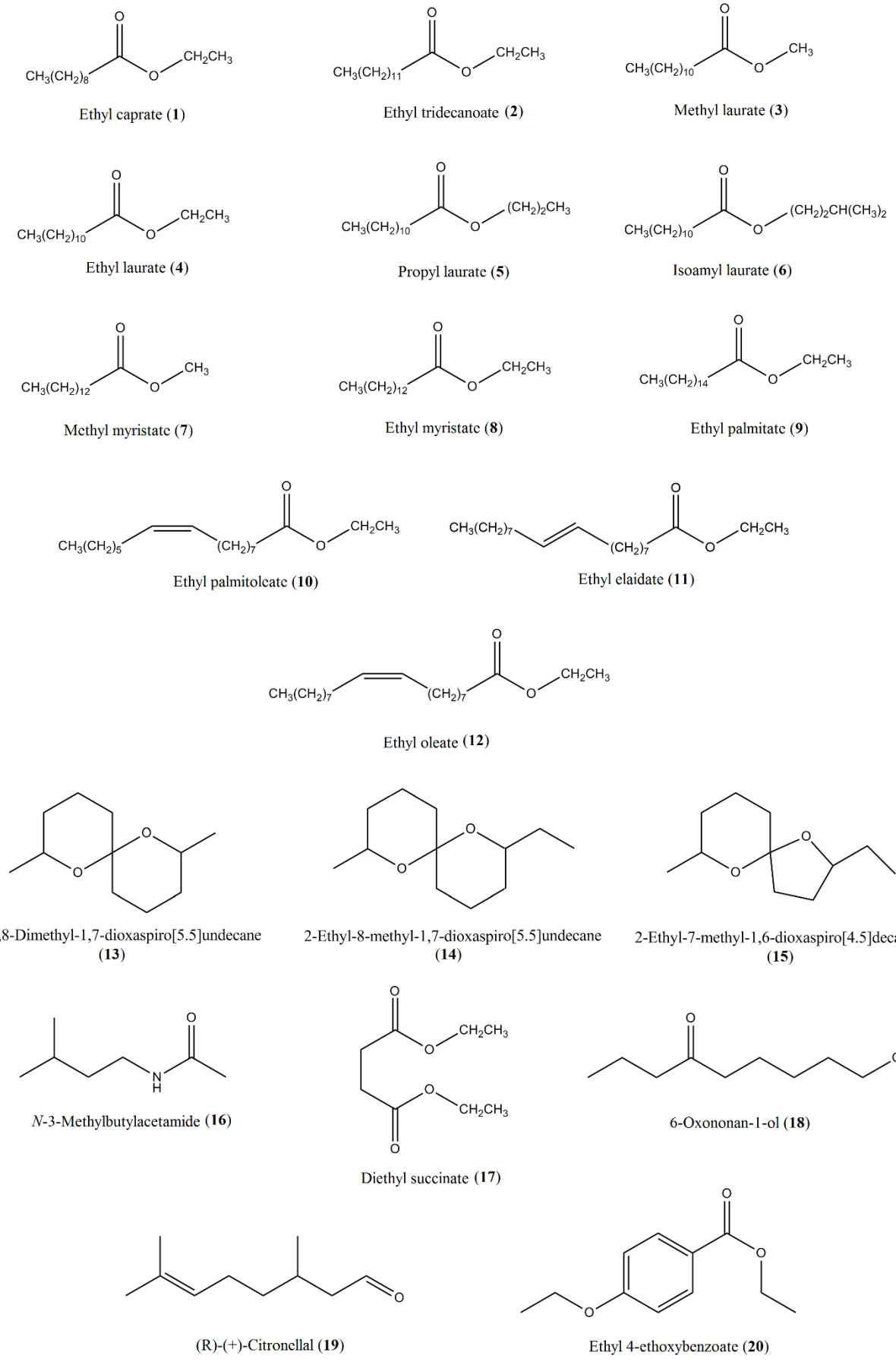


Figure 2.1. Structures of compounds identified through library and literature matching in chemical profiles.

2.2 Chemical Profiles

2.2.1 *B. kraussi*

Of the species tested, only the chemical profile for male *B. kraussi* had been previously reported[59]. This previous study identified compounds via GC-MS on a Hewlett-Packard 5970 Series GC-MS system using a non polar column, or a Finnigan Mat 1020 GC-MS. In the current study, compounds were identified via GC-MS on a Shimadzu GCMS-QP2010, using a mid-polarity phase column. Compounds detected in the current study included **13** (+++), **14** (+), **15** (+), **16** (+), **17** (+) and **18** (+). These compounds have been previously reported as major, moderate and minor components, and their identification served to confirm the methodology used in the current study was appropriate. Despite the more sensitive detection system, six previously reported compounds (2,8-dimethyl-1,7-dioxaspiro[5.5]undecan-3-ol, 2-methyl-6-pentyl-3,4-dihydro-2*H*-pyran, 2-hydroxyundecan-6-one, 3-methylbutan-1-ol and *N*-methylbutylpropanamide) were not detected in the current study. For the polar compounds (*i.e.* 2,8-dimethyl-1,7-dioxaspiro[5.5]undecan-3-ol and 3-methylbutan-1-ol), it is possible that these compounds were not extracted into the hydrocarbon solvent used in the current, and could be present in the ethanol extracts. For other components, it is likely that the quantities extracted were not sufficient for detection. It is therefore possible that the compounds are present in these samples, but have yet to be identified. Five additional compounds were detected only in the 2014 rectal gland extracts (methyl laurate, ethyl laurate, ethyl myristate, ethyl palmitate and ethyl palmitoleate), and were all present as minor components. In headspace collections, two compounds were identified: **13** (++) and **16** (+++).

For female *B. kraussi*, a total of thirteen compounds were identified as present in rectal gland extracts collected in both 2014 and 2016 (Table 2.1). Identified compounds included ten esters, **2** (+), **3** (+), **4** (+++), **5** (+), **6** (+), **8** (+++), **9** (++), **10** (++), **11** (+) and **12** (+), as well as **13** (+), **14** (+), and **16** (++). Ten compounds were identified in headspace collections (Table 2.1). Identified compounds included **2** (+), **3** (+), **4** (+++), **5** (+), **6** (+), **8** (++), **9** (+), **10** (+), **13** (+) and **16** (+). **7** was identified as a minor component in the 2016 samples only, in both rectal gland extracts and headspace collections.

Nine compounds were detected in both the male and female rectal gland extracts. These included the three spiroacetals (**13**, **14** and **15**), five esters (**3**, **4**, **8**, **9** and **10**) and **16**. The esters were only detected in the 2014 extracts for males. Spiroacetals were the most abundant compounds for males, whilst esters were the most abundant compounds for females. **13** and **16** were detected in headspace collections for both sexes. For males, these were the only compounds identified, and **13** was the major component. For females, these compounds were minor components.

2.2.2 *B. frauenfeldi*

For *B. frauenfeldi* males, two spiroacetal compounds were identified in rectal gland extracts collected in both 2014 and 2016: **13** (+) and **15** (+++). Additional minor components were present, but were not investigated further due to either incomplete spectra or hydrocarbon matches. Rectal gland extracts taken from 2014 were also very contaminated, with most minor peaks being matched to siloxane compounds. Only the major component, **15** (+++), was present in headspace collections.

In contrast, ten compounds were identified in female *B. frauenfeldi* rectal gland extracts collected in both 2014 and 2016, including the two spiroacetals identified in males (present as minor components), and eight esters; **3** (+), **4** (+++), **7** (+), **8** (+++), **9** (+++), **10** (+++), **11** (+) and **12** (+). Six esters were identified from headspace collections; **3** (+), **4** (+++), **7** (+), **8** (+++), **9** (+), and **10** (+).

For both males and females, additional minor components were identified in the 2014 rectal gland extracts. Only **1** and **2** in the female chemical profile could be identified. For the 2016 samples, fewer rectal gland extracts were taken for both sexes of *B. frauenfeldi*, and a smaller number of flies were used in headspace collections (20 of each sex as opposed to 30), due to an unfortunate placement of the fruit fly cages, which exposed the fruit flies to excessive morning sun, resulting in premature death of many of the flies. Additional flies were obtained, however the number was insufficient to last the 2 weeks of collection. As such, the quantity collected of these minor components may not have been sufficiently high for detection in the 2016 samples. Additional samples will need to be collected in order to confirm this.

2.2.3 *B. musae*

For *B. musae* males, six compounds were identified in rectal gland extracts collected in 2014 (Table 2.1), including **3** (+++), **4** (+++), **8** (+++), **9** (++), **10** (++) and **16** (++). No compounds were identified from the rectal gland extracts of males in the 2016 collections. Additional minor peaks in rectal gland extracts collected in 2014 were difficult to identify, with many matching to hydrocarbons in the spectral database. No compounds were detected from headspace collections.

For *B. musae* females, twelve compounds were identified in rectal gland extracts from both 2014 and 2016 (Table 2.1). Identified compounds included eight esters, **3** (+), **4** (+++), **5** (+), **7** (+), **8** (+++), **9** (++), **10** (++), **11** (+) and **12** (+), as well as **13** (+), **14** (+) and **16** (+). Two additional minor components, **1** and **2**, were identified in 2014 rectal gland extracts. Nine compounds were identified in headspace collections (Table 2.1). Identified compounds included **3** (+), **4** (+++), **5** (+), **7** (+), **8** (++), **9** (+), **10** (+), **13** (+) and **16** (+).

Six compounds were detected in the rectal gland extracts for males (2014 only) and females (2014 and 2016). These included **3**, **4**, **8**, **9**, **10** and **16**. **4**, **8**, **9** and **10** were detected in similar relative abundance levels. **3** and **16** were relatively more abundant in males.

No compounds were detected in 2016 rectal gland extracts or headspace collections for males. Given that this was not seen with females, and was present across all samples taken (which were collected on different days), it is unlikely that this was due to experimental error. Rectal gland extracts and headspace collections from *B. musae* were taken on days of heavy rain. Characteristic calling activity was seen during these collections, including rubbing of legs over abdomen and wing vibration. Even though calling behaviour was witnessed, the weather conditions may have resulted in suppression of released quantities. Additional rectal gland extracts and headspace collections would need to be taken to confirm this, ensuring that collections occurred during favourable weather conditions.

2.3 Comparison Between Species

Twenty compounds were tentatively identified as being present in the fruit fly species studies through the library databases NIST21 and NIST107. Four compounds (**13**, **14**, **15** and **18**) were unable to be tentatively assigned through the libraries. These compounds were tentatively assigned based on matches to literature mass spectra. Overall, it was found that males typically produced one major component, usually a spiroacetal, and had additional compounds present in their chemical profile. In contrast, females produced a wider range of compounds, including acetamides, spiroacetals and esters, with the latter being the major components in the chemical profile. The only exception was males of *B. musae*, which were found to produce a range of compounds in their chemical profile, predominantly esters. A number of additional minor components were identified in all chemical profiles. Some of these were tentatively identified as ester or spiroacetal compounds, based on fragmentation patterns. Others were difficult to identify, due to hydrocarbon matches or incomplete spectra. This is discussed further in Chapter 3.

A number of the compounds detected were compounds that have been previously reported as either present in the pheromone profiles of other fruit fly species, and/or capable of eliciting a behavioural response. Chapter 1 includes the description of **4** in rectal gland extracts of female *B. oleae*,[63] **4**, **8** and **9** in rectal gland extracts in male *B. jarvisi*,[38] and **16** in abdominal extracts of female *B. tryoni*.[58] The *E,E, E,Z* and *Z,Z* isomers of **13** have also be reported as present in many fruit fly pheromone profiles[37,38,58-60] (see Chapter 1). **3**, **7**, **8** and **9** have also been reported as minor components of the rectal gland extracts of female *B. oleae*.[63] **16** has been reported as present in many fruit fly pheromone profiles, and elicits female attraction in *Z. cucurbitae*, *B. dorsalis* (Hendel) and *B. carambolae*.[60,77,80] **18** has also been reported in the rectal gland extracts of male *B. carambolae*, and has been demonstrated to elicit a response from conspecific females.[80]

Three compounds were identified across both sexes, and most species, in the 2014 rectal gland extracts, and confirmed by comparison to authentic samples as *S*-(+)-Carvone, **19** and **20**. *S*-(+)-Carvone, the major constituent of caraway seed oil,[81] gave strong peaks in the 2014 samples, and was a major component for males of all three species. Given its absence from any 2016 GC-MS profiles, it is most likely a contaminant and was therefore not tested further. **19** and **20** were minor peaks in the 2014 samples, and were not identified in any 2016 rectal gland extracts. Citronellal is a natural product present in a variety of lemongrass species,[82] and is a common ingredient in mosquito repellants.[83,84] It is a reported alarm and aggregation pheromone for a variety of bee[85] and ant[86-88] species. It has also been reported as being attractive to *B. diversa* (Coiquillett) and *B. zonanta* (Saunders).[89] **20** is a floral volatile compound, reported as present in *Cordylandra burchellii*, *C. panapanari*, and *C. spiritu-sanctensis*.[90] There are no previous reports of these compounds being present in *Bactrocera* pheromone profiles. These compounds are here tentatively identified as present in the chemical profiles, however further investigation would be required to confirm this, most likely through additional rectal gland extracts from another fruit fly colony.

It was also seen that generally a higher number of compounds could be detected in 2014 rectal gland extracts than in 2016 rectal gland extracts. This may be because that flies used in the 2016 rectal gland extracts were from a later generation of laboratory-rearing than those used in the 2014 rectal gland extracts.[91] In some cases, multiple generations of laboratory-rearing can affect the sexual performance of the fruit fly. For example, mass-reared flies of *Ceratitis capitata* have been shown to have reduced sexual performance after 7 to 10 generations, in comparison to wild flies.[92] As such, the subsequent generations of laboratory-rearing may have affected the quality of flies used for 2016 rectal gland extracts and headspace collections. Additional rectal gland extracts and headspace collections from a recently established colony would be needed to confirm this.

It is interesting to note that although many of the compounds identified were detected in multiple chemical profiles, across both species and sex, the chemical profiles for each species and each sex were generally distinct from one another. Assuming the chemicals identified are pheromone components, this serves to reproductively isolate the species from others during mating, such that only members of the same species (and opposite sex) respond to the pheromones released.[12] In terms of lure application, this may mean that a blend of different pheromone components, rather than a single component, will be most effective. Indeed, this has been seen in *C. capitata*, where females were found to be attracted to the major male pheromone components individually, more attracted to a mix of the major components, and even more attracted to the entire pheromone blend.[62,93,94]

Chapter 3: Confirmation of Fruit Fly Components

This chapter presents studies on the identification of compounds tentatively identified in the GC-MS investigations, with the aim to confirm structures through comparison to authentic samples obtained commercially or synthetically with the GC retention times and MS fragmentation patterns. An additional aim was to obtain authentic samples of these compounds to allow preliminary fruit fly attractant testing of the pure compounds and mixtures.

3.1 Introduction

The use of GC-MS library databases has greatly aided preliminary identification of a vast number of volatile compounds, including those obtained as complex mixtures from natural product extracts.[11,57-60,62,64,65] For many compounds, preliminary identification occurs through matching experimental mass spectra to those in a library spectral databases. Comparison of GC retention times and MS fragmentation patterns to authentic samples then confirms compound identification. For other molecules, there are no matching library data, and elucidation has to be done through detailed mass spectral fragment analysis and comparison to authentic samples (obtained *via* synthesis or commercially).

In Chapter 2, 23 compounds (including esters and other compounds) were tentatively identified as being present in the fruit fly species studies through the library databases NIST21 and NIST107, while four compounds (**13**, **14**, **15** and **18**) were unable to be tentatively assigned through the libraries. These compounds were tentatively assigned based on matches to literature mass spectra as described below. A number of additional compounds were tentatively identified as unknown esters or spiroacetals, based on fragmentation patterns, as also described below.

3.2 Results and Discussion

3.2.1 Esters

A total of 19 compounds were tentatively identified as esters from the GC-MS library databases. These included methyl, ethyl and propyl esters. They were easily identifiable by the McLafferty fragment ion in the MS. The McLafferty rearrangement is characteristic of ester compounds, yielding fragments of 74 *m/z* (for methyl esters), 88 *m/z* (for ethyl esters) and 102 *m/z* (for propyl esters), and are commonly the base peak. The McLafferty rearrangement of an ethyl ester is shown in Figure 3.1. Of the 19 esters tentatively identified, nine were available commercially and were purchased (**1**, **2**, **3**, **4**, **6**, **7**, **8**, **9** and **12**). They were found to have identical GC-MS profiles to samples in the extracts, confirming their presence in the fruit fly extracts. The remaining ten esters (shown in Figure 3.2) were not available commercially, and were synthesised for comparison with the extracts.

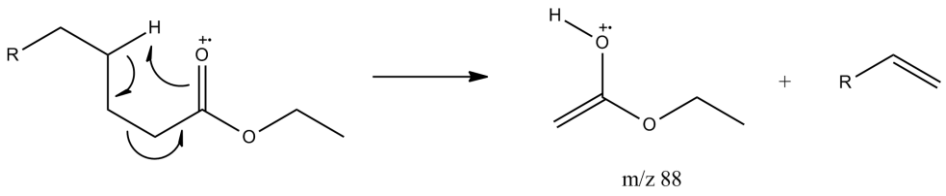
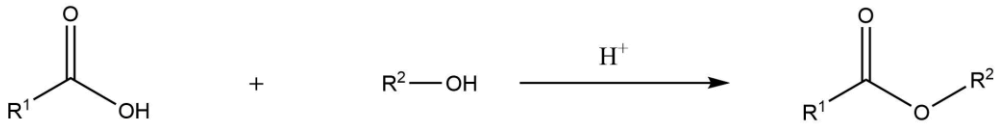


Figure 3.1. McLafferty rearrangement for an ethyl ester.

Ester synthesis was undertaken using the Fischer esterification reaction, involving an acid catalysed reaction between a carboxylic acid and an alcohol, shown in Figure 3.2. This procedure is known to work well for most esters, including long-chain esters.[95] Protonation of the carbonyl group activates the carbonyl carbon towards nucleophilic attack. The alcohol hydroxyl group subsequently attacks the carbonyl carbon, and rearrangement and loss of water leads to ester formation.[96,97] The advantage of this synthesis is in its simplicity, and the availability of starting materials. The reaction is reversible, and excess of either the carboxylic acid or alcohol is required to drive the reaction to completion. As such, an excess of alcohol was used in these reactions.



R^1 = $(CH_2)_6CH_3$, $(CH_2)_{10}CH_3$, $(CH_2)_{17}CH_3$, $(CH_2)_{20}CH_3$, $(CH_2)_7CH{=}CH(CH_2)_5CH_3$, $(CH_2)_7CH{=}CH(CH_2)_7CH_3$

R^2 = Et, Pr, *i*Pr

8 R^1 = $(CH_2)_{10}CH_3$, R^2 = Pr, 38%
10 R^1 = $(CH_2)_7CH{=}CH(CH_2)_5CH_3$, R^2 =Et, 19%
11 R^1 = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$, R^2 =Et, 23%
21 R^1 = $(CH_2)_6CH_3$, R^2 = *i*Pr, 0.52%
22 R^1 = $(CH_2)_6CH_3$, R^2 = Pr, 14%
23 R^1 = $(CH_2)_{10}CH_3$, R^2 = *i*Pr, 29%
24 R^1 = $(CH_2)_{17}CH_3$, R^2 = Et, 17%
25 R^1 = $(CH_2)_{20}CH_3$, R^2 = Et, 74%
26 R^1 = $(CH_2)_7CH{=}CH(CH_2)_5CH_3$, R^2 = *i*Pr, 24%
27 R^1 = $(CH_2)_7CH{=}CH(CH_2)_5CH_3$,, R^2 = Pr, 31%

Figure 3.2. General synthesis procedure for esters synthesised

Reaction progress was monitored using GC-MS, and the reaction was quenched once product formation was ≥ 90%. Overall, the reactions proceeded well, with the use of acid catalysis, heat, and an excess of alcohol giving high product yields in short periods of time (1.5-3.5 hrs). Significant product was lost during purification by distillation, hence the yields for these compounds were all below 40%. Given the small quantities needed for characterisation, the low yield did not hinder this work and so no investigations into improving the yields for these reactions were undertaken. Experimental NMR and mass spectra for all esters synthesised were consistent with those presented in the literature.[98-109] In cases where no literature spectral data were available, experimental spectra were consistent with that expected for the compound, based on chemical shifts, peak splitting patterns and chemical integrations.

All syntheses and purifications proceeded as expected, except for isopropyl laurate (**23**). **23** was isolated as a solid, however it has been reported as a colourless liquid,[102] and a yellow oil,[110] at room temperature. **23**, which was a liquid when impure, was purified by distillation, causing removal of solvent and isolation of the solid product. GC-MS and NMR showed that this solid was very pure, and was consistent with literature spectral data.[101,102]

For peaks suspected to be isopropyl or propyl esters, both isomers were synthesised. This was done as isopropyl and propyl esters are structural isomers, and will therefore produce very similar mass spectra, and likely will have similar retention times. It was found that the retention times for isopropyl caprylate (**21**), propyl caprylate (**22**), isopropyl oleate (**26**) and propyl oleate (**27**), were significantly different to the proposed peaks for these compounds in the chemical profiles. Conversely, isopropyl laurate (**23**) had a retention time only slightly earlier than the proposed compound peak, while **8** had a retention time slightly later. The mass spectrum for **8** (but not **23**) was also very similar to that seen in the chemical profiles. Re-running a gland extract suspected to contain **8** found that the retention times matched, and therefore **8** was positively identified as present in the chemical profiles. For the other peaks suspected to be **21**, **22**, **26** or **27**, it is likely that the unidentified compound is a propyl or isopropyl ester showing an incomplete mass spectrum. An example of this is shown in Figure 3.3. In this spectrum, the base peak of 43 *m/z* presumably corresponds to the propyl moiety, the ion at 61 *m/z* to the propyloxy moiety, whilst the McLafferty ion is seen at 102 *m/z*.

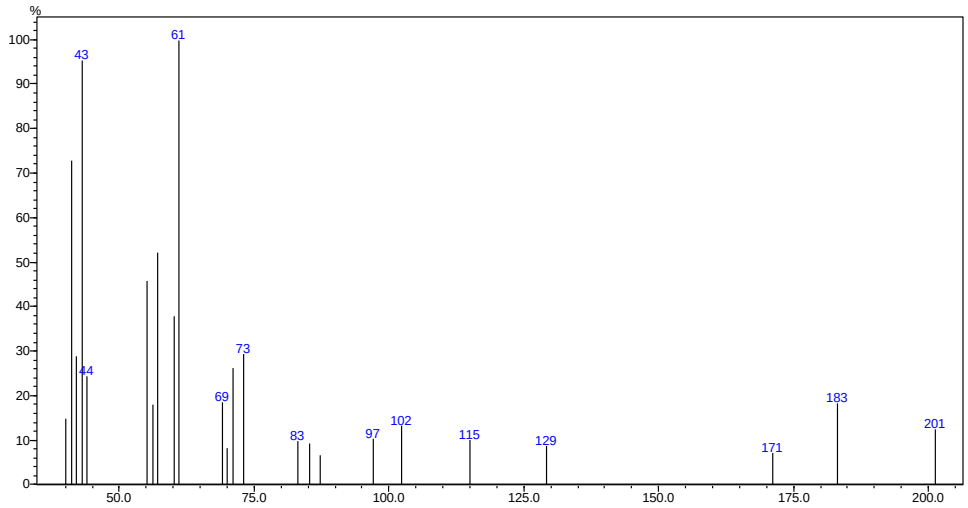


Figure 3.3. Mass spectrum of unknown compound in female *B. frauenfeldi* suspected to be a propyl ester.

For the ethyl esters, **10** and **11** were found to be present in the chemical profiles, based on GC-MS (retention time and mass spectra). Ethyl nonadecanoate (**24**) and ethyl behenate (**25**) were found to have dissimilar retention times to that of the proposed compound peaks. The peak suspected to be **24** had a retention time of 18.6 min in the chemical profiles, whilst the authentic sample had a retention

time of 24.8 min. For **25**, the peak had a retention time of 17.8 min in the chemical profiles, whilst the authentic sample had a retention time of 28.8 min. The mass spectra were similar between the unknown compound and the authentic samples, showing the McLafferty ion of 88 *m/z*, characteristic for ethyl esters. As such, these peaks are likely to be ethyl esters, but require further analysis for identification.

Of the ten esters synthesised, only three were found to be present in chemical profiles: **8**, **10** and **11**, based on GC-MS. For the other esters synthesised, in most cases the mass spectrum was similar to that of the unknown compound, however retention times were significantly different. As such, it is likely that these peaks are esters similar to those synthesised, and they would be of interest to investigate further in future studies.

3.2.2 Spiroacetals

Spiroacetals have been previously reported as present in the chemical profiles for a number of *Bactrocera* species.[11,37,38,58,59,65,111] They show characteristic fragmentation patterns in mass spectra, including a fragmentation 'doublet', which is typically the base peak for spiroacetals containing six-membered or seven-membered rings.[112] Three spiroacetals were identified by comparison to literature mass spectra (**13**, **14** and **15**). Some proposed spiroacetal peaks were not identified by comparison to literature mass spectra, and would require detailed fragmentation analysis for structure elucidation. The mass spectrum for one such compound is shown in Figure 3.4.

Spiroacetals were not commercially available and the synthesis of **13**, **14** and **15** (Figure 3.5) was therefore investigated. Spiroacetal synthesis was attempted using the procedure published by Doubský *et al*.[113] This procedure has been used to synthesise a wide range of spiroacetals, including spiroacetals of different ring sizes and substitution patterns, hence it was proposed that this procedure would work well for the spiroacetals of interest. The reaction involves two steps; first an α-alkynone intermediate is synthesised *via* the reaction between a lithiated terminal alkyne bearing a benzyl-protected hydroxyl group and a lactone. Deprotection, hydrogenation of the triple bond and subsequent ring closure yields the spiroacetal of interest. The reaction scheme is shown in Figure 3.6.

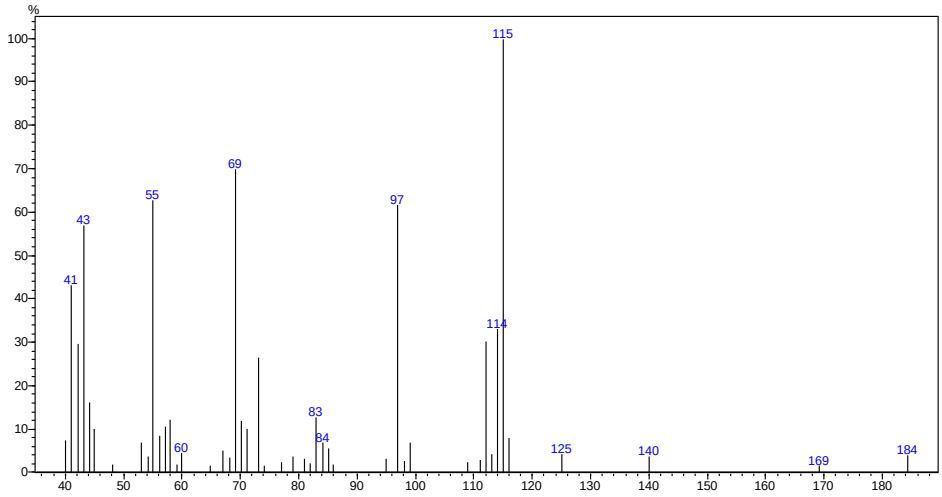
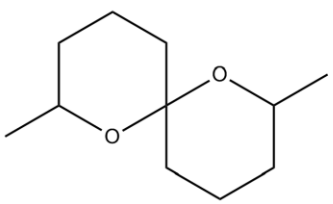
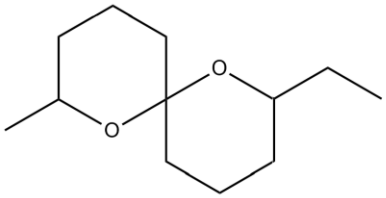


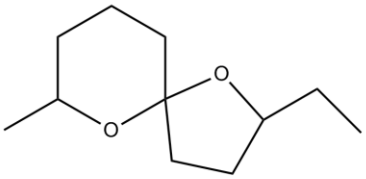
Figure 3.4. Mass spectrum for unknown spiroacetal present in chemical profiles of *B. kraussi.*



2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane
(**13**)



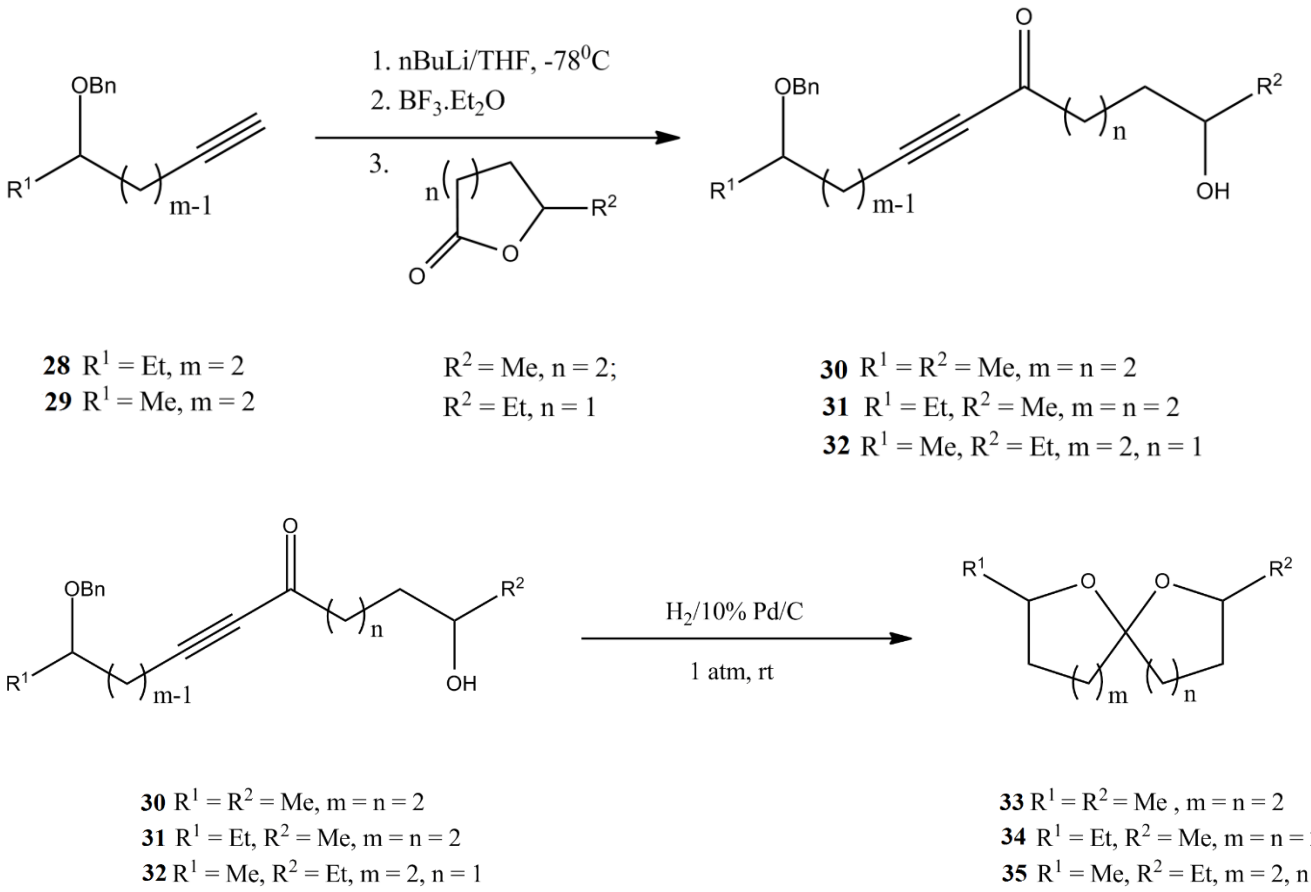
2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane
(**14**)



2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane
(**15**)

Figure 3.5. Structures for spiroacetals of interest.

Commercially available alkynols were first benzyl protected following the procedure outlined in Lindsay *et al.*[114] This procedure has not been used to synthesise the benzyl ethers in the current study, 2-benzyloxypent-4-yne (**28**) and 3-benzyloxyhex-5-yne (**29**), however it has been used to synthesise a range of benzyl ethers. The alkynols were first deprotonated using sodium hydride (NaH), followed by nucleophilic substitution with benzyl bromide (BnBr), in the presence of the catalyst tetrabutylammonium iodide (TBAI). This yielded the desired benzyl ethers in yields of approximately 70%. The reaction scheme is shown in Figure 3.7. Spectral data was found to be consistent with literature values for **29**,[115] whilst experimental spectral data was consistent with that expected for **28**, based on chemical shifts, peak splitting patterns and chemical integrations. The ^{1}H and ^{13}C NMR spectra for **28** are shown in Appendix E.



28 R^1 = Et, m = 2
29 R^1 = Me, m = 2

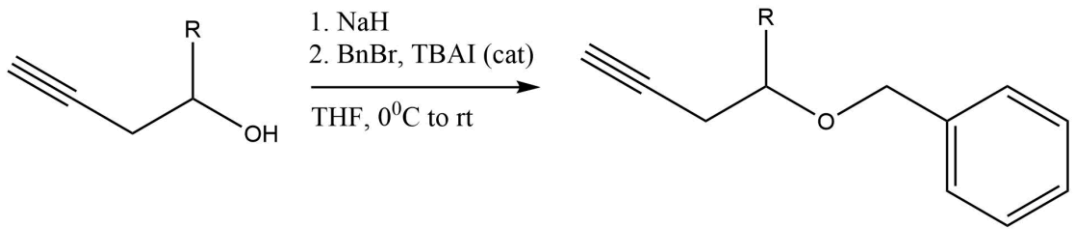
R^2 = Me, n = 2;
R^2 = Et, n = 1

30 $R^1 = R^2$ = Me, m = n = 2
31 R^1 = Et, R^2 = Me, m = n = 2
32 R^1 = Me, R^2 = Et, m = 2, n = 1

30 $R^1 = R^2$ = Me, m = n = 2
31 R^1 = Et, R^2 = Me, m = n = 2
32 R^1 = Me, R^2 = Et, m = 2, n = 1

33 $R^1 = R^2$ = Me , m = n = 2
34 R^1 = Et, R^2 = Me, m = n = 2
35 R^1 = Me, R^2 = Et, m = 2, n = 1

Figure 3.6. Reaction scheme for synthesis of spiroacetals.



28 R = CH_2CH_3, 70%
29 R = CH_3, 74%

Figure 3.7. Reaction scheme for benzyl protection of alkynols.

The first step of spiroacetal synthesis *via* the method outlined in Doubský *et al.*[113] involves the synthesis of alkynyltrifluoroborates *via* the reaction between stoichiometric quantities of the benzyl ether, *n*-butyllithium (*n*-BuLi) and boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2O$). The lactone is then added, which undergoes regioselective C-O ring cleavage, yielding the α-alkynone intermediate. The theorised mechanism for this reaction is shown in Figure 3.8. Formation of the intermediate was proposed to be characterised by a reduction in the R_f value on thin layer chromatography (TLC) when compared to the benzyl ether, due to the higher polarity of the product, and a possible colour change after the addition of *n*-BuLi, indicating the acetylide ion formation. Loss of the triplet associated with

the terminal alkyne hydrogen in the ^{1}H NMR spectrum would confirm the desired intermediate was formed.

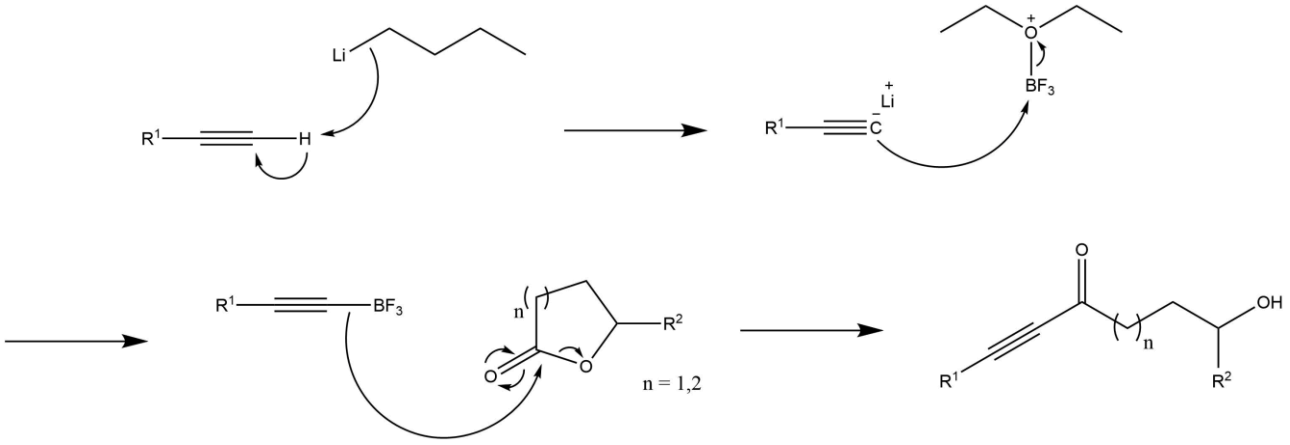


Figure 3.8. Theorised mechanism for α-alkynone synthesis.

Synthesis attempts using the two benzyl ethers (**28** and **29**) and δ-hexalactone proved quite difficult, with initial attempts only yielding recovered starting material upon workup. No observable colour change was seen after addition of *n*-BuLi, which suggested that *n*-BuLi was being quenched, possibly due to the presence of traces of water. As drying of THF with a sodium still is discouraged within Macquarie's research laboratories, a new bottle of THF from Sigma-Aldrich with a water content < 0.002% was used, subsequent to drying over a high loading of molecular sieves for at least 24 hours before use. This drying procedure has been shown in the literature to be sufficient in drying THF; a 20% w/v loading of 3Å molecular sieves in THF has been shown to reduce water content to approximately 15 ppm after 24 hours, and approximately 6 ppm after 48 hours.[116] Thus, water in the THF is unlikely to have been the cause of the lack of reaction. The reaction glassware was also oven dried overnight and flame dried under a constant stream of argon gas. Thus, wet glassware was also unlikely to be the cause. *n*-BuLi (1.6 M in hexanes) was titrated in order to determine the true concentration. This was determined to be 1.4 M. As such, it was concluded that the *n*-BuLi was active and was not the cause of the problem with this reaction. NMR analysis of 3-benzyloxyhex-5-yne (**2b**) revealed a significant water peak at 1.57 ppm, and so it was theorised that water present in **2b** may be quenching the reaction. **2b** was further dried under vacuum and stored in a desiccator. Repeating the reaction with this dried sample did not cause an improvement. No large water peak was seen with 2-benzyloxypent-4-yne (**2a**).

A structurally similar α-alkynone has been successfully synthesised by a colleague previously using this method[117] (Figure 3.9), and so to determine what might be preventing the synthesis undertaken by the candidate, this synthesis reaction was also performed. Whilst the compound synthesised is not one of interest in the current study, this synthesis served as a trouble-shooting exercise, and was

anticipated to help in identifying the problems related to this procedure. This reaction was carried out on the same scale used in previous attempts (0.50 g scale), but with a different benzyl ether, 3-benzyloxybut-1-yne. Upon addition of *n*-BuLi to the benzyl ether in dry THF, a yellow colour was observed. However, carrying through the reaction did not yield any α-alkynone. Two peaks were seen on GC-MS, with masses of 114 and 160, which corresponds to the molecular weight of the lactone (δ-hexalactone) and benzyl ether respectively. NMR analysis showed that the terminal alkyne peak was still present, indicating that the reaction did not proceed. This benzyl ether was very pure, as seen by GC-MS and NMR, with no water peak present, so it is difficult to say what the cause for the failed reaction is. The procedure outlined in Doubský *et al.*[113] used a larger amount of benzyl ether (8.00 mmol), whilst the reaction carried out by a colleague used a 1.00 g scale (approximately 6.00 mmol). It may be that the 0.5 g scale (approximately 3.00 mmol) used was too small, and somehow affected the success of the reaction, but this is unlikely. It is also possible that this reaction is very water sensitive, and very small amounts of water were sufficient in quenching the reaction. Given time constraints, and the small amount of starting benzyl ether left, investigating this reaction further was left for future studies.

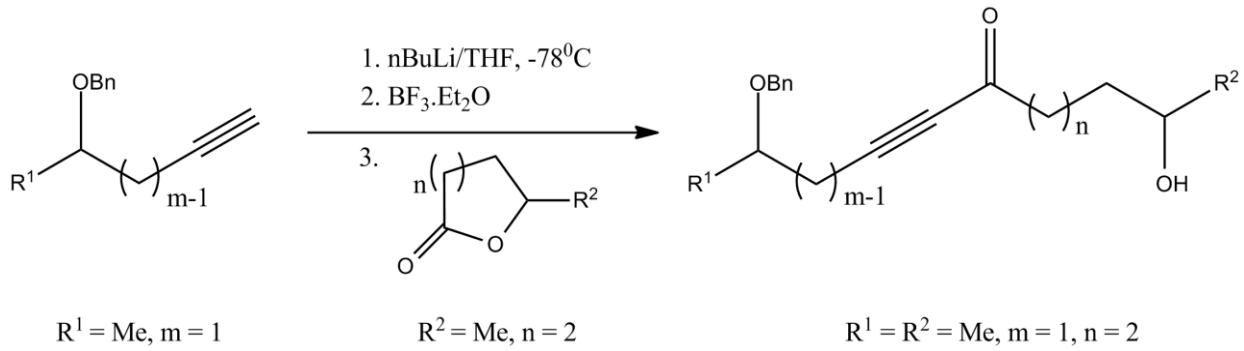


Figure 3.9. α-Alkynone synthesis previously undertaken by a colleague.

Alternative synthesis procedures could be investigated in future studies. It would be particularly of interest to investigate additional syntheses employing lactones and alkynols, given that they have now been acquired. A procedure published by Phillips *et al.*[118] is similar to the approach taken here, involving the reaction between a lithiated protected alkynol and a lactone, which after workup yields the spiroacetal. Three different methods for protecting the alkynol were investigated in this project (Figure 3.10). This procedure has only been used to synthesise 1,6-dioxaspiro[4.4]nonanes[118] and 1,6-dioxaspiro[4.5]decanes,[118-120] and yields were typically low to moderate. A procedure published by Cahill *et al.*[121] synthesised **13** and **14** starting from a lactone and a protected furanyl alcohol, with the furanyl group directing the stereochemistry of the spiroacetal. The procedure involved 9 steps, and gave moderate yields of 56% for **13** (Figure 3.11) and 54% for **14**.

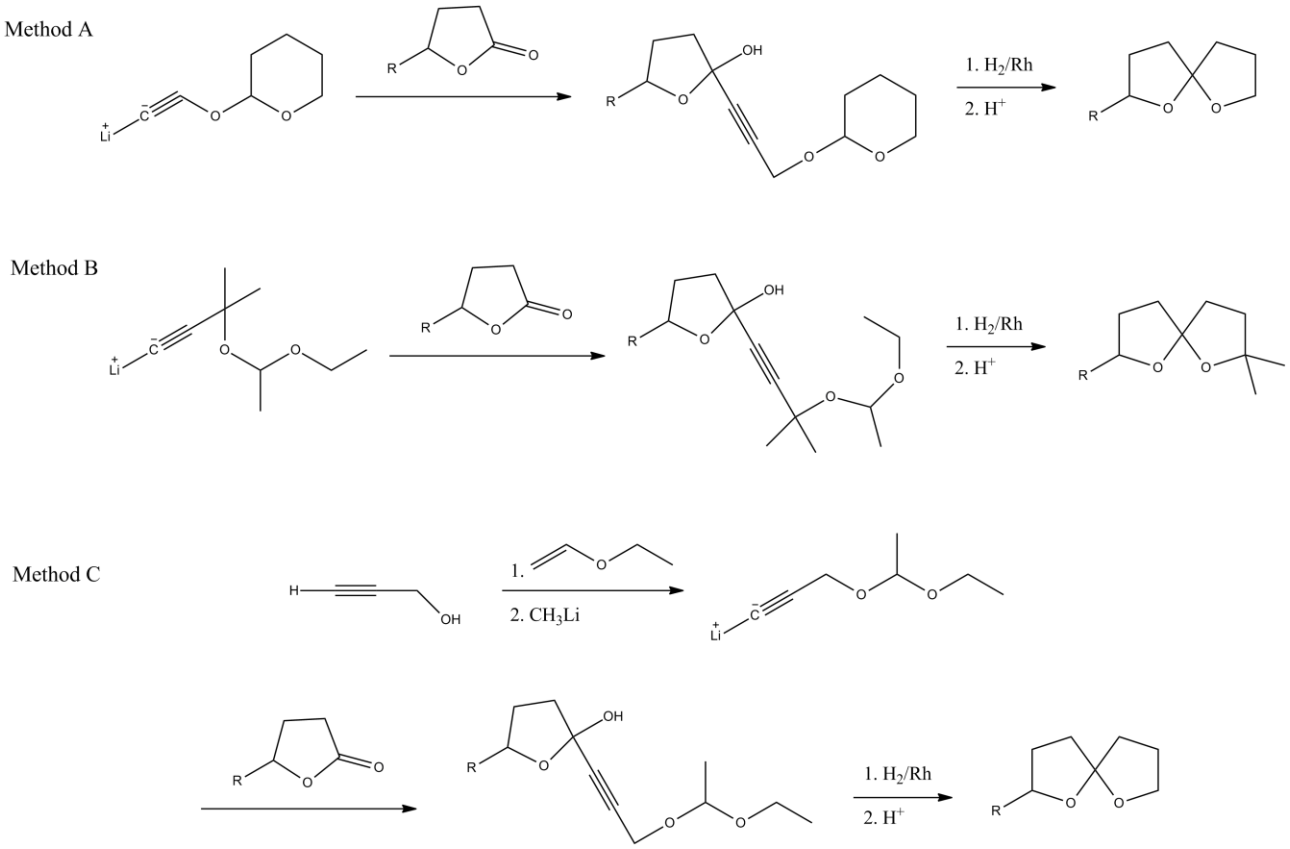


Figure 3.10. Reaction scheme for spiroacetal synthesis *via* Phillips *et al.*[118] Method A used a tetrohydropyran ether, method B a 1-ethoxyethyether, and method C involved the *in situ* generation of the 1-ethoxyethyether prior to methyllithium addition.

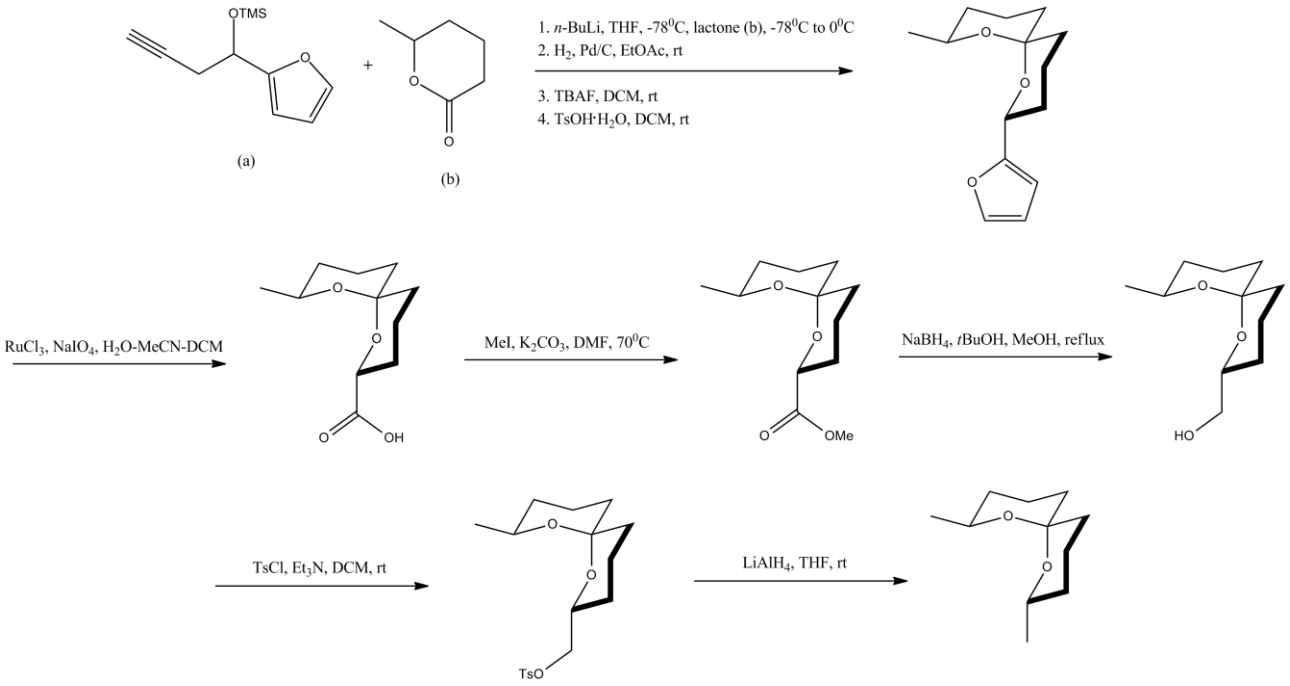


Figure 3.11. Reaction scheme for synthesis of **13** *via* Cahill *et al.*[121]

It would also be of interest to investigate previous syntheses of the spiroacetals investigated in the current work in future studies. All three spiroacetals of interest (**13**, **14** and **15**) have been synthesised *via* oxymercuration of a dienone (Figure 3.12), with reported yields ranging from moderate to high.[37,122] **13** and **15** have also be synthesised *via* metalated hydrazone intermediates[123] (Figure 3.13). The spiroacetals were isolated in yields of 71% and 92% respectively, with a mixture of the (*E*,*E*) and (*E*,*Z*) isomers obtained for **13**. The (*E*,*E*) isomer of **14** has been synthesised in 8 steps starting from (*S*)-(+)-lactate,[65] with an overall yield of 2.3%, and in 5 steps starting from (+)-1-nitro-2-propanol,[124] with an overall yield of 15.1% (Figure 3.14).

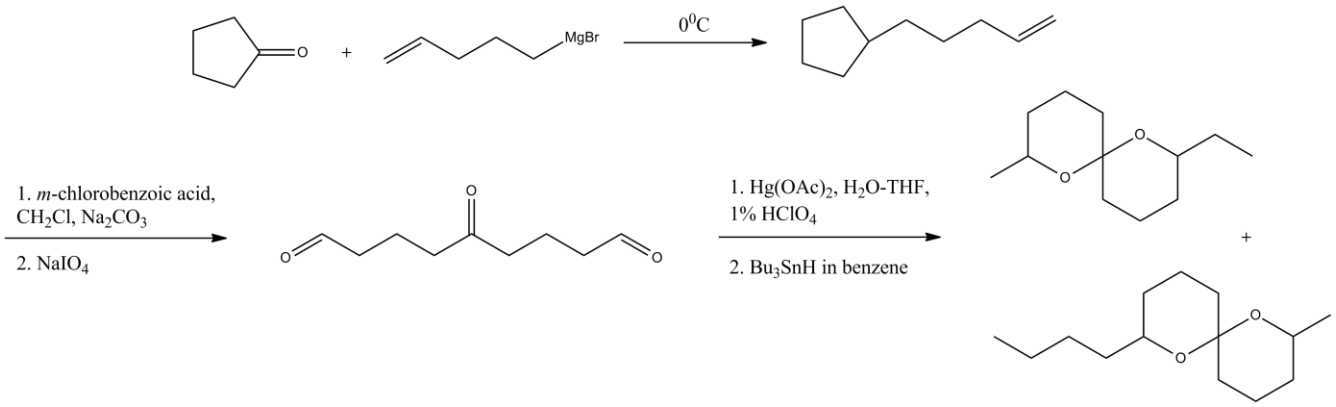


Figure 3.12. Synthesis of some spiroacetals *via* Kitching *et al.*[122]

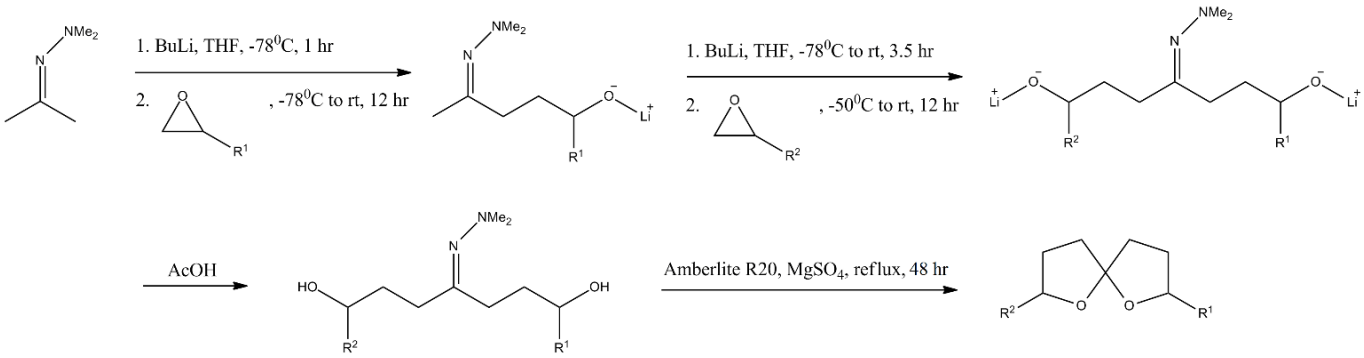


Figure 3.13. Synthesis of spiroacetals *via* Enders *et al.*[123]

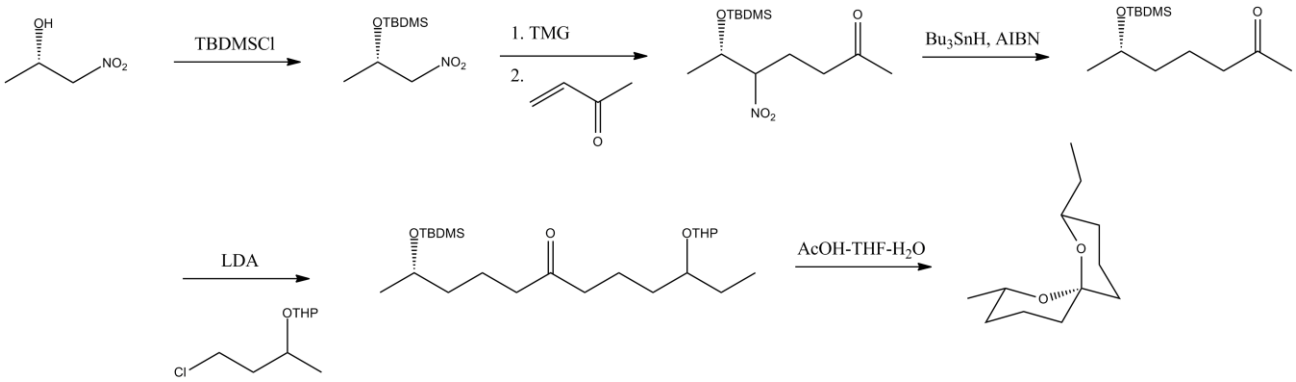


Figure 3.14. Synthesis of (*E*,*E*)-**14** *via* Kitayama.[124]

3.2.4 6-Oxononan-1-ol

6-Oxononan-1-ol was tentatively identified as present in the rectal gland extracts of male *B. kraussi* based on mass spectral fragmentation. As it was not commercially available, it was proposed to be synthesised *via* a procedure adapted from Singh *et al.*[125] This method was chosen due to its simplicity, moderate yields, and the availability of starting materials. The procedure involves the conjugate addition of 3-bromo-1-propanol and 1-hexen-3-one, catalysed by Zn(Cu) generated *in situ*. The reaction scheme is shown in Figure 3.15.

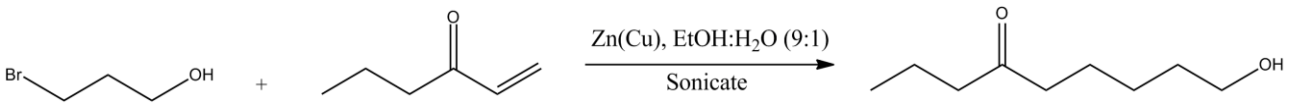


Figure 3.15. Reaction scheme for synthesis of **18**.

The reaction was monitored *via* GC-MS, and quenched once desired product formation (as determined by the retention time and molecular ion) ceased. GC-MS showed multiple peaks, including side products and unreacted starting material. For primary alkyl halide compounds, studies have shown their low reactivity in coupling reactions, leading to low yields and the generation of many side products,[126,127] as was the case in this synthesis.

Whilst yields may be small, these coupling reactions are known for their ease in product purification.[126,127] For this reaction, however, purification proved difficult. The crude mixture was purified twice by normal phase silica gel chromatography, eluting with a 0-10% gradient of ethyl acetate in *n*-hexane, as reported by Singh *et al.*[125] Very little product was obtained after the second elution (< 30 mg), however the desired compound could be identified, through similarity of retention times and mass spectral fragmentation. The desired product was not pure as evidenced by multiple peaks on the GC trace. Given that this compound is suspected to be an intermediate in the biosynthesis of a major pheromone component of *B. kraussi*,[59] it is unlikely that this compound would elicit a response from the fruit flies. As such, further attempts at synthesis and isolation were not undertaken, and the compound was tentatively identified as present in the male *B. kraussi* rectal gland extract

profiles based on matching to the literature mass spectra. Future isolation attempts could employ preparative GC to further purify the crude product.

Alternative synthesis procedures for this compound, including methods from Mitsudome *et al.*[128] (Figure 3.16) or Perkins *et al.*[77] (Figure 3.17) could also be investigated. The former method uses a Wacker oxidation process to oxidise internal olefins to carbonyl compounds, catalysed by $PdCl_2$/dimethylacetamide. Oxidation of 6-nonen-1-ol yielded a mixture of **18**, in 45% yield, and 7-oxononan-1-ol, in 47% yield. The two compounds were not separated. The latter method uses a multi-step reaction, starting with the synthesis of 1-propylcylcohexene *via* a Grignard reaction. This is then converted to 6-oxononan-1-al, followed by reduction to give 6-oxononan-1-ol (yield not reported).

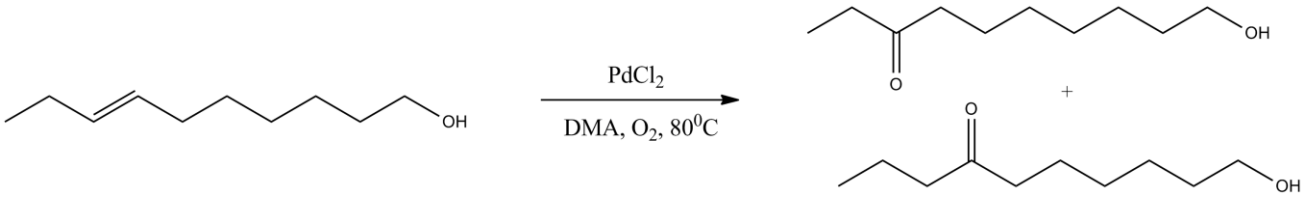


Figure 3.16. Reaction scheme for synthesis of **18** *via* Mistudome *et al.*[128]

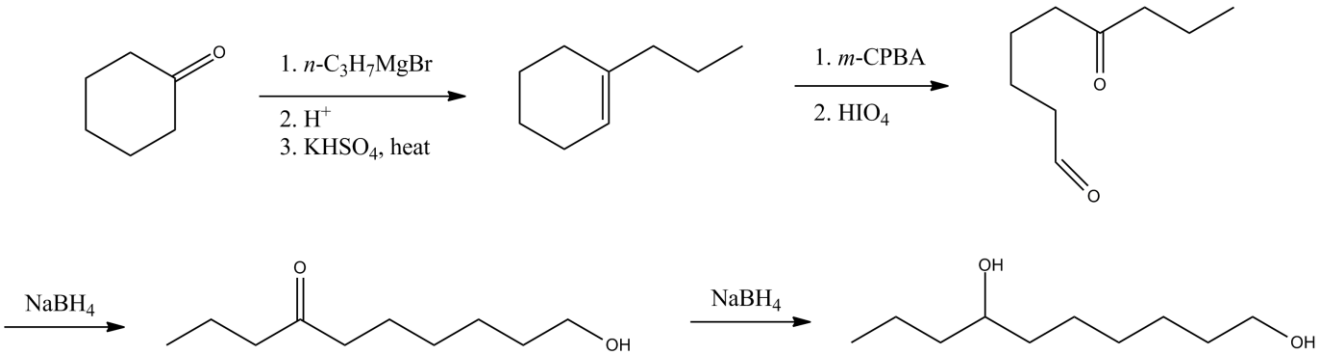


Figure 3.17. Reaction scheme for synthesis of **18** and 1,6-nonanediol *via* Perkins *et al.*[77]

3.2.5 Unknown Compounds

For all chemical profiles, it was found that there were a number of minor compounds present that were difficult to identify due to incomplete spectra. EI mass spectra generally includes intense fragment ion peaks and less intense molecular ion peaks.[67,129] The measurement of the amount of ions generates the spectra, which will often only include the most intense fragment ions. Weak ions will often not be seen, which can cause incomplete spectra. Spectral libraries will often only have a subset of the peaks for a given compound, hence incomplete spectra can yield matches to many different compounds, all with comparable similarity percentages. An example of this is shown in Table 3.1. Table 3.1 shows the library spectral matches for one mass spectrum (Figure 3.18) searched through

the spectral databases. As can be seen, the top ten spectral database matches are all compounds with a higher molecular weight than the largest fragment seen (except for diisoamylene).

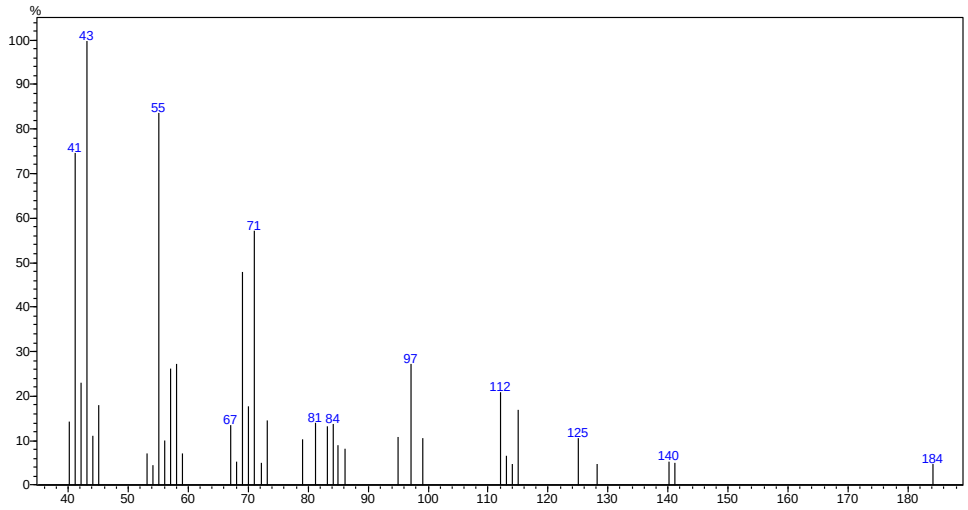


Figure 3.18. Mass spectrum of unknown compound from male *B. kraussi* searched through spectral databases, yielding results in Table 2.

Compound	Similarity (%)	Molecular Weight	Library
3,7-Dimethyloctan-1-ol	83	158	NIST21
2-Trifluoroacetoxytetradecane	82	310	NIST107
2-Trifluoroacetoxytridecane	82	296	NIST107
Diisoamylene	82	140	NIST107
[(Tetradecyloxy)methyl]oxirane	82	270	NIST107
Eicosan-1-ol	81	298	NIST107
3-Heptafluorobutyroxypentadecane	81	424	NIST107
2-Heptafluorobutyroxypentadecane	81	424	NIST21
6,10-Dimethylundecan-4-ol	81	200	NIST107
Tetracosan-1-ol	81	354	NIST107

Table 3.1. Top ten spectral database matches to searched mass spectrum (Figure 3.18).

In some cases, mass spectra searched through the spectral databases yielded the highest similarity results to compounds with lower molecular weights than the unknown compound. Searching the mass spectrum in Figure 3.19, for example, yielded the results shown in Table 3.2. As can be seen, the top ten spectral database matches are for compounds with lower molecular weights than the largest fragment ion seen (except for 2-decanyl propanoate).

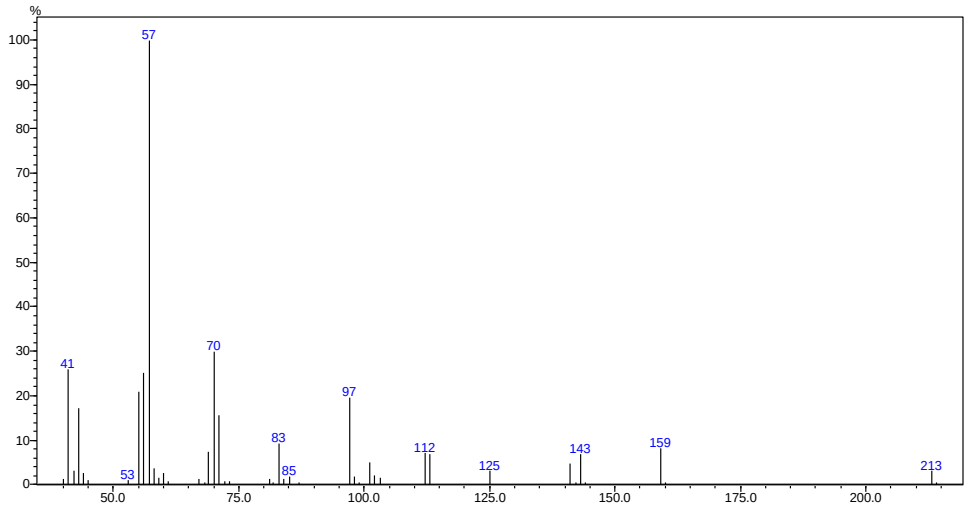


Figure 3.19. Mass spectrum of unknown compound from female *B. kraussi* searched through spectral databases, yielding results in Table 3.

Compound	Similarity (%)	Molecular Weight	Library
2-Ethylhexan-1-ol	86	130	NIST21
[[(2-Ethylhexyl)oxy)methyl]oxirane	84	186	NIST107
2-Ethylhexan-1-ol	84	130	NIST21
2-Propylpentan-1-ol	84	130	NIST107
2,6,7-Trimethyldecane	83	184	NIST107
2-Ethylhexan-1-ol	83	130	NIST107
2,4,4-Trimethylpentan-1-ol	83	130	NIST21
2-Decanyl propanoate	83	214	NIST107
5,5-Dimethylhex-1-ene	83	112	NIST107
(E)- 6-Methylundec-3-ene	83	168	NIST107

Table 3.2. Top ten spectral database matches to searched mass spectrum (Figure 3.19).

In other cases, very few fragment ions were seen. When very few fragment ions are seen, the mass spectrum generated can be matched to a wide variety of possible compounds, causing extreme difficulty in compound identification. When very few fragment ions are seen, the fragments can be easily matched to a very large set of mass spectra, as structurally similar compounds are likely to have similar fragment ions. Figure 3.20 shows an incomplete mass spectrum showing very few fragment ions.

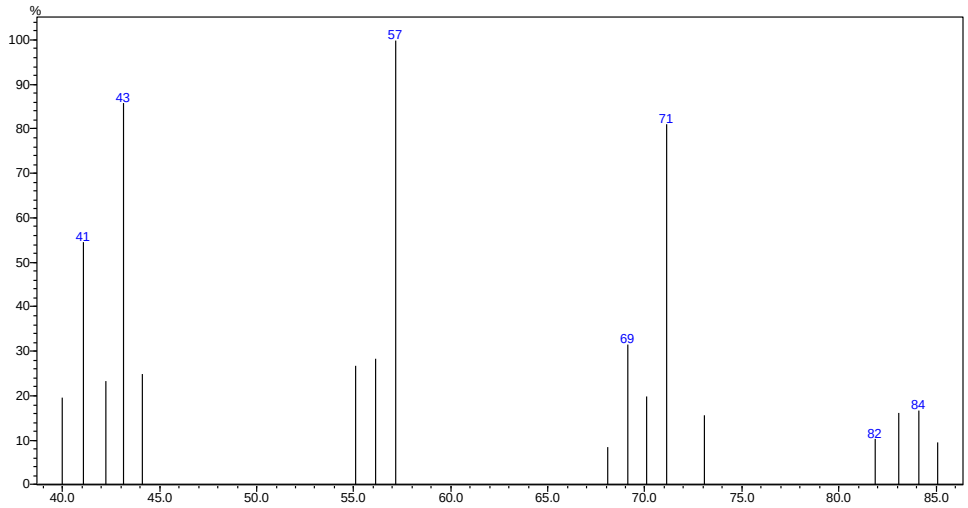


Figure 3.20. Incomplete mass spectrum from female *B. musae* showing very few fragment ions.

As can be seen from the examples above, there was much difficulty in matching incomplete spectra against a spectral database, especially in cases where the parent ion was not seen or very few fragment ions were seen. Isolation of these compounds from rectal gland extracts would likely be needed for identification, such as through preparative GC. Alternatively, chemical ionisation could be used in order to identify the parent ion, which when paired with EI mass spectra, and detailed analysis of fragmentation patterns, should aid in compound identification. Additional identification methods, including GC-infrared spectroscopy (IR) and NMR (as examples) could also be employed. Given the short time period of this project, further investigations into identifying minor components was left for future studies.

Chapter 4: Conclusions and Future Directions

Many fruit fly species are significant horticultural pests in Australia, and with the recent restrictions on the use of organophosphate insecticides, there is a strong need for the development of new lures for fruit fly monitoring and control. Sex pheromones serve as an interesting class of compounds to study for this purpose, given that *Bactrocera* are known to produce and release sex pheromones to attract the opposite sex. In this study, the chemical profiles of males and females of three *Bactrocera* pest species, *B. frauenfeldi, B. musae* and *B. kraussi,* were investigated.

Chemical profiles were determined by analysis of both rectal gland extracts and headspace collections. Compounds were identified *via* GC-MS, and were then confirmed by comparison of retention time and mass spectral fragmentation to authentic samples. Given the simplicity of the compounds identified, most were purchased, while synthesis of more complicated compounds, including long-chain esters and spiroacetals, was undertaken.

In total, 27 compounds were preliminarily identified in the chemical profiles, across males and females of all three species. Of these, 17 were confirmed by comparison to authentic samples, seven compounds were found not to be present, and three compounds (the spiroacetals) were tentatively identified. Overall, three major chemical groups were found to be present; esters, spiroacetals, and acetamides. In the rectal gland extracts, it was found that males typically produced one major component, usually a spiroacetal, along with additional compounds present in their chemical profile. In contrast, females produced a wider range of compounds, including acetamides, spiroacetals and esters, with the latter being the major components. The only exception was with males of *B. musae*, who were found to produce a range of compounds in their chemical profile, predominantly esters. This was only observed in rectal gland extracts from 2014. Fewer compounds were detected from headspace collections, and in both males and females, only the most abundant chemicals detected in the rectal gland extracts were also detected in the headspace collections. No additional compounds were detected. The results presented in this thesis also serve as the starting point in determining the complete chemical profiles, and determining the sex pheromone compounds, for *B. frauenfeldi*, *B. musae* and *B. kraussi.*

Future research should look at the biological testing of the compounds identified as present in the chemical profiles, to determine their attractant properties, and therefore presence as sex pheromones. Possible biological testing methods could include electroantennography (EAG), and cage and field trials. EAG involves placing the antenna of an insect between two electrodes. As a chemical stimulus is introduced to the antenna, and if there are receptors present to detect the stimulus, depolarisation of receptor neurons will occur, which is detected and recorded as a change in electrical potential.

Using EAG, multiple compounds can be rapidly screened in a shorter period of time and fewer flies are required, when compared with cage or field bioassays. The main disadvantage is that EAG does not provide information in regards to the behavioural response of a fruit fly to a compound; whilst a receptor to detect the compound may be present, this does not mean that the fruit fly will behaviourally respond, which is essential for an effective lure. Using EAG, compounds found to elicit responses can be further tested with cage and/or field trials to determine the biological response of the fruit fly to each compound. Furthermore, cage and field trials using mixtures of compounds, in different ratios, can also be tested, in order to identify optimal blends. This would allow for the identification of pheromones, and the evaluation of these compounds as potential fruit fly attractants for use with MAT, as female lures, or biosecurity and population monitoring.

Future research should also investigate elucidation of the components not yet fully identified from the rectal gland extracts. This could include methods such as preparative GC, as well as further syntheses. Peaks suspected to be hydrocarbons should be investigated further, possibly through alternative ionisation methods (*e.g.* chemical ionisation). This will allow for the complete chemical profiles of *B. frauenfeldi*, *B. kraussi*, and *B. musae* to be known. Furthermore, the chemical profiles for other important fruit fly species should be investigated using similar approaches undertaken in this project.

Chapter 5: Experimental Methods

5.1 Compound collection

Gland extractions*:* *n*-Hexane and ethanol extracts were obtained by the candidate using sexually mature males and females (13-25 days old) supplied from the Queensland Government Department of Agriculture, Fisheries and Forestry (Cairns) (collected May 2016), following literature procedures.[37] Flies were chilled on dry ice to kill, then dissected as follows; the abdomen was gently squeezed with tweezers such that the glands protruded slightly. Glands were gently pulled out with tweezers, and the secretory sac separated. Once 20 glands were collected, compounds were extracted into 200 μL of the desired solvent, by saturating the glands with solvent and leaving to stand for 10 min. Samples were stored at – 4°C. *n*-Hexane extracts for males and females of all species (collected September 2014) were supplied by the Behavioural Biology Research Group (within Macquarie University's Department of Biological Sciences).

Headspace collections*:* For headspace collections, 30 sexually mature males or females (13-25 days old) were placed into an air chamber, and charcoal-filtered air (flow rate of 1 L per min, air pulling system) was passed over the flies for a period of 2 hours, beginning 30 min before calling. Released volatiles were adsorbed onto traps made of Tenax adsorbent (Scientific Instrument Services, Inc, Tenax-GR Mesh 60/80) packed into Pasteur-like cartridges. Volatiles were extracted into 1 mL of solvent (*n*-hexane and ethanol). Samples were stored at – 4°C. The headspace apparatus used in this project is shown in Figure 5.1.

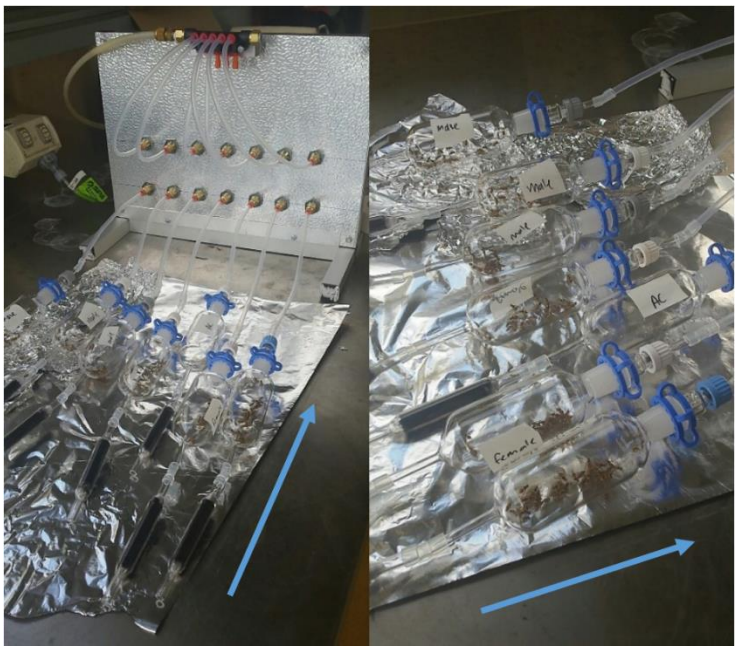


Figure 5.1. Headspace collection apparatus used in this study. Arrows indicate the direction of air flow.

5.2 GC-MS analysis of rectal gland extracts and headspace collections

Low resolution mass spectra were recorded on a Shimadzu GCMS-QP2010 instrument, using a capillary column with 35% diphenyl / 65% dimethyl polysiloxane as the stationary phase (30 m x 0.25 mm I.D. x 0.25 μm film thickness) and helium as a carrier gas. The temperature program was 50°C (4 min) to 250°C (6 min) at 10°C/min, with an injector temperature of 270°C. Mass spectra were recorded in EI mode (70 keV), scanning from 40 to 620 *m/z*. Mass spectra were analysed using the LabSolutions GCMSsolution software version 2.40.

5.3 General Procedures for Synthesis

All reagents were used as received from commercial suppliers. Benzyl bromide (reagent grade, 98%), $BF_3{\cdot}Et_2O$ (for synthesis), *n*-BuLi (1.6M in hexanes), 5-hexyn-3-ol (97%), 4-pentyn-2-ol (≥ 98%), δ-hexalactone (≥ 98%), γ-hexalactone (≥ 98%), 3-bromo-1-propanol (97%), copper (I) iodide (purum, ≥ 99.5%), and zinc (dust, < 10 μm, ≥ 98%) were purchased from Sigma Aldrich. 1-Hexen-3-one (90+%, stab. with 0.5% 4-methoxyphenol) was purchased from Alfa Aeser. All solvents were anhydrous or of ACS grade and used without further purification. THF was dried over 4Å molecular sieves. *n*-BuLi was titrated using the procedure outlined in Kofron and Baclawski.[130] Water and air sensitive reactions were carried out under an argon atmosphere using flame dried glassware.

Reaction progress was monitored through GC-MS (Shimadzu GCMS-QP2010 instrument, using the same procedure listed in Section 5.2) or with TLC using Merck TLC silica gel 60 F_{254} on aluminium sheets (0.2 mm) and visualised with ultraviolet light at 254 nm.

Flash column chromatography was performed using a Biotage Isolera Four over normal phase Merck 60 silica gel (40-60 μm) packed in a Biotage cartridge. Solvents were removed under reduced pressure using a Büchi Rotavapor R-200 equipped with a Büchi Vacuum Controller V-800, Büchi Recirculating Chiller F-105 set to a temperature of – 10°C, and Büchi B-490 heating bath set to a temperature of 40°C. Mixtures were further dried under high vacuum using an Alcatel Pascal 2005SD vacuum pump equipped with a Vacuubrand DCP 3000 + VSP 3000 vacuum gauge set.

NMR spectra were recorded on either a Bruker AVANCE-400 instrument (^{1}H NMR: 400 MHz, ^{13}C NMR: 101 MHz) or a Bruker AVANCE-600 instrument equipped with a cryoprobe (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) using $CDCl_3$. The ^{1}H chemical shifts were referenced to the residual protonated solvent peaks at δH 7.26 for chloroform-d. ^{13}C chemical shifts were referenced to the central solvent peaks of bulk solvent at δC 77.16 for chloroform-d. *J* values are given in Hz. The following abbreviations are used to describe the NMR data – singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), and multiplet (m).

5.4 Synthesis

5.4.1 General Method for Synthesis of Esters (8, 10, 11, 21-27)

To alcohol (10 mL, 134 equiv.) was added carboxylic acid (1.0 g, 1.0 equiv.) and concentrated sulfuric acid (2-3 drops). The solution was heated at reflux, and reaction progress was monitored *via* GC-MS. After cooling, the crude product was separated between diethyl ether (10 mL) and sodium bicarbonate (10 mL, 5% *w/v*). The organic layer was washed with sodium bicarbonate (3 x 10 mL), and dried over Na_2SO_4. Solvent was removed under reduced pressure, yielding the crude product. The crude product was purified by distillation to yield the pure ester.

Propyl laurate (**8**): reaction mixture was refluxed for 1.5 hours. Colourless liquid, 440 mg, 1.8 mmol, 38% overall yield. ^{1}H NMR (600 MHz, $CDCl_3$): 0.87 (3H, t, *J* = 7.0 Hz, $CH_2C\mathbf{H_3}$), 0.93 (3H, t, *J* = 7.4 Hz, $OCH_2CH_2C\mathbf{H_3}$), 1.25-1.29 (16H, m, $C\mathbf{H_2}$), 1.57-1.66 (4H, m, $C\mathbf{H_2}CH_2COOPr$, $CH_3C\mathbf{H_2}CH_2OCO$), 2.29 (2H, t, *J* = 7.5 Hz, $C\mathbf{H_2}COOPr$), 4.02 (2H, t, *J* = 6.7 Hz, $C\mathbf{H_2}OCO$) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): 10.5 (CH_3), 14.2 (CH_3), 22.1 (CH_2), 22.8 (CH_2), 25.1 (CH_2), 29.3 (CH_2), 29.40 (CH_2), 29.47 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 32.0 (CH_2), 34.5 (CH_2), 65.9 (OCH_2), 174.1 (C=O) ppm. GC-MS (EI) *m/z* (% of base peak): 242 ($M^{\cdot+}$, 4.3), 213 (1.4), 201 ($M^{\cdot+}$ - $CH_2CH_2CH_3$, 27.5), 183 ($M^{\cdot+}$ - $OCH_2CH_2CH_3$, 25.7), 171 (6.6), 157 (6.8), 143 (3.3), 129 (8.7), 115 (21.8), 102 (32.5), 97 (7.7), 85 (12.2), 73 (39.3), 61 (100), 57 (30.9), 43 (80.2). Experimental spectra were consistent with literature data.[103]

Ethyl palmitoleate (**10**): reaction mixture was refluxed for 1.5 hours. Synthesised half scale. Colourless liquid, 100 mg, 0.35 mmol, 19% overall yield. ^{1}H NMR (400 MHz, $CDCl_3$): 0.88 (3H, t, *J* = 6.9 Hz, $CH_2C\mathbf{H_3}$), 1.23-1.30 (19H, m, $C\mathbf{H_2}$), 1.59-1.63 (2H, m, $C\mathbf{H_2}CH_2COOEt$), 1.98-2.01 (4H, m, $C\mathbf{H_2}CH{=}CHC\mathbf{H_2}$), 2.28 (2H, t, *J* = 7.5 Hz, $C\mathbf{H_2}COOEt$), 4.11 (2H, q, *J* = 7.2 Hz, $OC\mathbf{H_2}CH_3$), 5.32-5.35 (2H, m, C**H**=C**H**) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): 14.2 (CH_3), 14.4 (CH_3), 22.8 (CH_2), 25.1 (CH_2), 27.30 (CH_2), 27.36 (CH_2), 29.1 (CH_2), 29.23 (CH_2), 29.26 (CH_2), 29.3 (CH_2), 29.82 (CH_2), 29.87 (CH_2), 31.9 (CH_2), 34.5 (CH_2), 60.2 (OCH_2), 129.9 (CH), 130.1 (CH), 174.0 (C=O) ppm. GC-MS (EI) *m/z* (% of base peak): 282 ($M^{\cdot+}$, 3.8), 236 ($M^{\cdot+}$ - OCH_2CH_3, 14.3), 218 (1.4), 207 (1.4), 194 ($M^{\cdot+}$ - $CH_2COOCH_2CH_3$, 15.0), 179 (1.65), 165 (2.8), 152 ($M^{\cdot+}$ - $(CH_2)_4COOCH_2CH_3$, 14.9), 138 (6.9), 123 (11.9), 101 (31.8), 88 (50.6), 83 (44.9), 69 (64.1), 55 (100), 41 (81.8). Although no literature spectral data were available, experimental spectral data were consistent with that expected for the title compound, based on chemical shifts in NMR and fragmentation patterns in GC-MS.

Ethyl elaidate (**11**): reaction mixture was refluxed for 1.5 hours. Synthesised half scale. Colourless liquid, 110 mg, 0.40 mmol, 23% overall yield. ^{1}H NMR (400 MHz, $CDCl_3$): 0.87 (3H, t, *J* = 6.7 Hz, $CH_2CH_2C\mathbf{H_3}$), 1.23-1.28 (23H, m, CH_2), 1.57-1.60 (2H, m, $C\mathbf{H_2}CH_2COOEt$), 1.95-1.96 (4H, m,

C**H₂**CH=CHC**H₂**), 2.27 (2H, t, *J* = 7.6 Hz, C**H₂**COOEt), 4.11 (2H, q, *J* = 7.1 Hz, OC**H₂**CH_3), 5.36-5.28 (2H, m, C**H**=C**H**) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): 14.2 (CH_3), 14.4 (CH_3), 22.8 (CH_2), 25.1 (CH_2), 29.1 (CH_2), 29.30 (CH_2), 29.36 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 29.8 (CH_2), 29.8 (CH_2), 32.0 (CH_2), 32.73 (CH_2), 32.78 (CH_2), 34.5 (CH_2), 60.3 (OCH_2), 130.4 (CH), 130.6 (CH), 174.0 (C=O) ppm. GC-MS (EI) *m/z* (% of base peak): 310 ($M^{\cdot+}$, 3.5), 281 ($M^{\cdot+}$ - CH_2CH_3, 0.25), 264 ($M^{\cdot+}$ - OCH_2CH_3, 16.2), 222 (11.3), 180 (11.2), 155 (7.0), 138 (5.6), 123 (13.5), 111 (20.6), 97 (38.6), 88 (45.6), 83 (49.0), 69 (69.0), 55 (100), 41 (76.4). Experimental spectra were consistent with literature data.[107]

Isopropyl caprylate (**21**): reaction mixture was refluxed for 3 hours. Colourless liquid, 7.8 mg, 0.042 mmol, 0.52% overall yield. ^{1}H NMR (400 MHz, $CDCl_3$): 0.87 (3H, t, *J* = 6.8 Hz, CH_2C**H₃**), 1.22 (6H, d, *J* = 6.3 Hz, OCH(C**H₃**)₂), 1.27-1.29 (8H, m, C**H₂**), 1.58-1.62 (2H, m, C**H₂**CH_2COO*i*Pr), 2.25 (2H, t, *J* = 7.5 Hz, C**H₂**COO*i*Pr), 4.9-5.0 (1H, m, OC**H**$(CH_3)_2$) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): 14.2 (CH_3), 21.9 (OCH(**C**H_3)₂), 22.7 (CH_2), 25.1 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 31.8 (CH_2), 34.8 (CH_2), 67.4 (OCH), 173.6 (C=O) ppm. GC-MS (EI) *m/z* (% of base peak): 187 ($M^{\cdot+}$, 0.5), 171 (1.0), 145 ($M^{\cdot+}$ - $CH(CH_3)_2$, 18.2), 127 ($M^{\cdot+}$ - $OCH(CH_3)_2$, 32.9), 115 (4.6), 102 (33.2), 97 (3.7), 84 (16.4), 73 (25.5), 60 (66.2), 57 (54.0), 43 (100). Experimental spectra were consistent with literature data.[98,99]

Propyl caprylate (**22**): reaction mixture was refluxed for 1.5 hours. Colourless liquid, 190 mg, 1.0 mmol, 14% overall yield. ^{1}H NMR (600 MHz, $CDCl_3$): 0.87 (3H, t, *J* = 7.0 Hz, CH_2C**H₃**), 0.93 (3H, t, *J* = 7.4 Hz, OCH_2CH_2C**H₃**), 1.26-1.30 (8H, m, C**H₂**), 1.56-1.66 (4H, m, C**H₂**CH_2COOPr, CH_3C**H₂**CH_2OCO), 2.28 (2H, t, *J* = 7.6 Hz, C**H₂**COOPr) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): 10.5 (CH_3), 14.2 (CH_3), 22.2 (CH_2), 22.8 (CH_2), 25.1 (CH_2), 29.3 (CH_2), 29.40 (CH_2), 29.47 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 32.0 (CH_2), 34.5 (CH_2), 65.9 (OCH_2), 174.1 (C=O) ppm. GC-MS (EI) *m/z* (% of base peak): 187 ($M^{\cdot+}$, 1.0), 157 (1.3), 145 ($M^{\cdot+}$ - $CH_2CH_2CH_3$, 43.3), 127 ($M^{\cdot+}$ - $OCH_2CH_2CH_3$, 58.3), 115 (11.3), 102 (26.5), 97 (5.0), 87 (13.1), 83 (10.5), 73 (34.1), 61 (100), 57 (62.5), 43 (79.9). Although no literature spectral data were available, experimental spectral data were consistent with that expected for the title compound, based on chemical shifts in NMR and fragmentation patterns in GC-MS.

Isopropyl laurate (**23**): reaction mixture was refluxed for 3.5 hours. White, waxy solid, 340 mg, 1.4 mmol, 29% overall yield, mp 175-185°C (no lit). ^{1}H NMR (400 MHz, $CDCl_3$): 0.87 (3H, t, *J* = 6.8 Hz, CH_2C**H₃**), 1.22 (6H, d, *J* = 6.2 Hz, OCH(C**H₃**)₂), 1.25-1.28 (16H, m, C**H₂**), 1.58-1.62 (2H, m, C**H₂**CH_2COO*i*Pr), 2.25 (2H, t, *J* = 7.6 Hz, C**H₂**COO*i*Pr), 4.97-5.03 (1H, m, OC**H**$(CH_3)_2$) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): 14.5 (CH_3), 22.2 (CH_2), 23.1 (CH_2), 25.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 32.3 (CH_2), 35.1 (CH_2), 67.7 (OCH), 173.8 (C=O) ppm. GC-MS (EI) *m/z* (% of base peak): 242 ($M^{\cdot+}$, 0.9), 200 ($M^{\cdot+}$ - $CH(CH_3)_2$, 26.8), 183 ($M^{\cdot+}$ - $OCH(CH_3)_2$, 17.8),

171 (3.1), 157 (8.4), 143 (3.6), 129 (10.1), 115 (7.0), 102 (51.0), 97 (10.3), 85 (16.8), 73 (28.1), 60 (70.0), 57 (43.3), 43 (100). Experimental spectra were consistent with literature data.[101,102]

Ethyl nonadecanoate (**24**): reaction mixture was refluxed for 1.5 hours. Crude product deemed to be sufficiently pure (≥ 99% by GC-MS and NMR). Synthesised half scale. White, waxy solid, 92 mg, 0.28 mmol, 17% overall yield, mp 36-37°C (lit. 35°C[131], 37-38°C[95]). ^{1}H NMR (400 MHz, $CDCl_3$): 0.87 (3H, t, *J* = 7.0 Hz, $CH_2C\mathbf{H_3}$), 1.23-1.27 (33H, m, $C\mathbf{H_2}$), 1.57-1.62 (2H, m, $C\mathbf{H_2}CH_2COOEt$), 2.28 (2H, t, *J* = 7.7 Hz, $C\mathbf{H_2}COOEt$), 4.12 (2H, q, *J* = 7.1 Hz, $OC\mathbf{H_2}CH_3$) ppm. Not seen: $OCH_2C\mathbf{H_3}$. ^{13}C NMR (101 MHz, $CDCl_3$): 14.2 (CH_3), 14.4 (CH_3), 22.8 (CH_2), 25.1 (CH_2), 29.30 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 29.8 (CH_2), 32.0 (CH_2), 34.5 (CH_2), 48.9 (CH_2), 62.5 (OCH_2) ppm. Not seen: C=O. GC-MS (EI) *m/z* (% of base peak): 326 ($M^{\cdot+}$, 9.6), 297 (1.0), 283 ($M^{\cdot+}$ - $CH_2CH_2CH_3$, 5.8), 269 (2.4), 255 (1.7), 241 (2.6), 227 (4.0), 213 (4.1), 199 (2.8), 185 (1.9), 157 (13.6), 143 (4.9), 129 (2.5), 115 (5.9), 101 (53.5), 88 ($M^{\cdot+}$ - $(CH_2)_{16}CH_3$, 100), 70 (21.5), 55 (28.9), 43 (48.9). Experimental ^{1}H NMR spectra were consistent with literature data,[105] which also did not show OCH_2CH_3. No literature data available for ^{13}C NMR or GC-MS.

Ethyl behenate (**25**): Reaction mixture was refluxed for 1.5 hours. Crude product was purified by recrystallisation from ethyl acetate. White solid, 730 mg, 2.0 mmol, 74% overall yield, mp 49 °C (lit. 48-49 °C[95,131]). ^{1}H NMR (400 MHz, $CDCl_3$): 0.87 (3H, t, *J* = 6.8 Hz, $CH_2C\mathbf{H_3}$), 1.08 (3H, t, *J* = 6.9 Hz, $OCH_2C\mathbf{H_3}$), 1.22-1.26 (36H, m, $C\mathbf{H_2}$), 1.57-1.62 (2H, m, $C\mathbf{H_2}CH_2COOEt$), 2.27 (2H, t, *J* = 7.4 Hz, $C\mathbf{H_2}COOEt$), 4.11 (2H, q, *J* = 7.1 Hz, $OC\mathbf{H_2}CH_3$) ppm. Not seen: $C\mathbf{H_3}CH_2O$. ^{13}C NMR (101 MHz, $CDCl_3$): 14.4 (CH_3), 14.5 (CH_3), 18.6 (CH_2), 22.0 (CH_2), 23.0 (CH_2), 25.30 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 32.2 (CH_2), 34.7 (CH_2), 58.8 (OCH_2) ppm. Not seen: C=O. GC-MS (EI) *m/z* (% of base peak): 368 ($M^{\cdot+}$, 9.5), 339 (0.5), 325 (3.6), 311 (0.8), 297 (0.5), 283 ($M^{\cdot+}$ - $CH_2CH_2CH_3$, 1.1), 269 (2.4), 255 (1.2), 241 (0.7), 227 (1.3), 213 (3.3), 199 (1.5), 185 (0.8), 157 (10.8), 143 (5.7), 129 (2.1), 101 (46.7), 88 ($M^{\cdot+}$ - $(CH_2)_{19}CH_3$, 100), 69 (17.2), 57 (39.1), 43 (54.2). Experimental spectra were consistent with literature data.[106]

Isopropyl oleate (**26**): reaction mixture was refluxed for 2 hours. Pale yellow liquid, 260 mg, 0.79 mmol, 24% overall yield. ^{1}H NMR (400 MHz, $CDCl_3$): 0.87 (3H, t, $CH_2C\mathbf{H_3}$), 1.26-1.29 (18H, m, $C\mathbf{H_2}$), 1.58-1.61 (6H, m, $OCH(C\mathbf{H_3})_2$), 1.56-1.62 (4H, m, $C\mathbf{H_2}CH_2COO$*i*Pr, $C\mathbf{H_2}CH_2COO$*i*Pr), 1.97-2.01 (4H, m, $C\mathbf{H_2}CH{=}CHC\mathbf{H_2}$), 2.24 (2H, t, $C\mathbf{H_2}COO$*i*Pr), 4.96-5.02 (2H, m, $OC\mathbf{H}(CH_3)_2$), 5.29-5.38 (2H, m, $C\mathbf{H}{=}C\mathbf{H}$) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): 14.2 (CH_3), 21.9 (CH_2), 22.8 (CH_2), 25.1 (CH_2), 25.4 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 32.0 (CH_2), 34.8 (CH_2), 35.3 (CH_2), 67.4 ($O\mathbf{C}H(CH_3)_2$), 129.9 (CH), 130.1 (CH), 173.6 (C=O) ppm. GC-MS (EI) *m/z* (% of base peak): 324 ($M^{\cdot+}$, 4.4), 282 (2.2), 265 ($M^{\cdot+}$ - $OCH(CH_3)_2$, 20.6), 245 (3.5),

222 (5.5), 207 (2.7), 193 (3.2), 179 (5.0), 165 (6.2), 151 (6.0), 125 (11.6), 111 (28.8), 97 (47.0), 83 (63.5), 69 (73.2), 55 (91.6), 43 (100). Experimental spectra were consistent with literature data.[108,109]

Propyl oleate (**27**): reaction mixture was refluxed for 2 hours. Colourless liquid, 360 mg, 1.2 mmol, 31% overall yield. ^{1}H NMR (600 MHz, $CDCl_3$): 0.88 (3H, t, *J* = 6.9 Hz, $CH_2C\mathbf{H_3}$), 0.94 (3H, t, *J* = 7.4 Hz, $OCH_2CH_2C\mathbf{H_3}$), 1.27-1.33 (20H, m, C**H**$_2$), 1.59-1.68 (4H, m, C**H**$_2CH_2COOPr$), 1.99-2.03 (4H, m, C**H**$_2CH{=}CHC\mathbf{H_2}$), 2.28 (2H, t, *J* = 7.5 Hz, C**H**$_2COOPr$), 4.03 (2H, t, *J* = 6.6 Hz, $OC\mathbf{H_2}CH_2CH_3$), 5.31-5.37 (2H, m, C**H**=C**H**) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): 10.5 (CH_3), 14.2 (CH_3), 22.1 (CH_2), 22.8 (CH_2), 25.1 (CH_2), 27.30 (CH_2), 27.36 (CH_2), 29.24 (CH_2), 29.27 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 32.0 (CH_2), 34.5 (CH_2), 65.9 (OCH_2), 129.9 (CH), 130.1 (CH), 174.1 (C=O) ppm. Not seen: 1x CH_2. GC-MS (EI) *m/z* (% of base peak): 324 ($M^{\cdot+}$, 4.0), 283 (1.1), 264 ($M^{\cdot+}$ - $OCH_2CH_2CH_3$, 20.5), 246 (6.6), 222 (12.7), 207 (2.0), 193 (1.8), 180 (10.8), 169 (6.7), 152 (6.4), 123 (11.6), 111 (22.9), 97 (47.4), 83 (61.7), 69 (72.2), 55 (88.6), 43 (100). Experimental ^{13}C NMR spectra were consistent with literature data,[109] no literature data available for ^{1}H NMR or GC-MS.

5.4.2 General Method for Synthesis of Protected Alkynols (28-29)

In an oven dried round bottom flask, NaH (60% dispersion in mineral oil, 2.0 equiv.) was added, and the flask was capped with a septum and flushed with argon gas. Dry THF (30.0 mL) was added, and the resulting suspension cooled to 0 °C. Alkynol (2.0 g, 1.0 equiv.) was added dropwise, and the reaction mixture stirred for 40 min. TBAI (0.05 equiv.) was added in one portion, followed by dropwise addition of BnBr (1.1 equiv). The reaction mixture was stirred at room temperature overnight, cooled to 0°C, and quenched by slow addition of aqueous saturated NH_4Cl (30 mL). Crude product was extracted with diethyl ether (3 x 50 mL), and the combined organic layers were washed with saturated brine (3 x 20 mL), dried over Na_2SO_4, and solvent removed under reduced pressure. Crude product was purified by flash column chromatography (0-10% ethyl acetate in *n*-hexane), and dried under vacuum to yield the pure benzyl ether.

2-Benzyloxypent-4-yne (**28**): colourless liquid, 3.0 g, 17 mmol, 70% overall yield. ^{1}H NMR (600 MHz, $CDCl_3$): 1.32 (3H, d, *J* = 6.1 Hz, $CHC\mathbf{H_3}$), 2.02 (1H, t, **H**C≡C), 2.36-2.53 (2H, m, HC≡C-C**H**$_2$), 3.69-3.72 (1H, m, O-C**H**, 4.58 (2H, s, O-C**H**$_2$-Ar), 7.27-7.37 (5H, m, Ar**H**) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): 19.6 (CH_3), 26.1 (CH_2), 70.1 (CH_2-O), 70.8 (CH-O), 73.3 (CH), 81.3 (CH), 127.7 (ArC), 128.5 (ArC), 138.6 (ArC). Although no literature spectral data were available, experimental spectral data were consistent with that expected for the title compound, based on chemical shifts in NMR.

3-Benzyloxyhex-5-yne (**29**): colourless liquid, 2.8 g, 15 mmol, 74% overall yield. ^{1}H NMR (400 MHz, $CDCl_3$): 0.95 (3H, t, *J* = 7.4 Hz, $CH_2C\mathbf{H_3}$), 1.63-1.75 (2H, m, C**H**$_2CH_3$), 2.00 (1H, t, *J* = 2.6 Hz, **H**C≡C), 2.38-2.51 (2H, m, HC≡C-C**H**$_2$), 3.48-3.51 (1H, m, O-C**H**), 4.52-4.66 (2H, dd, *J* = 11.6 Hz,

O-C**H₂**-Ar), 7.27-7.38 (5H, m, Ar**H**) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): 9.6 (CH_3), 23.5 (CH_2), 26.7 (CH_2), 62.5 (CH_2-O), 69.9 (CHO), 71.4 (CH), 78.5 (CH), 127.7 (ArC), 127.8 (ArC), 128.4 (ArC). Experimental spectra were consistent with literature data for ^{1}H NMR,[115] no literature spectra available for ^{13}C NMR.

5.4.3 General Method for Synthesis of α-Alkynones (30-32)

To a solution of alkyne (0.5 g, 1.0 equiv) in dry THF (8.0 mL) at -78°C, *n*-BuLi (1.1 equiv.) was slowly added, maintained an argon atmosphere. The reaction mixture was stirred for 20 min, warmed to approximately -30°C, and held at this temperature for approximately 10 min. The reaction mixture was then cooled to -78°C, and $BF_3{\cdot}Et_2O$ (1.1 equiv.) was added dropwise. Stirring was continued for 10 min, and lactone (1.0 equiv.) was added in one portion *via* syringe. The resulting mixture was warmed to room temperature within 1 hour, and reaction progress was monitored *via* TLC (20% ethyl acetate in *n*-hexane). Upon completion, a solution of saturated NH_4Cl-NH_3 (60% aq) 2:1 (1.5 mL) was added, then the mixture was poured into water (16 mL), and extracted with diethyl ether (4 x 8 mL). The combined ethereal layers were washed with saturated brine (2 x 8 mL), dried over Na_2SO_4, and evaporated to yield to crude product. The crude material was purified by flash column chromatography (eluted with 0-10% ethyl acetate in *n*-hexane) to yield the alkynone.

5.4.4 Synthesis of 6-oxononan-1-ol (18)

In a round bottom flask, a mixture of ethanol-water (9:1, 24 mL), zinc dust (1.0 g, 16 mmol), copper(I) iodide (0.9 g, 4.8 mmol), 3-bromo-1-propanol (880 mg, 6.4 mmol) in ethanol (4 mL) and 1-hexen-3-one (620 mg, 6.4 mmol) in ethanol (4 mL) at 0°C were combined. The reaction mixture was sonicated for 7.5 hours, and reaction progress was monitored *via* GC-MS. The reaction mixture was then quenched with brine, filtered, and solvent removed under reduced pressure. The crude product was taken up in diethyl ether (50 mL), washed with water (2 x 20 mL), and brine (2 x 20 mL), and dried over Na_2SO_4. Solvent was removed under reduced pressure, yielding the crude product. The crude product was purified by flash column chromatography (eluted twice with 0-10% ethyl acetate in *n*-hexane). Very little product was obtained after purification, hence no pure compound was recovered.

GC-MS (EI) *m/z* (% of base peak): 158 ($M^{\cdot+}$, 1.1), 140 ($M^{\cdot+}$ - H_2O, 2.1), 115 (8.2), 112 (3.5), 99 (7.3), 97 (26.1), 86 (32.0), 79 (10.0), 73 (11.4), 71 (66.9), 69 (70.1), 58 (52.1), 55 (34.7), 43 (100), 41 (72.5). Experimental spectra were consistent with literature data.[77]

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Appendices

Appendix A: Representative GC-MS traces for rectal gland extracts (collected September 2014)

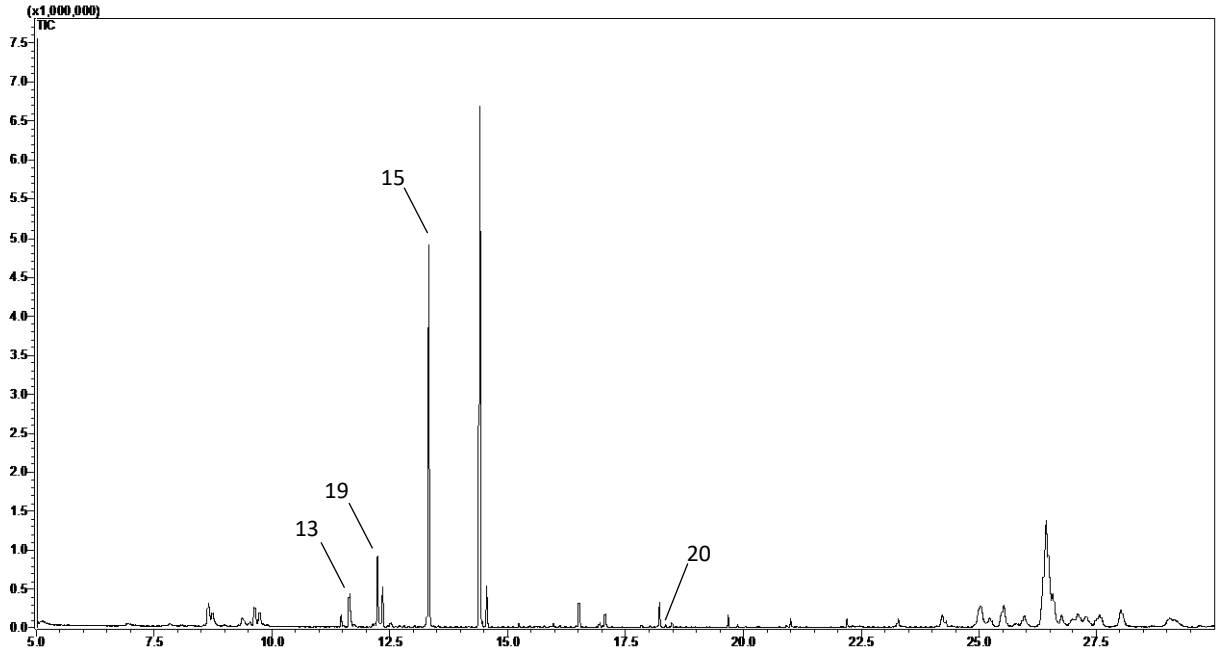
Appendix B: Representative GC-MS traces for rectal gland extracts (collected May 2016)

Appendix C: Representative GC-MS traces for headspace collections (collected May 2016)

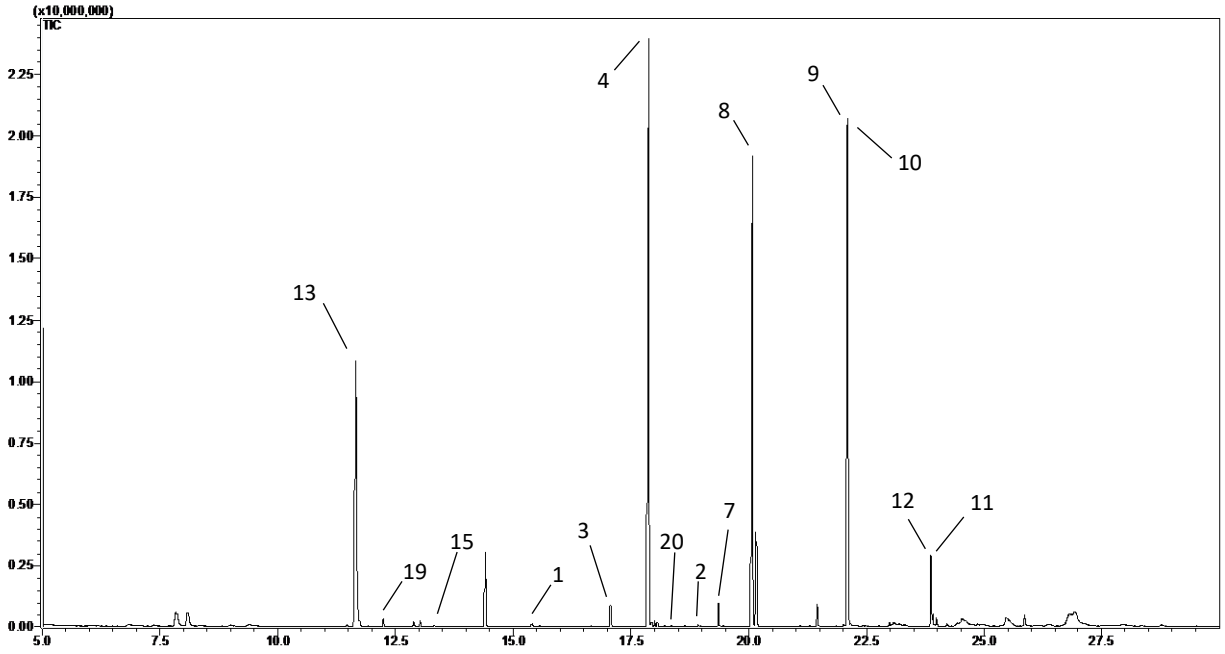
Appendix D: Representative GC-MS trace for headspace blanks (collected May 2016)

Appendix E: ^{1}H and ^{13}C NMR for 2-benzyloxypent-4-yne

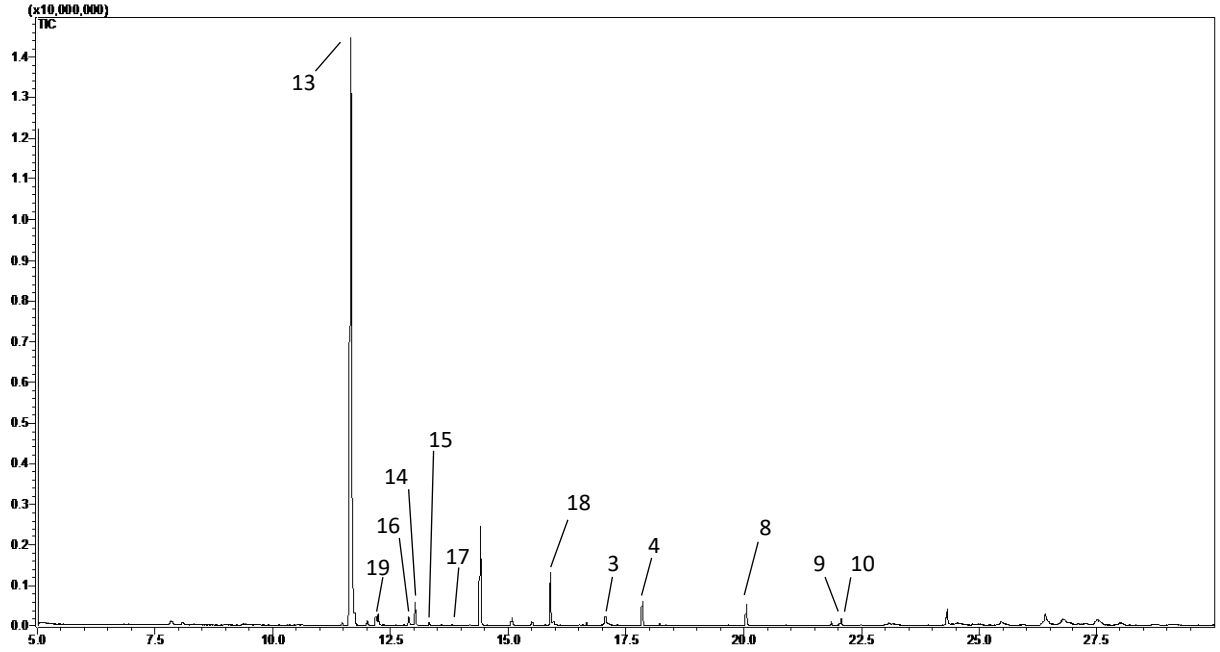
Appendix A: Representative GC-MS traces for rectal gland extracts (collected September 2014)



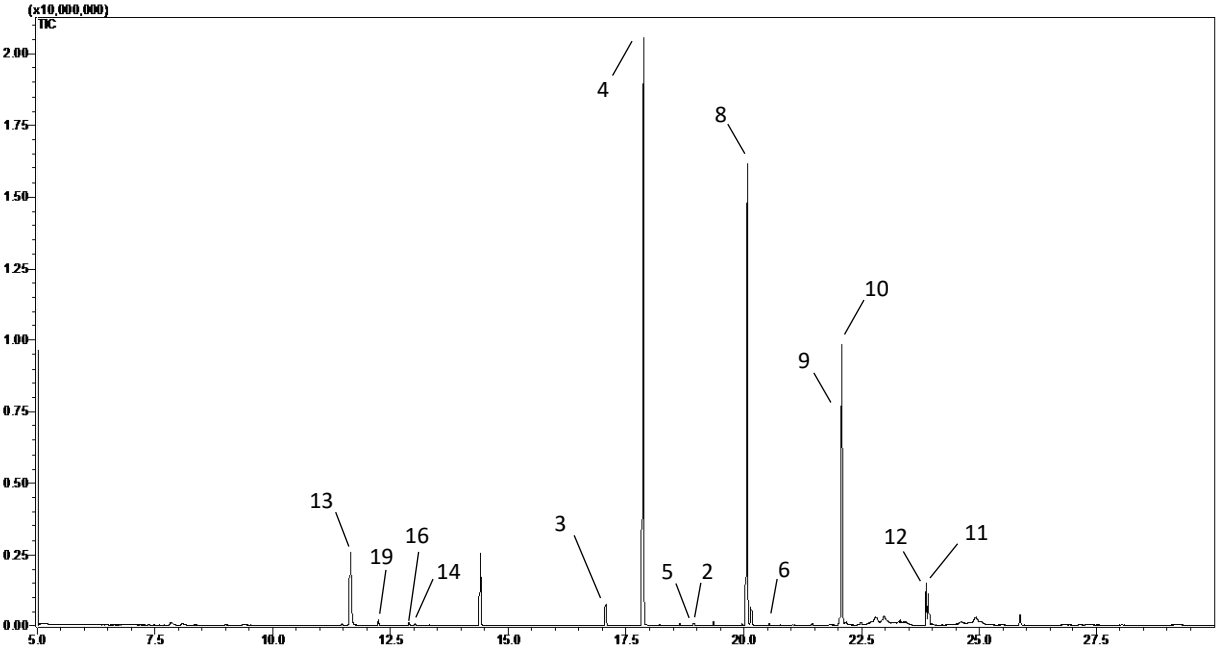
A1: Male *B. frauenfeldi*



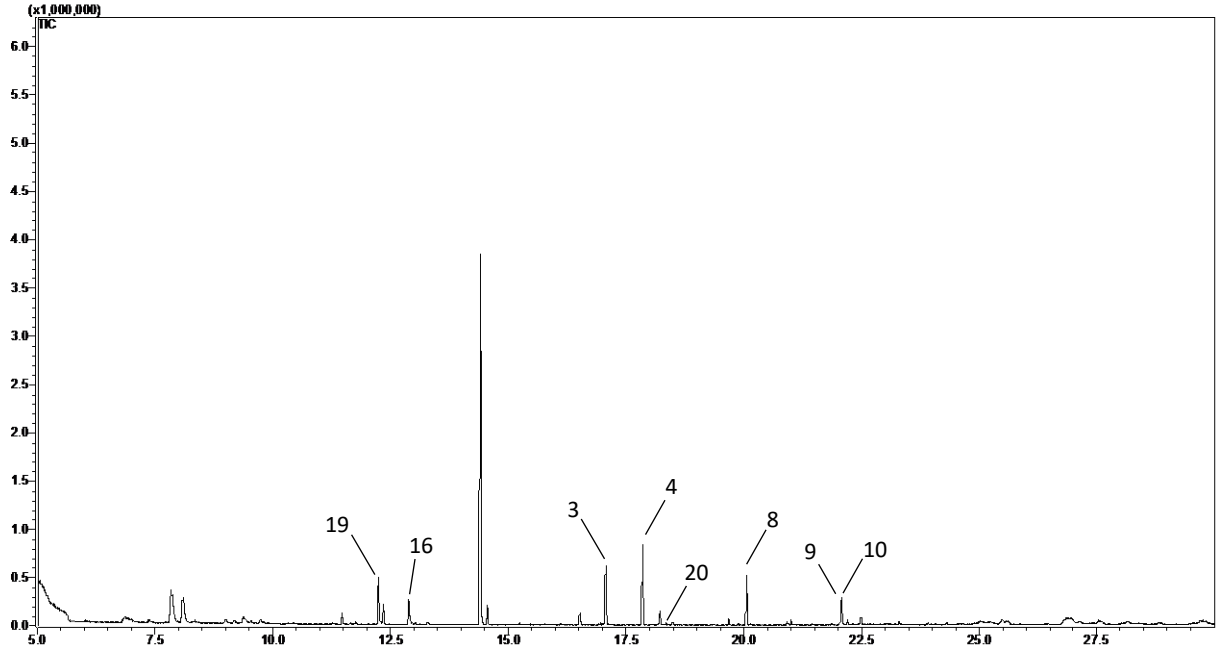
A2: Female *B. frauenfeldi*



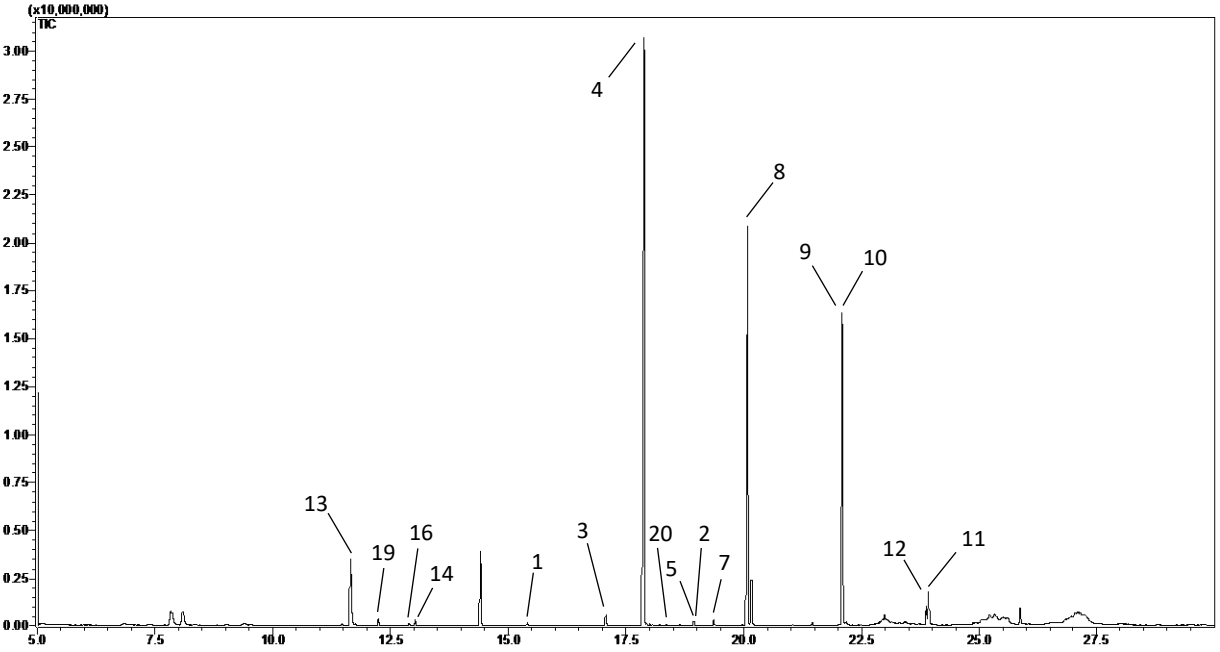
A3: Male *B. kraussi*



A4: Female *B. kraussi*

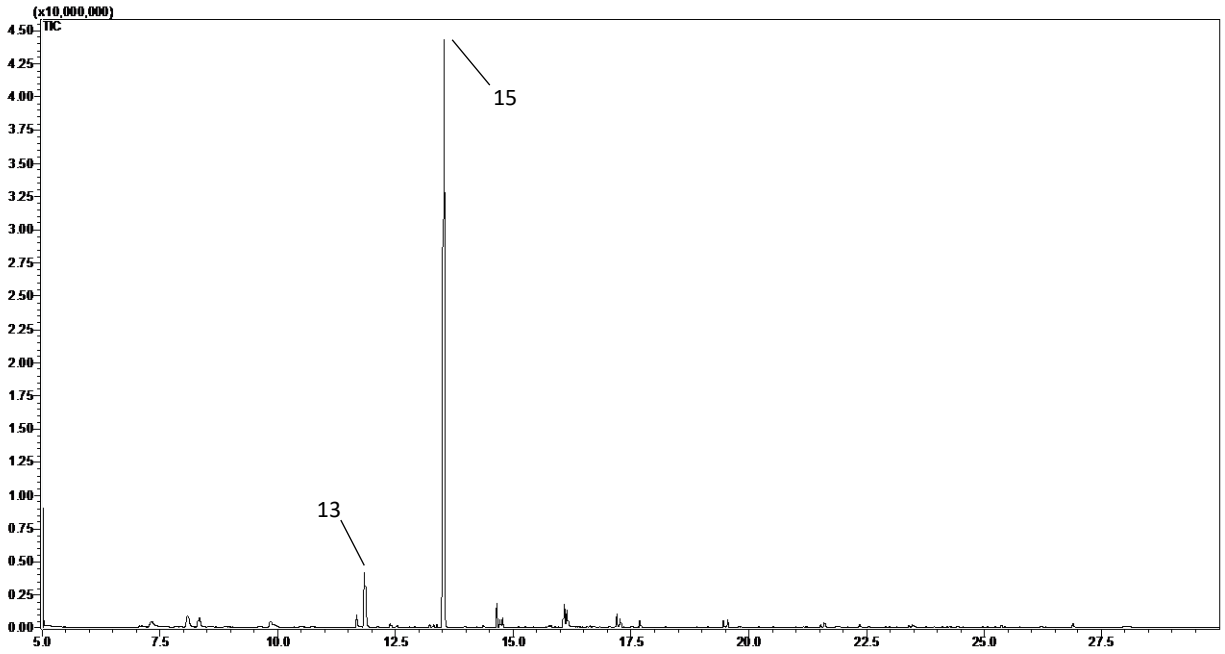


A5: Male *B. musae*

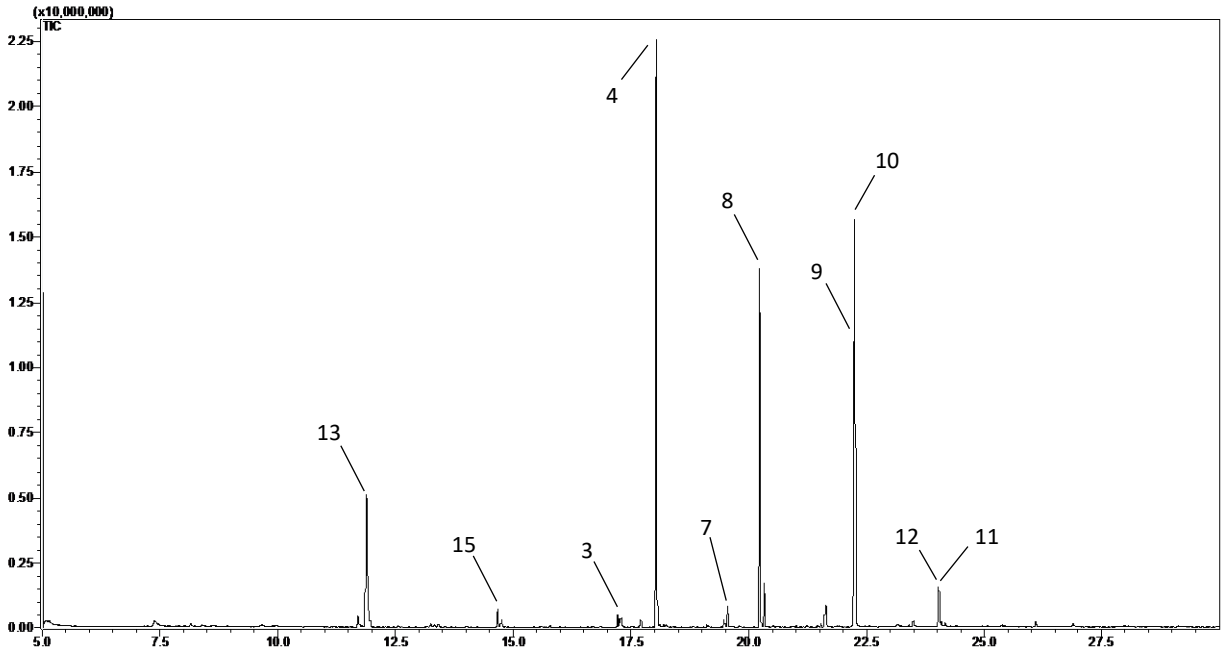


A6: Female *B. musae*

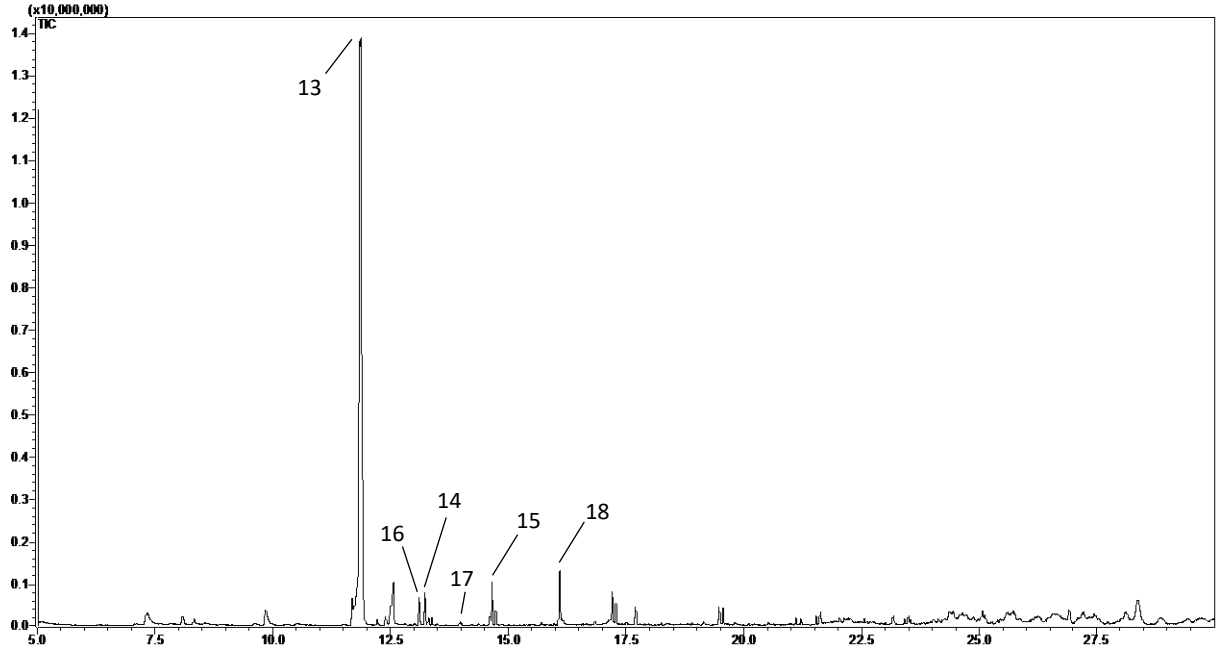
Appendix B: Representative GC-MS traces for rectal gland extracts (collected May 2016)



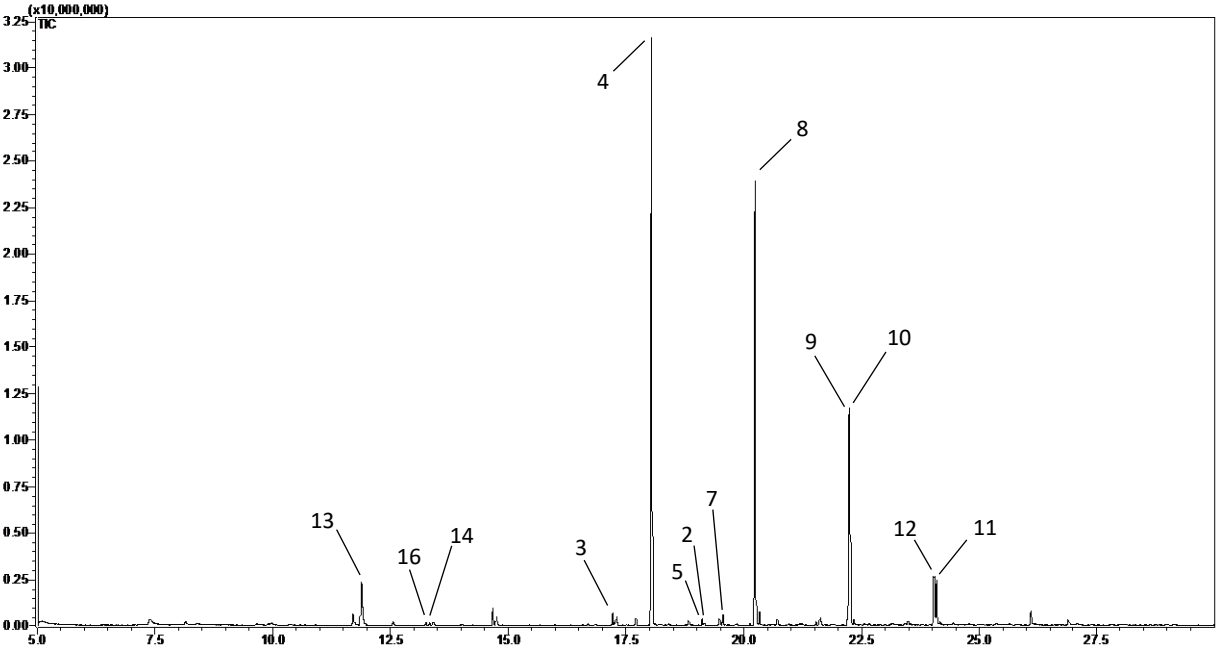
B1: Male *B. frauenfeldi*



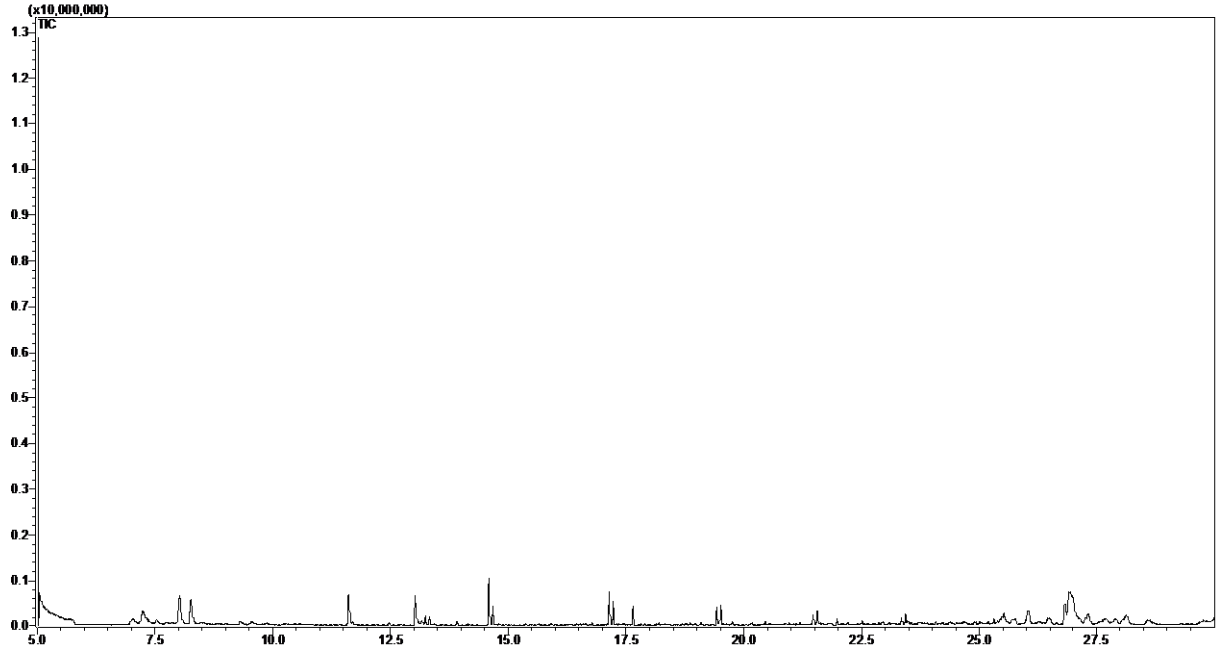
B2: Female *B. frauenfeldi*



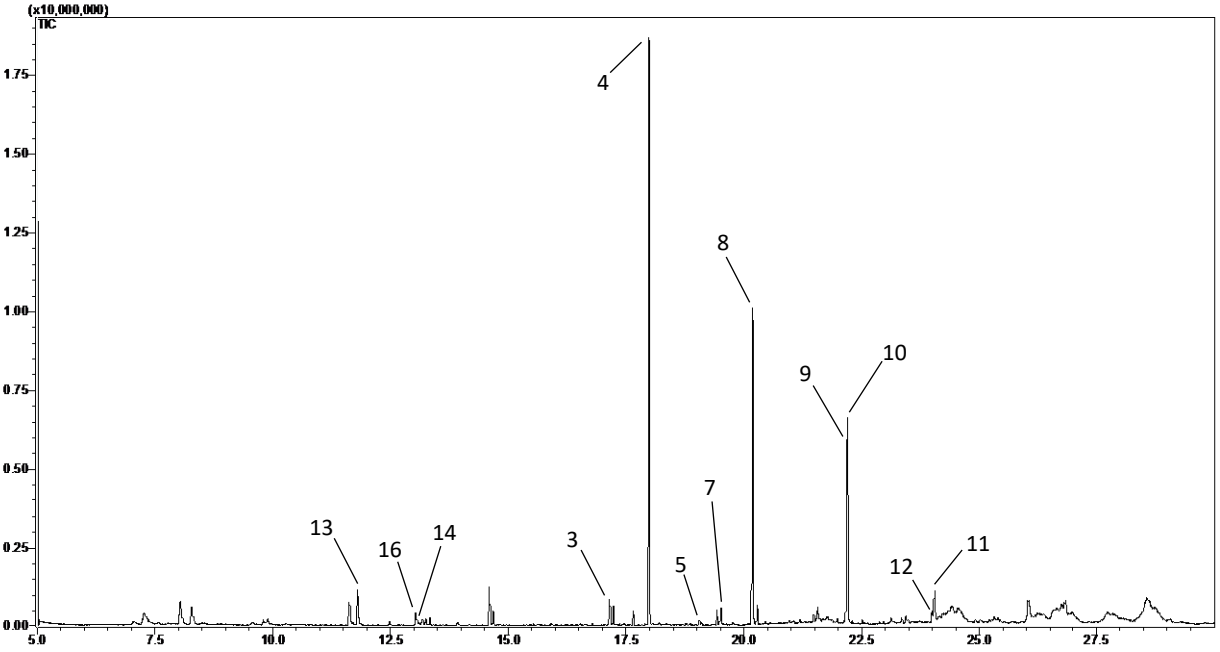
B3: Male *B. kraussi*



B4: Female *B. kraussi*

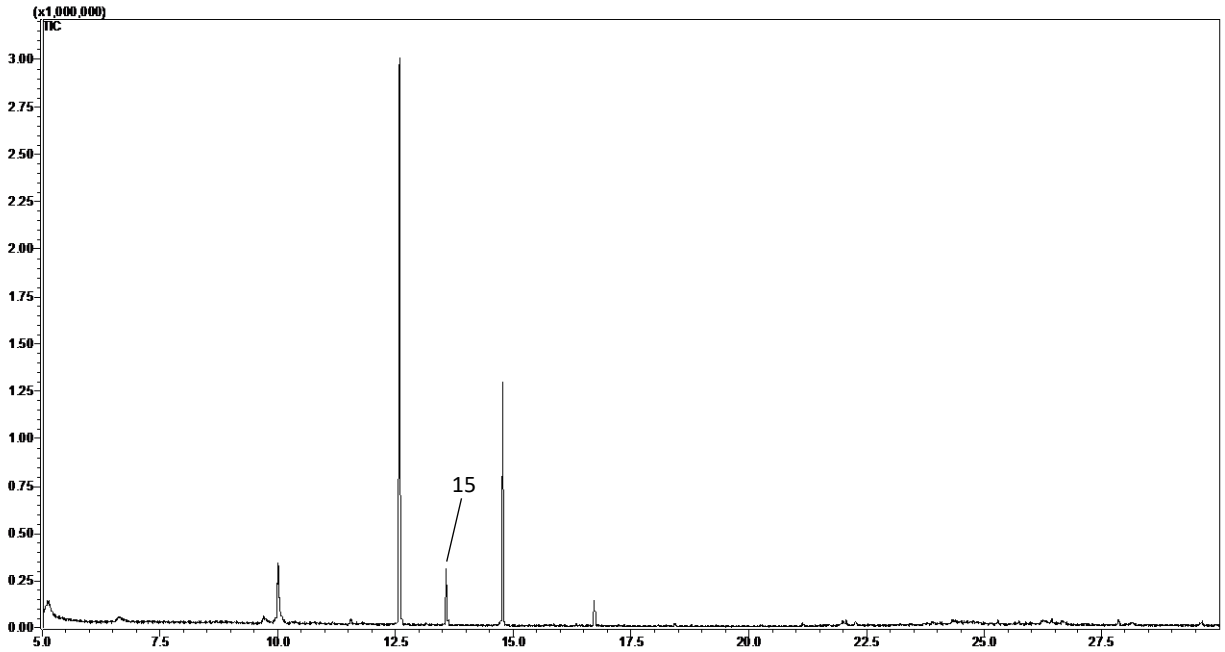


B5: Male *B. musae*

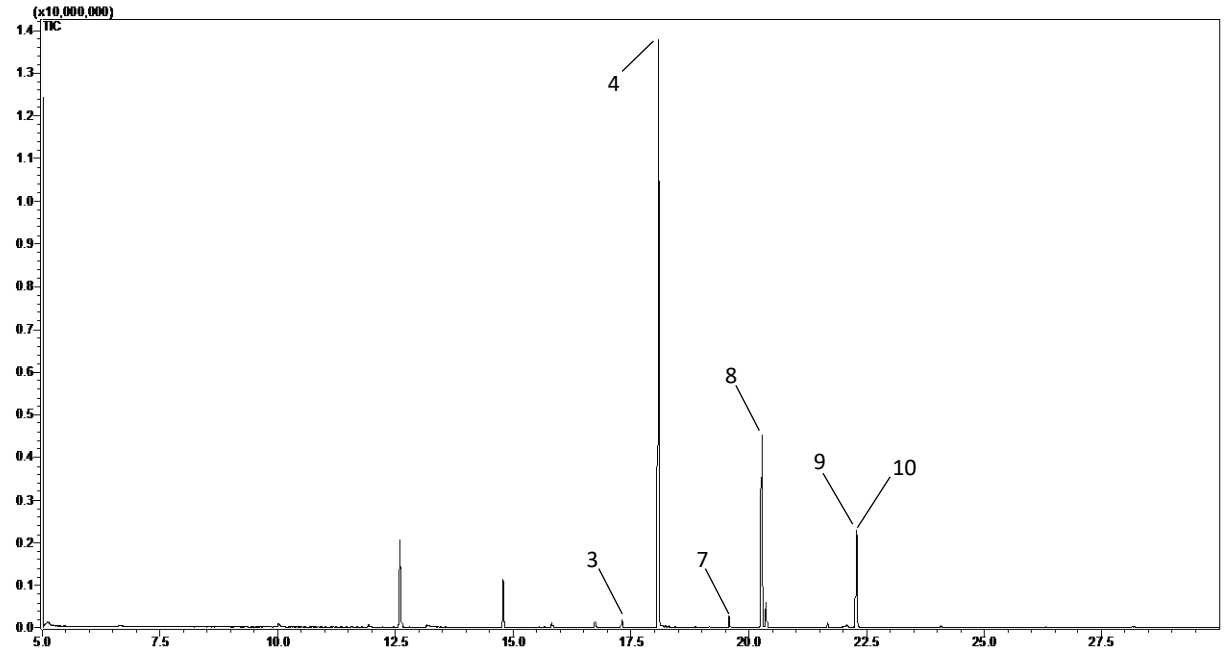


B6: Female *B. musae*

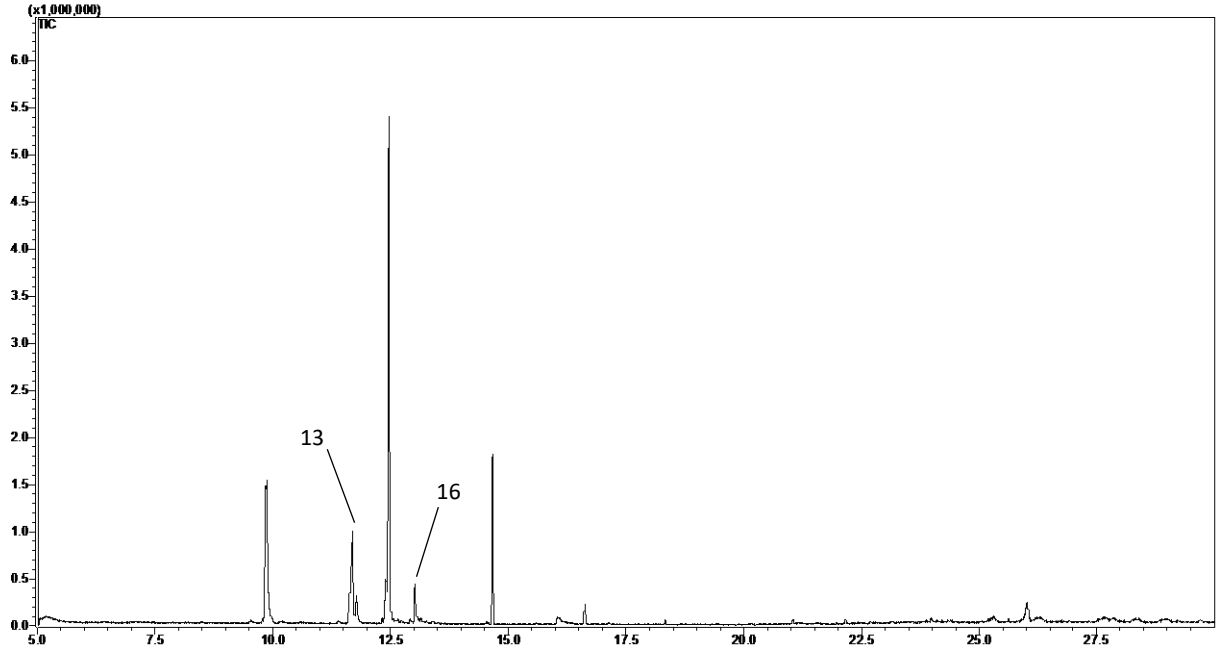
Appendix C: Representative GC-MS traces for headspace collections (collected May 2016)



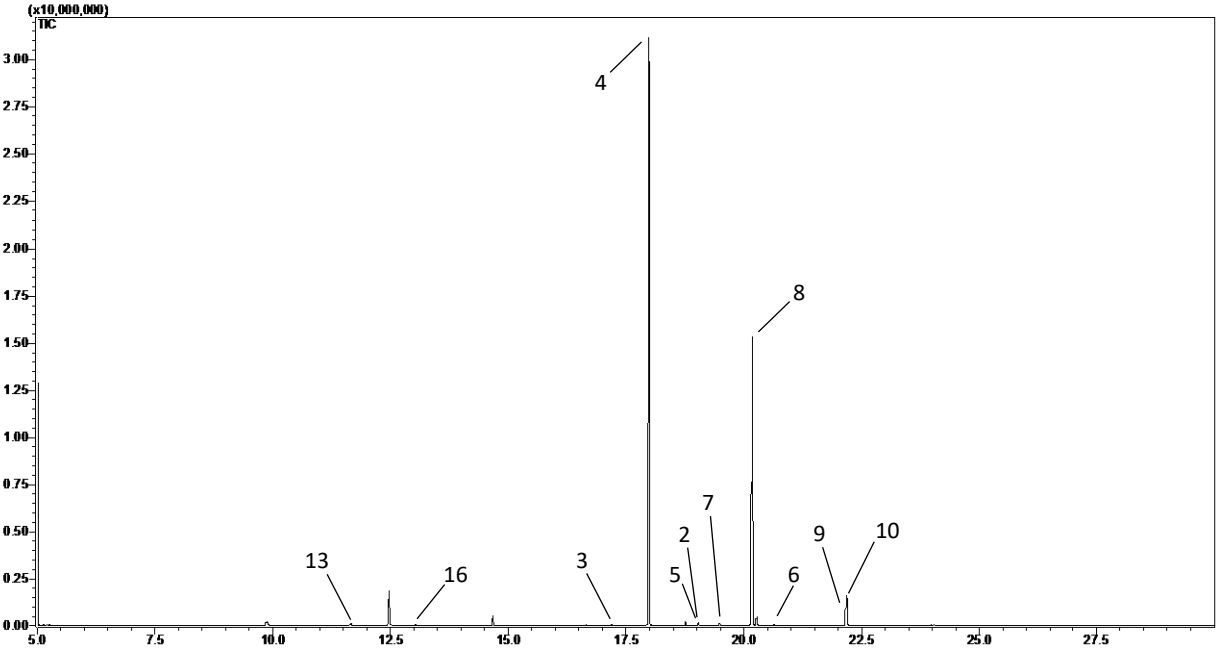
C1: Male *B. frauenfeldi*



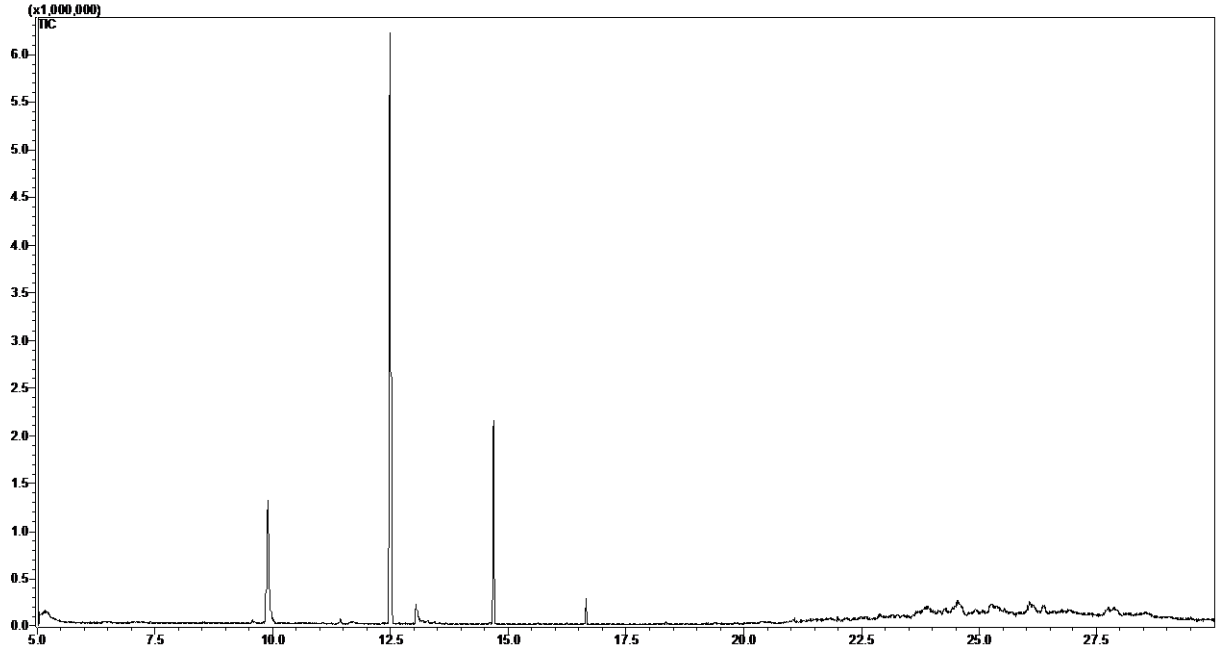
C2: Female *B. frauenfeldi*



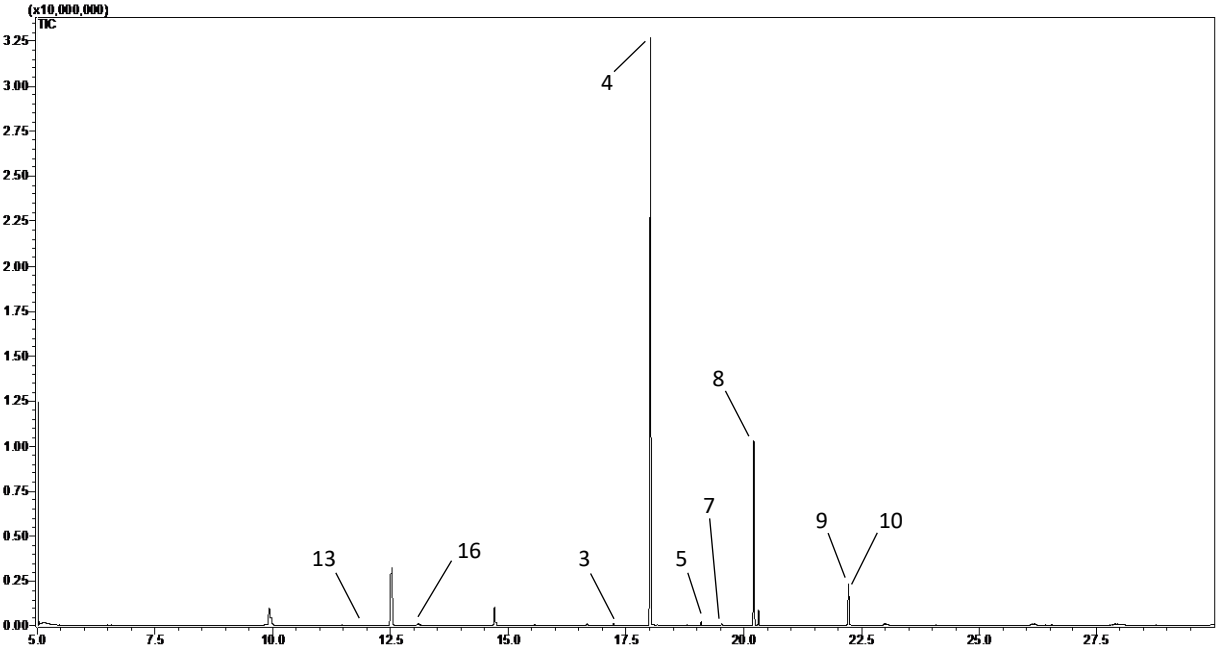
C3: Male *B. kraussi*



C4: Female *B. kraussi*

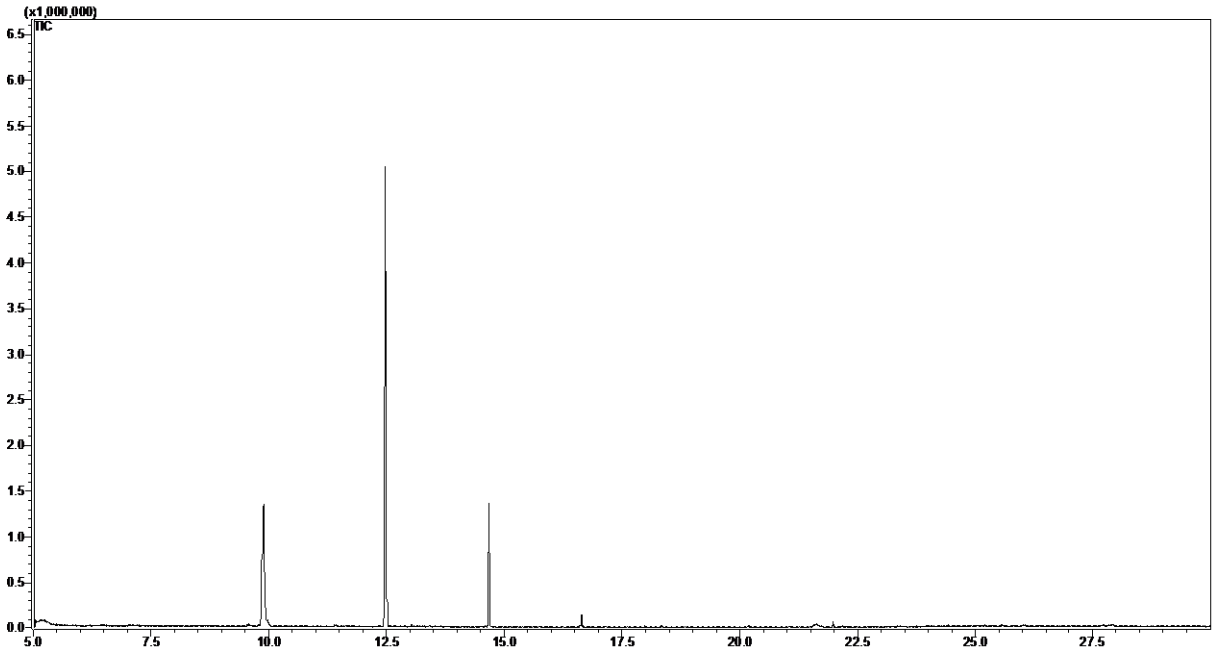


C5: Male *B. musae*



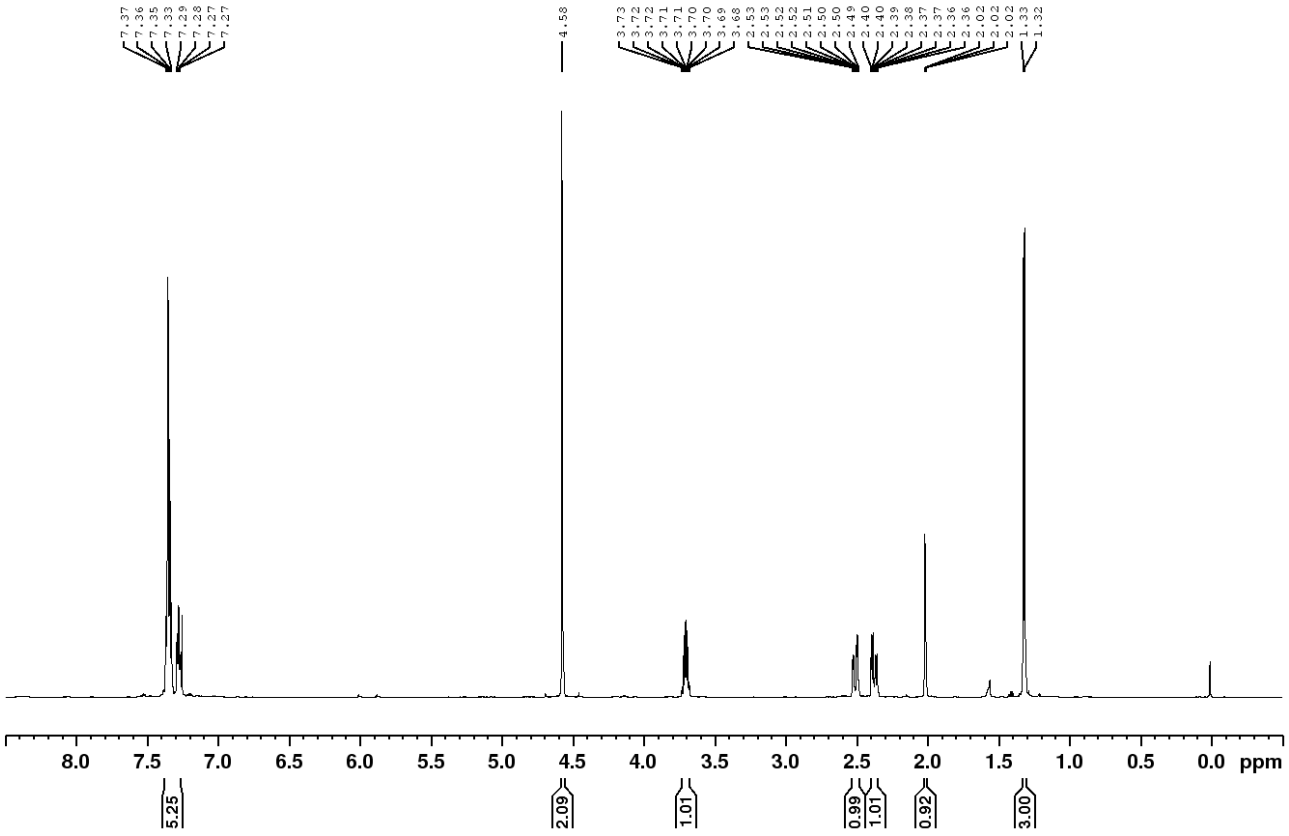
C6: Female *B. musae*

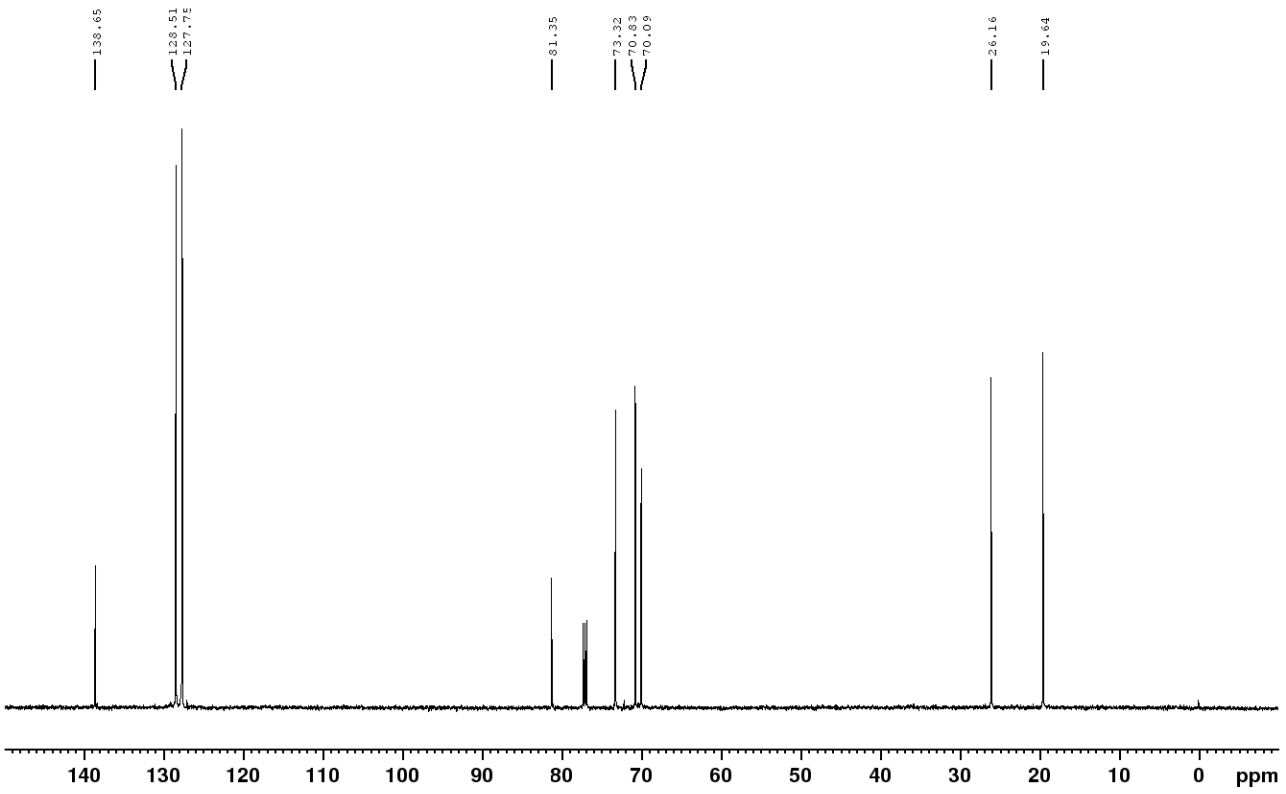
Appendix D: Representative GC-MS traces for headspace blanks (collected May 2016)



D1: Air control sample

Appendix E: ^{1}H and ^{13}C NMR for 2-benzyloxypent-4-yne

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128.51
127.75
81.35
73.32
70.83
70.09
26.16
19.64
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm