MICROSCALE STRUCTURE ELUCIDATION OF THE MONOTERPENES IN THE FRASS OF HYLOTRUPES BAJULUS (L) (COLEOPTERA: CERAMBYCIDAE), AND THEIR ROLE IN OVIPOSITION ATTRACTION.

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by

Martin Douglas Higgs

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C O N T E N T S

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ABSTRACT

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MICROSCALE STRUCTURE ELUCIDATION OF THE MONOTERPENES IN THE FRASS OF HYLOTRUPES BAJULUS (L) (COLEOPTERA: CERAMBYCIDAE), AND THEIR ROLE IN OVIPOSITION ATTRACTION.

by Martin Douglas Higgs

The work reported in this thesis describes microscale structural elucidation studies on organic extracts of insect origin. Techniques were established for microscale chemical analysis including gas chromatography, abstractor post-loops, syringe reactions and micro-ozonolysis. A high sensitivity gas chromatography-mass spectroscopy system was developed to operate in parallel with an existing one. An all-glass micropreparative splitter and trapping system was designed and used in conjunction with ultraviolet and proton Fourier transform NMR spectroscopy at the microgram level.

Behavioural studies on H. bajulus (L) (Coleoptera; Cerambycidae) indicated the operation of chemical factors in sexual and oviposition behaviour. The mono-oxygenated monoterpenes identified by chemical and spectroscopic analysis of frass were (-) verbenone, p-cymene-8-ol, myrtenol, trans-pinocarveol, cis-pin-3-en-2-ol, α-terpineol, terpinen-4-ol and 3,6,6-trimethylcyclohepta-2,4-dienone. Physiological activity was followed during purification procedures by electroantennography.

Statistical analysis of the bioassays with (-)-verbenone and p-cymene-8-ol implicated both these compounds in oviposition attraction. Solid sample gas liquid chromatography showed male and female adult beetles to have different volitile constituents.

1. INTRODUCTION

1.1 Insecticidal Pest Control

For over a quarter of a century the widespread use of synthetic insecticides has helped eradicate many insect pests, resulting in better control of insect-borne disease and crop damage. In recent years the short term gains obtained from using insecticides have been overshadowed by the realization that their indiscriminate use has not only controlled many insect pests, but also destroyed many beneficial species with the resulting adverse effect on the environment. A further disadvantage has been the need for a continual search for new insecticides caused by the development of insecticidal resistance in species by natural selection.

1.2 Hormonal Control of Insect Pests

The search for more selective methods of control has led to considerable interest in chemical and biochemical factors governing the behaviour of insects. One area of interest has been the hormonal mechanisms underlying insect behaviour, particularly the juvenile and moulting hormones[1]. Juvenile hormones which regulate the length of the larval stage have been found to be of three basic structural types[2a]. Although these hormones present a new method of control, their field application has been made difficult by two factors. They can only be produced by multi-step, highly stereoselective syntheses, and secondly they are rapidly degraded by ultra-violet light[2b]. A recent publication has

suggested that even with these natural hormones, high levels of insect resistance may develop due to selective pressures[3]. To overcome these problems a considerable amount of research has been done to find compounds capable of mimicing the natural hormonal activity. Many of the synthetic compounds which have been field tested to determine their suitability for pest control have a higher activity than the natural hormone.

1.3 Insect Pheromones as an aid to Pest Control

Another area of interest has been concerned with the role of volatile compounds in insect communication[4,5]. These compounds or mixtures of compounds which stimulate behavioural responses fall into two basic categories: (i) secretions of insect origin which produce responses including mating, aggregation, foraging and alarm within a single species, to which the term pheromone is applied; (ii) volatile constituents of plant- or animal-host utilized by insects in searching food and oviposition sites. A further group of compounds are those used for chemical communication between different species. If one or both interacting species have evolved chemical signals, or responses to cope with the other species, then the substances involved are referred to as allomones[6].

Pheromones are secreted from exocrine glands as liquids and transmitted either as liquids or gases to another animal of the same species. They either evoke an immediate behavioural response which Wilson and Bossert term a releaser effect[7], or else they have a more subtle influence which is referred to as a primer effect. Primer

substances activate the chemioreceptors in such a way as to alter the physiology of the receiving organs, probably through the endocrine systems, so that the animal in future displays a different response.

1.4 'Active space' of Pheromones

As a pheromone is released into the atmosphere it rapidly diffuses out, resulting in a concentration gradient being established from the point of emission. There is a zone around the point of emission within which the pheromone concentration is above that required to produce a physiological response. Termed the 'active space' by Wilson and Bossert, this whole zone is considered to be the signal to which the insect responds[7]. The shape of this active space is dependent upon the point of the emission and whether the pheromone is released into a wind or still air. If, for example, the attractant is released from the top of a tree in still air, then the active space is spherical. On the other hand release into a wind produces an ellipsoidal space having its long axis aligned down wind. Under the conditions of light wind a rate of emission of as little as 1 µg/sec of sex attractant is capable of forming an ellipsoidal active space with the major axis several kilometres in length. In the case of the silkworm moth (Bombyx mori) a concentration of as little as 100 molecules per cubic centimetre of air is capable of causing a response in the male.

1.5 Types of Pheromone systems

The lepidoptera pheromonal systems are among the simplest to be found, the majority of species having a single component sex

pheromone. However, synergists have been found in secretions of some species[4]. At the other end of the scale social insects, particularly ants and bees, have evolved highly complex multi-component systems which govern all aspects of these insects social behaviour. The chemical communication systems developed by many species of Coleoptera which have been investigated to date appear to fall between these two regions.

An example of the complex relationships which can exist between insects in the order Coleoptera and their host is given by the bark-boring beetles of the Scolytidae family which are a serious forest pest in North America. The females of Dendroctonus frontalis Zimm initiate the attack on a suitable host. The pioneer beetles release pheromones which act in combination with volatile terpenes of the tree resin to signal suitability for mass attack. This predominantly attracts males of the species, which then compensate and redress the population balance by releasing a male inhibitor[9,10]. Recent work has shown that the chemical communication of this species in particular and in the family as a whole is even more complex. The pheromones appear to be multi-functional, the behavioural responses being dependent upon concentration[11].

1.6 Method of Application of Pheromones in Pest Control

The potential economic and environmental importance of biological pest control is at present undergoing experimental evaluation[12,13]. Traps baited with sex pheromone and containing conventional insecticides have proved in field tests to be an

effective means of controlling the House fly (Musca domestica)[14]. Another method which has been successfully used, particularly with the Lepidoptera, is the 'confusion technique' where normal mating behaviour is disrupted by allowing small quantities of synthetic sex pheromone to diffuse from an inert matrix[15].

1.7 Introduction to the Cerambycidae

The Cerambycidae beetles cause damage to both living and dead wood of deciduous and coniferous trees. Members of this large family are commonly known as longhorn beetles because of their long slender antennae. Throughout this family the male is the active agent in sex location. In many species the male is attracted to the female, over long distances, by a scent produced in glands located in the metasternum near the hind coxae[16]. Both sexes stridulate when captured, and as a result this is regarded as a defence mechanism. It has also been suggested that in some species stridulation also plays a role in sex location[17].

Other studies on species within this family have involved determining the nutritional factors and the method by which the wood components are utilised during larval development. Results have shown that a few species, particularly those whose larvae live in fresh deciduous wood, appear to be without symbiots. The great majority of Cerambycids, however, have symbiotic yeasts present in the larval mycotome which encircles the midgut[18,19].

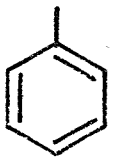
1.8 Defence secretions from the sub-family Cerambycinae

Work recently published on three species of Cerambycids has

shown the presence of volatilesof glandular origin which appear in each case to have a defensive function. Species of the Australian genera Stenocentrus McKeown and Syllis Roscoe are known to use chemical methods of defence: they are often referred to as 'stinking longhorns'. The defence secretions of Stenocentrus ostricilla (Newman) and Syllitus grammicus (Newman) have been found to be almost identical in chemical composition[20]. The secretion is released from paired mandibular glands and consists of toluene, the major component, and o-cresol (fig. 1a, (1) and (2)).

Two other species belonging to the sub-family Cerambycinae have also been shown to use chemical factors for defence, the origin in both cases being a pair of metasternal glands with openings on the distal edge of the metasternum. The defence secretion of the common eucalypt longhorn, Phorocantha semipunctata (f) consists of five volatile components (fig. 1b, (3-7)) the most abundant of which is 2-hydroxy-6-methyl-benzaldehyde (3), the second most abundant component, phorocanthol (4) has been shown to be (5-ethylcyclopent-1-enyl) methanol. The other three components are all aldehydes related to (4)[21].

The well known European musk beetle, Aromia moschata (L) was reported by Hollande in 1909 to secrete salicylaldehyde, emanating from paired metasternal glands[22]. More recently the secretion has been shown to contain the monoterpenes: cis and trans rose oxide and γ and δ iridodial in the ratio of 6 : 1 : 14 : 3 (fig. 1c, (8-11))[23].



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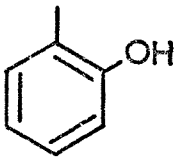

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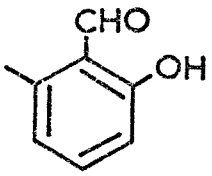
Fig Ia



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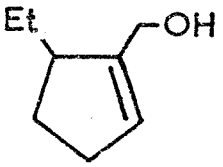

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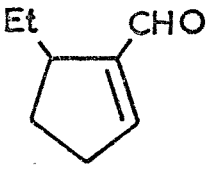

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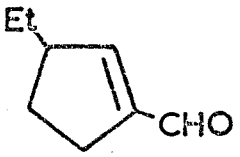

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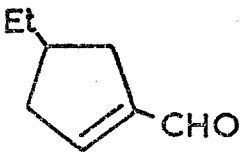

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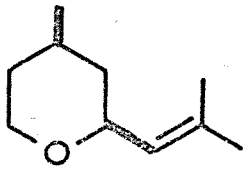
Fig Ib



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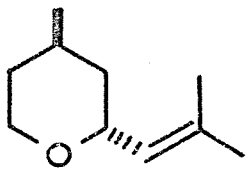

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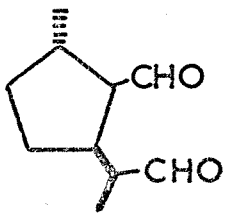

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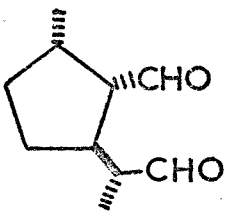
Fig Ic



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1.9 Introduction to Hylotrupes bajulus (L)

Hylotrupes bajulus (L) (Coleoptera: Cerambycidae), a member of the sub-family Cerambycinae is unusual in that it has largely become a domestic pest (fig. 2). The larvae live in the sapwood of softwood species (e.g. Abies, Picea and Pinus) used extensively as building timbers and can cause damage so severe as to produce total loss of the woods load bearing properties. Particularly important as a pest in several European countries, it has become established in temperate regions throughout the world.

Ingestion of the wood by the larvae leads to extensive tunnelling, forming galleries tightly packed with frass. This is a mixture of fecal pellets and 'wood flour', a fine structureless powder. Often the only visible sign of infestation is slight blistering of the surface of the wood caused by the pressure of the frass in the galleries contained by a thin but intact outer skin of wood. More usually an infestation is not observed until the appearance of flight holes.

The larval stage lasts between three and eleven years, the length of time being dependent upon temperature, humidity and the condition of the wood. Emergence occurs in July and August after pupation in a chamber cut out by the larvae just below the surface of the wood. The males, which start to emerge first, have a life span of about sixteen days, double that for females. Mating takes place almost as soon as the female emerges, after which the female searches for suitable cracks in the host wood for egg laying. The female

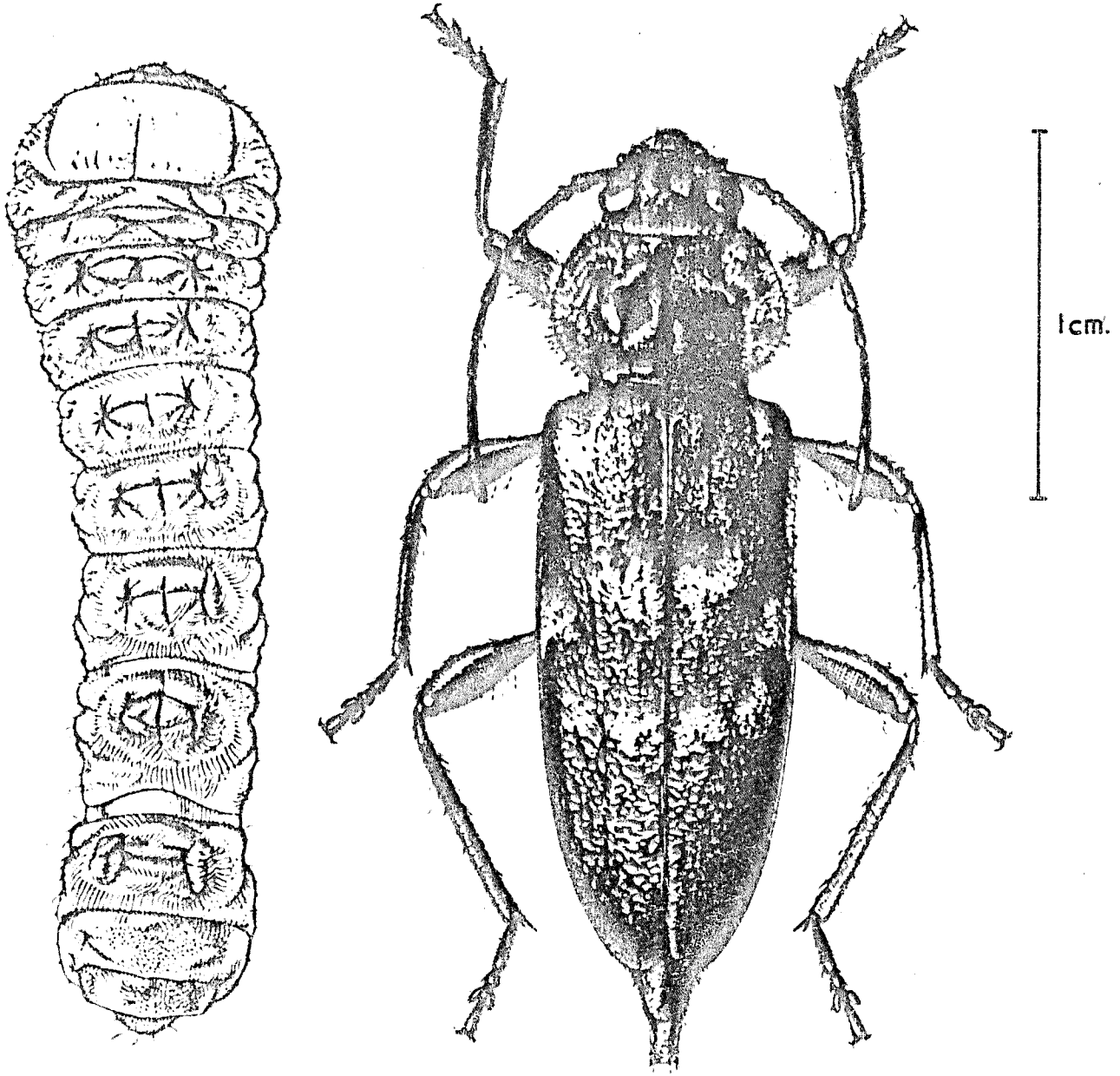


Fig 2. larva & adult ♀
of H. bajulus.

lays an average of 200 eggs in three or four batches over a period of several days[24].

Hylotrupes bajulus is frequently referred to as the House Longhorn beetle. Although first reported in Britain in 1795, it has never been a widespread pest here. The summers appear to be too cool and the winters severe for its successful development. The increased incidence of infestations over the last thirty years is thought to be due to a combination of warmer roof spaces caused by a combination of central heating and poor roof insulation. The increased use of imported timber for use in the building industry has also added to the risk of new infestations.

1.10 Previous Studies on Hylotrupes bajulus (L)

Hylotrupes bajulus differs from other longhorn beetles in that it is able to develop in wood from which both the inner and outer bark have been removed, even when the wood is old and dry. It is this ability which has allowed it to become a successful domestic pest of building timbers[18].

In common with other Cerambycids the majority of previous studies have been concerned with nutritional factors. Several publications have dealt with wood utilisation by the larvae of H. bajulus[25-27]. A particularly thorough study has been published by Seifert who cultured larvae in Pinus sylvestris from which resinous material had been removed by Soxhlet extraction[28]. Analysis of wood and frass showed that 34.6% cellulose, 20.1%

pentose and 19.7% hexose were consumed by the larvae. It was also demonstrated for the first time that the larvae are capable of digesting lignin to the extent of 5.5%. Although the presence of cellulase, amylase and lichenase in the larval gut has been known for some time[29], the process by which the lignin is metabolised is uncertain. Larvae appear to prefer the outer sapwood which has a higher protein content than other parts of the tree, a value of 0.2% protein by weight of wood is the lower limit for larval development[27]. The type of protein and the presence of the B vitamins also appear to be important. The incorporation of either into very old wood can once again make it suitable for larval development[30]. Impregnation of culture wood with the antibiotics such as aureomycin, terramycin and penicillin have little or no effect on larval development[31].

Heartwood is generally resistant to attack but only β-thujoplicin of the major phenolic compounds proved toxic. The origin of this resistance has been postulated as low molecular heartwood specific lignin, since larval development in heartwood can only be sustained after treatment with dilute alkali[26,27]. The inhibition of larval development by resins and essential oils has long been known[32]. However, little work has been done on the role these play as host-attractants. On the basis of a small number of oviposition experiments with the Pine essential oils, the host-wood monoterpene hydrocarbons α-pinene, β-pinene, car-3-ene and car-4-ene have been demonstrated to be attractive to females of H. bajulus. The oxygenated monoterpenes appear to be neutral in effect or repellant. The autoxidation of the surface hydrocarbons has

therefore been suggested as a possible reason for the loss of host-wood attraction with time[33].

The other area of interest with H. bajulus is more directly related to its importance as a pest and methods of control. A variety of methods have been tested for control and protection of wood including oily and water soluble preservatives, hot air and irradiation methods[34,35].

1.11 Microscale Studies of Insect Secretions

The small quantity of material available from individual insects has been the major obstacle in the identification of chemicals used in insect communication. Much of the early work relied upon the large scale extraction of thousands of insects (e.g. 300 μg of the sex pheromone of the Pine Emperor moth Nudaurelia cytherea (Fabr.) was isolated from 35,000 virgin females[36]), followed by a difficult and time consuming purification procedure. Development of microscale techniques and an ever increasing sensitivity of instrumentation have done much to facilitate structural identification at this level of concentration.

The successful interfacing of a gas liquid chromatograph (GC) to a mass spectrometer has meant that the components of complex natural mixture can be identified with little or no purification of extracts[37,38]. Infra-red spectroscopy (IR) has also been successfully coupled directly to a gas chromatograph using a rapid scan spectrometers fitted with light pipes[39,40].

1.12 Microscale reaction techniques for structural elucidation

A considerable amount of information can be gained on unknown structures by GC techniques alone. One of the simplest to use is that of retention time relationships, e.g. Kovats Index, which is particularly useful for aliphatic systems[41]. A serious limitation of this method is that it can only be applied under isothermal conditions. Another method for calculating Retention Indices has been devised which is capable of being used under isothermal and linear temperature programming conditions[42].

A number of methods have been described for the GC identification of functional groups[43]. Many standard reactions have been modified for use on micrograms of material enabling not only identification, but also interconversion of functional groups[44,45]. On-column reactions have also proved effective for the formation of volatile silyl ether derivatives of compounds containing hydroxyl and carboxylic groups[46]. Direct on-column hydrogenation of unsaturated compounds has proved a feasible technique using 'neutral' 1% palladium on Gas Chrom P placed in a heated pre-column[47]. The reaction proceeds cleanly with very little observed hydrogenolysis. A variation of this method is 'carbon skeleton chromatography'; using the same catalyst required for hydrogenation at 300°C plus a hydrogen carrier gas it is possible to remove functional groups and multiple bonds from molecules. The hydrocarbon so formed provides a useful method for identifying the carbon skeleton of the original compound[48].

Alternatively, subtractor loops fitted to the GC column as a pre- or post-loop provide a qualitative identification by selective removal of specific functional groups[48,49]. A recent extension of this work has been the application of colour reactions to characterize functional groups of compounds eluting from a GC column[50].

An off-column technique which has also been adapted for use on a microscale is ozonolysis[51]. When used in conjunction with GC and GC-MS this method allows direct identification of double bond positions. The carbonyl compounds (C_1-C_5) which are often difficult to separate from solvent peaks can be chromatographed as their 2,4-dinitrophenylhydrazones[52].

It is always easier to work with pure compounds and several micro preparative systems have been described for efficient trapping of small quantities of material[53-55]. Many other microscale techniques have been described for use with a wide variety of structural types[56,57].

1.13 Bioassay and Electrophysiological techniques

When working with physiologically active compounds it is important to be able to follow the activity during isolation and purification procedures. Laboratory bioassays provided the most detailed information on activity but they are often difficult to set-up and time consuming to run, usually requiring statistical analysis. Rapid screening on a qualitative basis for routine work is possible with an electroantennograph (EAG) which measures

electrical responses of the antennal nerve produced by chemical stimuli [58]. When interfaced with a GC, physiological activity of complex mixtures can readily be associated with specific groups of compounds[59]. The method can be used more specifically as demonstrated by Roelofs[60] who determined the position and configuration of the double bond in the Codling moth sex pheromone by EAG alone.

1.14 Present Studies

The object of present studies has been to set up and develop techniques for the structural elucidation of compounds on a microscale and by use of these to establish the possible role of chemical factors in the behaviour of H. bajulus.

During observations of the beetles behaviour it was noted that males were strongly attracted to females over a distance of two or three centimetres, but were strongly repulsed by other males at the same distance. Females in close proximity, on the other hand, did not react to one another and were generally inactive until after copulation. When given a choice between fresh culture blocks and previously infested ones, the female would generally lay the majority of eggs in the frass filled blocks. These initial studies suggested that chemical factors were involved in at least two areas of this insect's behaviour.

Comparison of GC traces of extracts of both host-wood, Pinus sylvestris and frass revealed that the monoterpene hydrocarbons, which are characteristic of pine wood, were completely absent in the frass. In the latter case, a group of low molecular weight

compounds are present which are not found in any significant amount in the wood extract. Biological activity of the frass extract was followed during purification procedures with an electroantennograph[61], major activity being associated with the low molecular weight fraction. This group yielded nine mono-oxygenated monoterpenes. The structures of these compounds were identified by reaction GC, GC-MS and Fourier transform nuclear magnetic resonance spectroscopy (FT-NMR). Pure compounds were obtained using an all glass splitter and solvent trapping system developed during this work.

2. SEPARATION AND MICROSCALE REACTION TECHNIQUES

2.1 Gas Liquid Chromatography

The successful analysis of a complex natural mixture is initially dependent upon the efficiency of separation into its individual components. Gas liquid chromatography is a technique capable of such separations on a routine basis, even when the constituents are of a varied nature. Successful operation with very small quantities of material is dependent upon the ability of the chromatograph to maintain stability and sensitivity over long periods of time. A further requirement, particularly with small scale work, is the ease with which the instrument can be modified for use with other techniques.

The Pye Unicam series 104 gas liquid chromatograph which was used throughout these studies satisfied these criteria. The heated dual flame ionization detector (FID) produced a uniform and stable response, even under conditions of temperature programming and high sensitivity. The dual detector capability proved particularly effective under these conditions, the 'difference' chromatogram obtained by electronically subtracting the column bleed from the recorded signal producing a steady base line. This enabled many components normally lost amongst the background bleed to be readily seen. Although the chromatograph functioned well for most requirements, it still showed instability when used for the analysis of nanogram quantities of material. This instability which was caused by the continual elution of material from the columns, was

found to come from two sources. High molecular weight impurities in the nitrogen carrier gas which were continually being swept onto the column was one of the factors involved. Purification of the gases by passing them through traps containing activated 13X molecular sieve was sufficient to eliminate this source of column contamination, although periodic reactivation of the molecular sieves proved necessary. Bleed from the rubber septum used in the injection port was the second source of column contamination. Silicone septa backed with PTFE removed this problem, and had the additional advantage of a longer life time, requiring replacement only after fifteen to twenty injections against three to five for the rubber septum.

The problem of column contamination is also an important factor in mass spectroscopy where high column bleed causes a severe reduction in the level of sensitivity at which the spectrometer can be effectively operated. The removal of extraneous sources of column contamination is therefore critical in this technique, especially when compounds are available in very small amounts.

2.1.1 Column Requirements for Gas Chromatography

In an attempt to reduce the number of columns required for routine work, while retaining both analytical and preparative capability, columns of each stationary phase to be used were packed in matched pairs. One column was fitted with a glass/metal seal (Pye Unicam, Cambridge) to connect the column to the detector. The second had a 6 mm o.d. ground glass end which was fitted to the

detector with a 'column connector' (Pye Unicam, Cambridge). Paired columns of this type were used for dual column analytical work to produce chromatograms from which bleed has been removed. The open ended column was also used for micro-preparative GC by connecting it to the splitter with a 0.25 in. Swagelok union, bored out to 7 mm i.d. Besides the advantageous reduction in the number of columns required, this system enabled identical separations to be obtained for both analytical and preparative GC.

Gas-tight seals for the connectors used with open-ended columns were originally made with Viton or silicone 'O' rings. Neither of these was found to be completely suitable, mainly because of the low maximum temperature at which they could operate. Viton 'O' rings were better in this respect than silicone 'O' rings and could be used up to 300°C for short periods. Silicone 'O' rings which commercially replaced these had a temperature limit of 200°C, above which they softened considerably, often leading to gas leaks from the seals. None of these shortcomings were experienced with graphite ferrules which have a longer life span and can be used up to 400°C (S.G.E., Techmation). The ferrules are capable of maintaining gas-tight seals even when frequently changed from one column to another. Although they are designed for Swagelok unions and could not be fitted to the Pye Unicam 'column connectors', their use was extended to dual column analytical work by replacing the 'column connector' thread with one cut from a 0.25 in. Swagelok union.

2.1.2 Column Supports and Stationary Phases

The variety and complexity of extracts from natural sources requires the use of columns of the highest efficiency and resolution. For ease of comparison of extracts over a period of time, it is also desirable that columns can be repacked reproducibly. In the present studies, chromatographic work was undertaken with 2 mm and 4 mm i.d. columns. The properties of these columns are dependent upon several factors, including the type and quality of support, the manner in which it was coated and the method of packing.

The support used initially was Diatomite C (J.J's (Chromatography) Ltd. Kings Lynn), a diatomaceous earth equivalent to Chromosorb W, which had been acid washed and HMDS treated. These columns did not have particularly high efficiencies and the best value achieved for the HETP (height equivalent to a theoretical plate) was 0.9 mm. Another disadvantage was bad peak tailing which was particularly evident when the support was coated with one of the high quality OV stationary phases. A considerable improvement was obtained using another diatomaceous earth Diatomite CLQ (J.J's (Chromatography) Ltd. Kings Lynn) which has been both acid and alkali washed followed by DMCS treatment, a more effective and permanent method of removing active sites than HMDS treatment. Since the quality of this support was standardised by the manufacturer, it was possible to pack columns over a period of time which had identical characteristics.

Many standard texts on GC technique include a section on stationary phases, their characteristics and recommended solvents for

coating supports. In several instances the use of these solvents produced mediocre columns, while others produced columns with the expected efficiency. This problem disappeared when the solvent recommended by manufacturers for their particular product was used. Although the reasons for this are not fully understood it is thought that some polymers differ slightly in their overall structure depending on the process by which they were synthesised. These differences in structure affect the solution conformation of the polymer and the manner by which it is deposited on the support. This in turn affects the efficiency with which the column operates[63].

Once coated, the support was thoroughly dried and resieved to remove lumps and fines. The columns were then packed under pressure and conditioned at the maximum working temperature for the recommended period with the carrier gas flow rate set at half its normal value[64]. This method routinely produced 4 mm i.d. columns with an HETP of 0.6 mm, and 2 mm i.d. columns with an HETP of 0.42 mm. Periodically it was observed that columns would begin to lose efficiency and resolution, usually when crude extracts were chromatographed. This was associated with the build-up of polymeric material at the point of injection and replacement of the column packing in this area returned the column to its previous performance.

2.2 Solid Sampling Gas Liquid Chromatography

Direct solid sampling of the volatiles from the tissues of H. bajulus by previously reported methods was not possible because of the size of the insect[65,66]. The method of Bergström was, however,

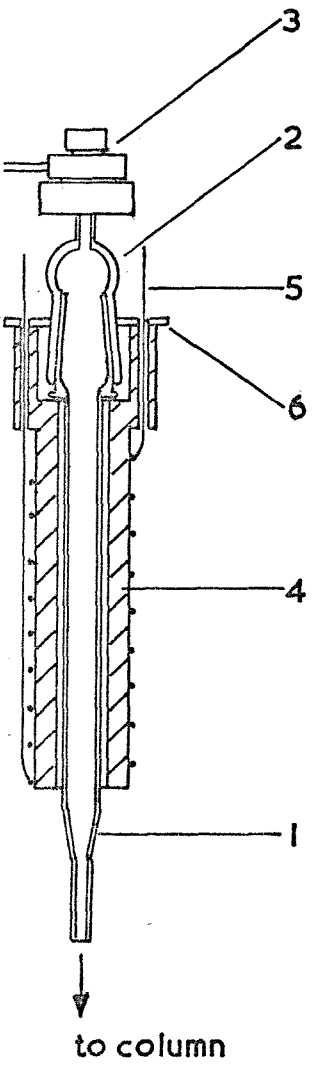


Fig 3. Pre-column and heater block for solid sample GC: Scale x$\frac{1}{2}$

1. Pre-column
2. B10 cone and socket
3. Injection head
4. 'Malanite' heater block
5. Heater wire connected to variable 12V supply
6. Brass retaining ring

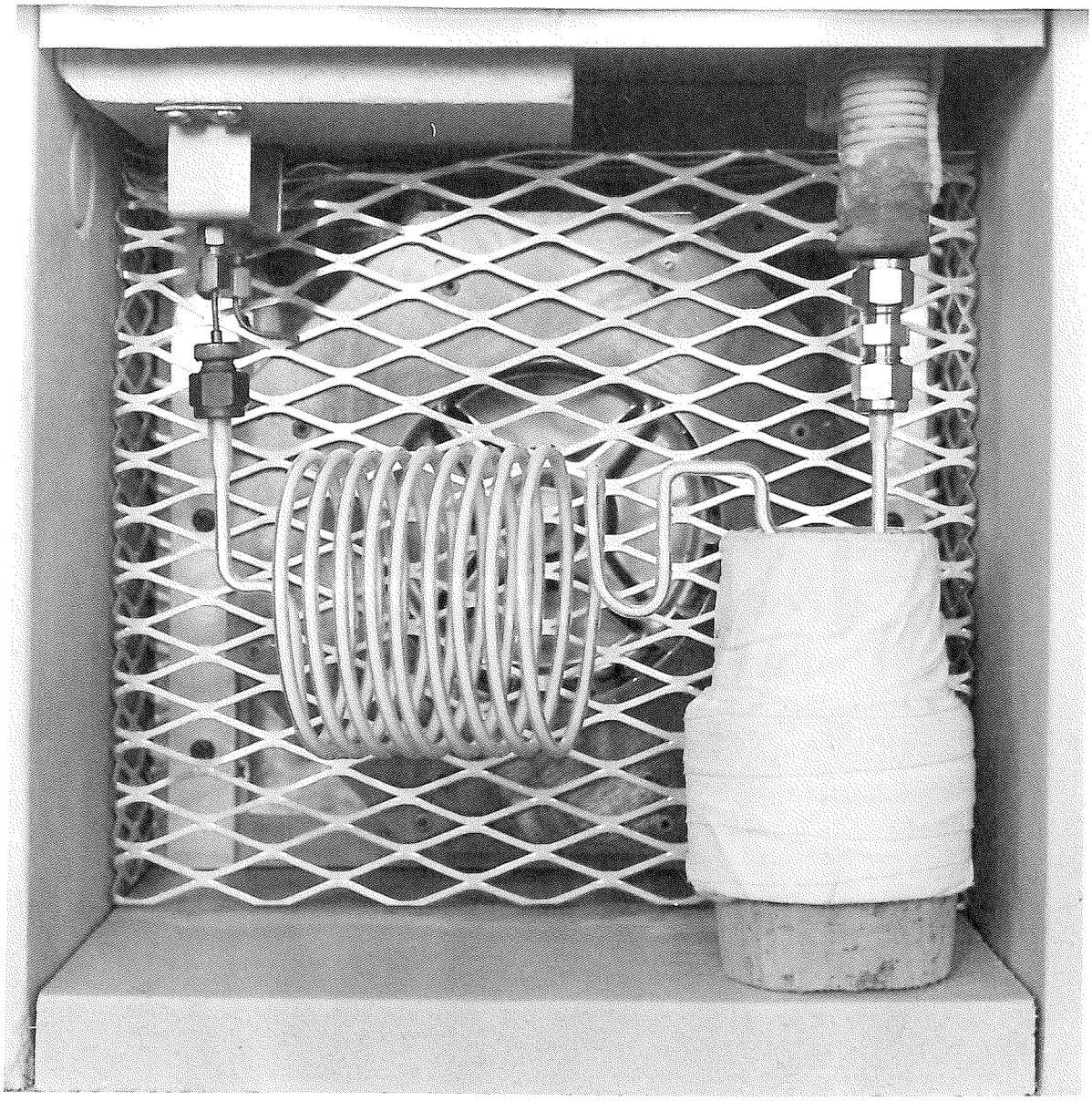


Fig 4. Bergström column in GC oven with pre-column and heater

specifically designed for use with bulky objects. The original system required some modification for use with the Pye 104 chromatograph. Changes were also made to enable the graphite ferrules to be used for gas-tight seals in place of those originally described, which could not be used above 200°C and required frequent replacement.

The beetles were killed by gradually freezing with solid carbon dioxide and then placed in the pre-column (fig. 3), this was connected to the column and the whole assembly was then fitted in the oven (fig. 4). The pre-column was heated for five minutes at 110°C, the lowest temperature necessary to achieve complete removal of volatiles. The volatilised compounds were swept onto the column by the nitrogen carrier gas and trapped as a 'plug' of material by cooling the 'U' shaped part of the column with dry ice/acetone. At the end of a five minute period the pre-column and heater were replaced with a length of 6 mm o.d. glass tubing. This prevented the contamination of the column with high molecular weight cuticle components which were found to volatilise during temperature programming at temperatures above 120°C. Once deposited on the column many of these compounds were extremely difficult to remove leading to high column bleed during temperature programming. After the carrier gas flow had restabilised, the coolant was removed and the oven heated ballistically to the required starting temperature, in this case 80°C.

A period of four minutes was necessary for complete stabilisation of the carrier gas flow rate. It was important that this was achieved

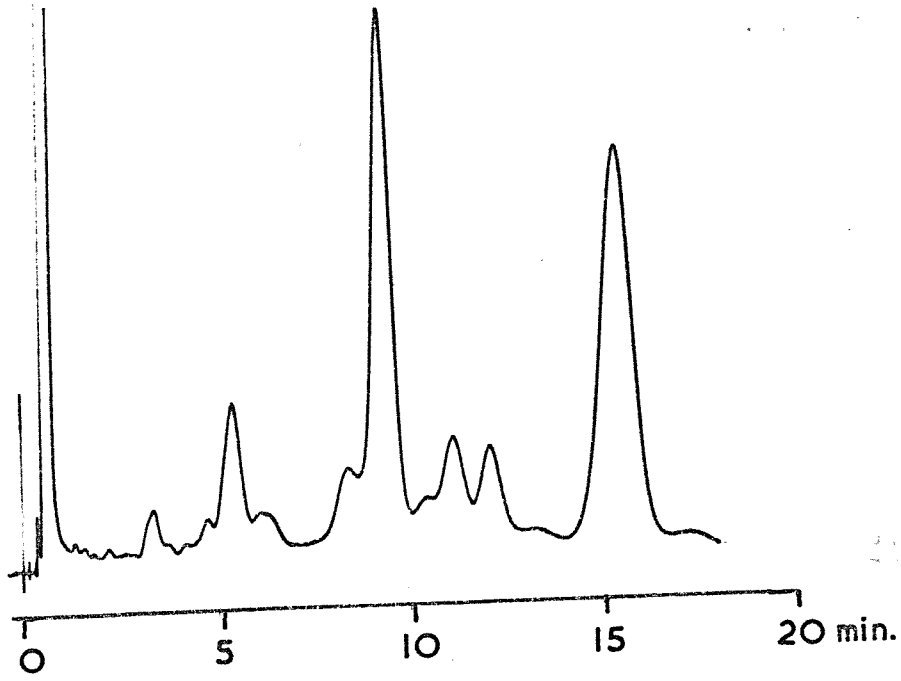
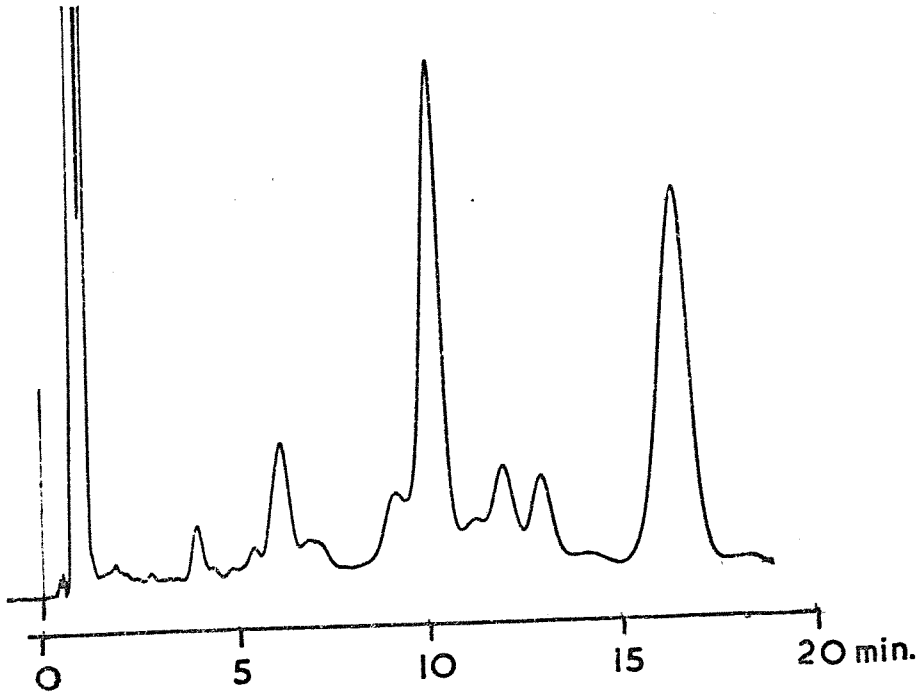


Fig 5. Sample injected directly onto the column



Sample trapped onto the column by cooling

if reproducible chromatograms were to be obtained, since an unstable flow rate affects not only retention times but also column efficiency. Separations of test mixtures trapped on the column by cooling were comparable to those obtained by direct injection, although retention times were changed (fig. 5).

2.3 Micro-ozonolysis for Double Bond Determination

One of the most difficult problems in structural elucidation is the positioning of double bonds. Though this can often be achieved by spectroscopic techniques, the analysis of the ozonolysis products still remains the only reliable method in certain cases. The difficulties presented by olefinic bonds are even greater when structural elucidation is determined on a microscale. In this situation many of the spectroscopic methods can no longer be applied and ozonolysis becomes the only definitive method which can be used.

Although commercially available ozone generators can be used for microscale applications, they have the disadvantage of producing quantities of ozone far in excess of the amount required for microgram samples. This excess ozone must therefore be vented to the atmosphere using a stream splitter. It is therefore more desirable to use a method capable of a production rate appropriate to the small sample size and several methods have been described for this purpose[45,50,51,65].

The simplest method for micro-ozonolysis is that of Beroza and Bierl[51]. Of the two generators described in this paper, the flow-through ozoniser (fig. 6) was the easiest to construct and use.

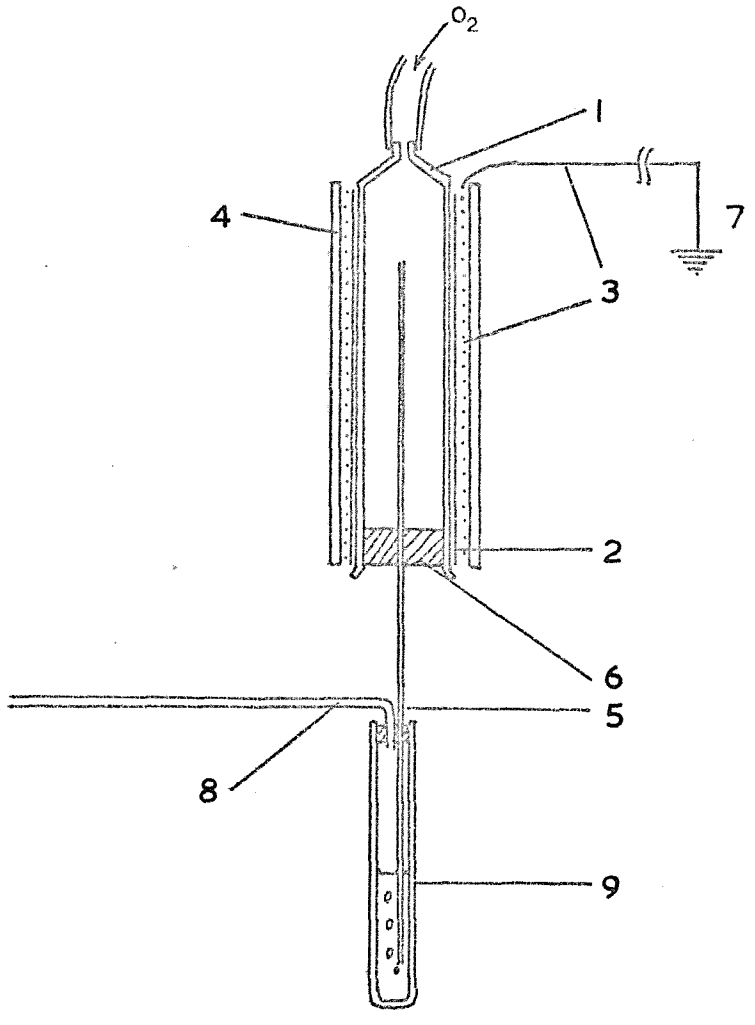


Fig 6. Flow-through micro-ozoniser.

1. 5 ml glass syringe barrel
2. Aluminium foil
3. 25 turns of 22 SWG copper wire
4. Insulating polythene tube
5. 180 x 2 mm o.d. stainless steel tube
6. Cork
7. Earthed wire
8. Gas outlet tube
9. Reaction vessel

This apparatus had considerable versatility and could be used to determine the position of ethylenic bonds on samples in the microgram to milligram range.

Problems frequently encountered during ozonolysis include those of artifacts and products from side reactions. Trials with oct-2-ene using ordinary grade oxygen showed the quality of this gas to be insufficient for small scale work. These impurities were completely eliminated by replacing this gas with Research Grade Oxygen (BOC, Southampton). Side-reaction products were minimised by operating at -60°C, and by removing excess ozone by purging the solution briefly before reducing the ozonides with a small crystal of freshly recrystallised triphenyl phosphine. Removing excess ozone from solution was found to be particularly important when the products were to be stored for any length of time. Trace amounts of ozone remaining in solution rapidly led to degradation of the carbonyl compounds, even at -20°C.

Identification of the ozonolysis products by GC and GC-MS was possible for sample sizes down to 1 μg, oct-2-ene, dihydromyrcene, geraniol and farnesol yielding the expected products (fig. 7). The low molecular weight carbonyl compounds (C_1-C_5) were identified as their 2,4-dinitrophenylhydrazones (2,4-DNP)[52]. These were formed by adding a dilute aqueous solution of the 2,4-dinitrophenylhydrazine reagent in slight excess, indicated by a slight colouration of the aqueous layer. The 2,4-DNP's present in the organic layer were then analysed by GC using an OV-1 column. The ratio of the carbonyl

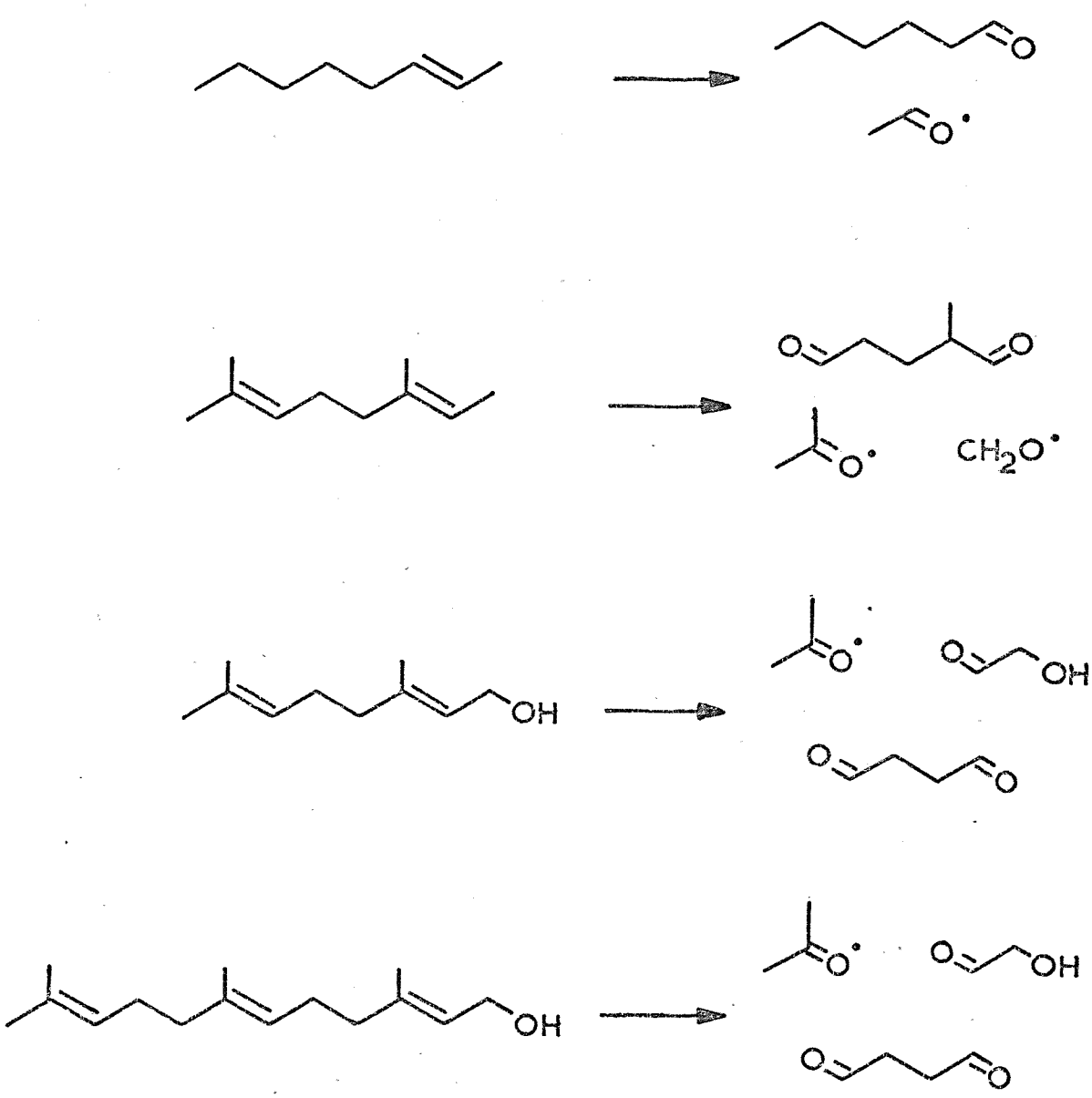


Fig 7. Products from ozonolisis of test compounds

• Identified as their 2,4-DNP derivatives

compounds formed when the carbon fragments were small was generally found to differ from the theoretical value. The reason for this appeared to be differential loss of the C_1-C_3 carbonyl compounds, which in every case were present in smaller amounts than expected.

2.4 Functional Group Identification by Subtractor Loops

Selective subtraction of compounds separated by GC using reaction post-loops can yield a considerable amount of information on the nature of functional groups present[43,48]. The post-loops consist of a 12 cm length of 2 mm i.d. glass tubing looped once and placed between the end of the column and the detector. The mixture to be analysed was first run with a blank post-loop and a comparison made with the chromatographic trace obtained with the various subtractor loops (results presented in Table 1).

While the results obtained are not absolutely definitive, they demonstrate the type of information to be gained. Zinc oxide which can be used in post-loops to subtract carboxylic acids proved to be less reliable than previously reported as it also removed many alcohols and aldehydes[48]. The boric acid loop did behave with test compounds as previously reported. Tertiary alcohols, which do not form borate esters[67] and are therefore not subtracted by the post-loop, can be identified as their dehydration products with a boric acid pre-column. However, this method was not used extensively as the GC column became saturated with borate esters of primary and secondary alcohols, leading rapidly to deterioration in column performance. As a post-loop, boric acid reproducibly subtracted

primary and secondary alcohols. Tertiary alcohol could normally be detected by a distorted peak shape, coupled with a slight change in retention time. Two drawbacks have to be remembered when interpreting results from the boric acid loop, firstly there is a tendency for some terpene aldehydes and ketones to be subtracted. Secondly the removal of allylic alcohols is poor unless they possess a terminal double bond.

The most effective agents for subtracting aldehydes and

TABLE 1

Efficiency of Post-Loops: % Subtraction

	20% FFAP	5% o-Dianisidine	1:20 Boric Acid	1:10 Zinc Oxide
Hexanal	73	100	2	21
Heptan-4-one	36	0	0	0
Hexyl Acetate	28	15	3	1
Octan-2-one	25	15	0	2
Hexanol	29	14	100	60*
Pentadecane	0	0	0	0
Citronellal	74	100	100	53*
Neral	64	88	19*	0
Verbenone	7	22	14	14
Geraniol	0	0	30*	5
Octanoic Acid	0	0	0	100

* GC Peak shape changed

ketones are o-dianisidine and benzidine respectively. However, both these aromatic amines are carcinogenic which makes their use less desirable. An alternative method for removing carbonyl compounds uses loops containing sodium metabisulphite, which has an operating range of 50-110°C[68]. Sodium metabisulphite was found to be ineffective for the carbonyl compounds listed in Table 1 over the whole of its working range. Even if this reagent had been effective, its application would have been restricted because of its low maximum operating temperature. The commercially available stationary phase FFAP (J.J's (Chromatography), Kings Lynn), at a 20% loading, has also been reported to subtract aldehyde though the mechanism by which this occurs is not known[69]. The aldehydes used as standards were only partially subtracted in the temperature range 75-200°C, and 60-70% appeared to be the maximum subtraction efficiency which could be attained. This value would have been acceptable provided it had been reproducible, however, the level of subtraction for a particular compound varied considerably from one GC run to another. Finally the o-dianisidine loop was tested and found to reproducibly subtract aldehydes with a high efficiency and because of this it was used in preference to the FFAP loop. Regular operation was possible up to 175°C, and even up to 200°C for short periods. The loop gave rise to considerable background bleed at these high temperatures resulting in rapid deterioration. Packing the last quarter of the loop with uncoated support minimised both these problems.

The boric acid and o-dianisidine post-loops were effective

for determining alcohol and aldehyde functionalities. The zinc oxide loop was less useful because of the manner in which it affected compounds other than carboxylic acids. No effective method was found for identifying ketones by subtraction since the bisulphite post-loop was not effective and benzidine was not readily available.

2.5 Column and Syringe Reactions

The interconversion of functional groups is a normal aid to structural elucidation of unknown compounds. Many of these reactions can be used to the same effect on a microscale with little modification to the procedure. These reactions are superior to subtractor loops as they not only identify the functional group but form a product which can be analysed by GC-MS. This greatly increases the amount of information available from which a structural assignment can be made. Although the reactions can be applied when several compounds in a mixture have the same functional group, the changes which occur sometimes restrict analysis to a qualitative assignment. The reactions used successfully were silylation, bromination and reduction of carbonyl compounds with sodium borohydride. A summary of these results can be seen in Table 2.

2.5.1 On-column Silylation

The on-column formation of trimethylsilyl derivatives is a useful way of increasing the volatility of carboxylic acids, phenols

and alcohols, making analysis possible by GC-MS of compounds which are either too involatile or too polar to pass through the GC-MS interface. A further advantage is the strong ion in the mass spectrum resulting from the loss of a methyl group from the parent ion of the silyl ether. This facilitates the calculation of the molecular weight of the compound from which the ether has been formed, often extremely difficult to determine for acids and alcohols which rarely have a significant parent ion.

Stationary phases used for this procedure must be inert to the silating reagent and the two most frequently used in these studies were XE-60, a methyl silicone gum containing a cyano side chain and the high quality methyl silicone gum OV-1. Mixtures of compounds with similar molecular weights were normally analysed qualitatively with an XE-60 column. Unfortunately the low polarity silyl ethers were difficult to resolve, eluting from the column with unreacted reagent. Samples for GC-MS were formed on an OV-1 column, where separation according to molecular weight allowed the ethers to be analysed free from sample or reagent.

The method as originally reported required 40 µl of 'Silyl 8', a commercial product containing three silylating reagents[46]. The percentage conversion with this reagent was rather variable and the large excess of reagent frequently obscured derivative peaks. Experiments with the individual components of 'Silyl 8' indicated N,O-bis-(trimethylsilyl)-acetamide (BSA) as the most suitable single component reagent, 0.1 µl proving sufficient under the

TABLE 2

Results of On-Column and Syringe Reactions with Monoterpenes

Compound	Silylation	Bromination	$NaBH_4$ Reduction
Camphor	-	-	-
Carvone	-	+	+
Cuminic Aldehyde	-	/	+
p-Cymene-8-ol	/	/	-
Geraniol	+	+	-
Menthol	+	-	-
Myrtenal	-	+	+
Pinane	-	-	-
α-Pinene	-	+	-
Pin-3-en-2-ol	/	+	-
α-Terpineol	-	+	-
iso-Piperitenone	-	+	+

+ Reactive: / Partially Reactive: - Unreactive

conditions used for samples up to 50 µg in size. For reproducible results both the column and reagent syringe had to be conditioned by passing 25 µl aliquots of BSA through the column at 150°C until only the reagent peak was observed on the chromatogram. To avoid coating the detector with silicone, which would result in considerable loss in sensitivity, the conditioning was performed with the column

disconnected from the detector. The highest conversions to silyl ethers were attained with column temperatures of 120°C and above, the BSA being injected five seconds after the sample. For very volatile samples which would elute too rapidly at this temperature, partial conversion was achieved by lowering the temperature to 110°C. Below this limit the conversion to silyl ethers with BSA was in the order of five to ten per cent.

Primary and secondary alcohols were readily converted to their silyl ethers with yields usually greater than 90%. Tertiary alcohols tested did not form ethers very easily and the yield was generally in the order of five per cent. Certain tertiary allylic and aromatic alcohols such as cis-pin-3-en-2-ol and p-cymene-8-ol were exceptions to this rule as their silyl ethers were produced in yields of 60-70%.

2.5.2 Bromination and Sodium Borohydride Reduction as Syringe reactions

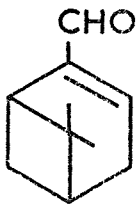
Two schemes have been published for functional group analysis on a microscale. The method of Hoff and Feit[44] brought the sample vapour into contact with the reagent which was spread over the wall of a 2 ml syringe. The disadvantage of this method is that it is restricted to samples with an appreciable vapour pressure. Difficulties were also presented by the type of sample handled in the course of this work, where the few micrograms of compound available were usually dissolved in 200-300 µls of solvent.

The method devised by Fredricks and Taylor[45] has a more

general application as the reactions are carried out in a 10 µl syringe. A general procedure was adopted for the compounds listed in Table 2 whereby the sample was introduced into the syringe, followed by 0.5 µl of reagent. The two were then mixed for the duration of the reaction period by barrel action. The sodium borohydride reduction as described in this paper did not initially work for the terpene ketones tested. Although the carbonyl compounds disappeared from the GC trace, no peaks corresponding to the alcohols could be found. However, yields of alcohols up to 50% were obtained when the reaction was worked-up in the syringe using 1 µl of distilled water spread by barrel action for a two minute period. It appears that the intermediate borate esters formed by the terpene ketones are more stable than those of the carbonyl compounds tested by Friedricks and Taylor. That it was not a problem of technique was demonstrated by the reduction of nonan-2-one to the alcohol in the absence of the aqueous work-up.

Reduction of solutions of carbonyl compounds more concentrated than 1 µg/µl were too vigorous for syringe work, invariably resulting in loss of sample from the syringe. In these cases a reaction vessel was constructed from a glass tube drawn down to a point, mixing of sample and reagent being achieved using a 10 µl syringe in a pumping mode. The only drawback of this reaction technique was the tendency for the sodium borohydride to reduce conjugated aldehydes and ketones to the saturated alcohol[70]. In the case of carvone five products were obtained, three saturated and two unsaturated in approximately equal amounts.

The results from this reaction were reproducible and allowed unequivocal identification of carbonyl compounds. Comparison of these results with those from the o-dianisidine post-loop permitted the identification of both aldehydes and ketones. A further investigation of the reaction products could lead, in some cases, to more information on the compound's structure. The products of compounds such as myrtenal and cuminic aldehyde (fig. 8; 12 and 13) give one peak by GC containing a single compound or enantiomers, while compounds such as iso-piperitenone and pinocarvone (fig. 8; 14 and 15) give rise to epimeric products. It is even possible to distinguish between the unhindered monocyclic and hindered bicyclic compounds. The epimeric products of the former are normally in the ratio of approximately 1 : 1, a value of 9 : 1 is more typical of bicylic compounds such as pinocarvone.



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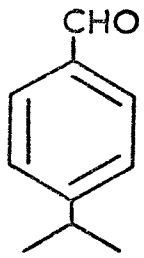

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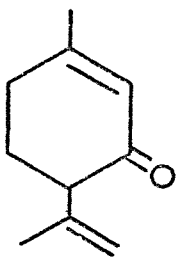

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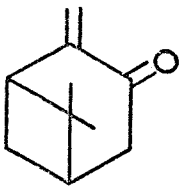
Fig 8.



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Although bromination was not used by Fredricks and Taylor, it was readily modified for use with their syringe technique. Compounds containing ethylenic bonds were reproducibly removed by 0.5 µl of a one per cent solution of bromine in water. Hoff and Feit[44] noted in their paper that many other compounds were affected by the reaction, particularly alcohols and aldehydes. These side-reactions were minimised in the syringe technique by expelling the bromine water as soon as it had become colourless. If the reaction was allowed to proceed beyond this point other components were increasingly affected. However, only unsaturated compounds ever reacted with 100% yield.

These two syringe reactions proved a reliable method for microscale identification. There appeared to be no adverse effect upon the columns performance with the 1-10 µg samples used. Column deterioration might be more rapid if larger amounts of reagent were required.

2.6 Thin Layer Chromatography

Although generally useful in microscale structural studies, thin layer chromatography could not be used to any great extent because of the small quantity and volatility of the compounds under investigation. However, one application which was found useful was the *in situ* formation of the 2,4-dinitrophenylhydrazones (2,4-DNP) of the isolated carbonyl compounds for comparison with synthetic samples.

The 2,4-DNP was made by first spotting the plate with

0.5-1 µg of the isolated carbonyl compounds followed by the reagent, the 2,4-DNP formation was indicated by a change in colour of the spot. This was then run against the 2,4-DNP of a synthetic sample in the two solvent systems recommended for these derivatives[71]. The small amount of acid present does not appear to affect the elution of the 2,4-DNP's, nor does the more polar unreacted 2,4-dinitrophenylhydrazine. The intense colour of these derivatives allows a further correlation of natural and synthetic samples using very small quantities of material.

2.7 All-Glass Micropreparative Splitter and Trapping System

It is frequently necessary to isolate pure compounds for complete identification by spectroscopy and chemical analysis. Preparative GC is a technique eminently suited for the direct isolation of pure compounds from a complex mixture. Unfortunately many commercially designed systems have poor recovery efficiencies and are generally not capable of trapping at the microgram level.

A number of highly efficient splitter and trapping systems have been described for microscale work[53-55]. However, none could be adapted conveniently to the requirements of present work with a Pye 104 chromatograph. One of the drawbacks of these designs is that they have hot metal surfaces with which the column effluent to be trapped comes into contact. This considerably increases the risk of artifacts caused by metal catalysed decompositions.

A splitter and trapping system was therefore designed

during the course of the present studies[72], this had to meet several criteria:

(1) it must be cheap to construct and easy to use.

(2) have a high trapping efficiency for a variety of compounds over the milligram to nanogram range.

(3) prevent contamination of consecutive fractions by pre-condensation of the compound in the splitter.

(4) cause minimum degradation of the trapped sample by thermal or catalytic processes at the splitter surfaces.

(5) produce minimal changes in column efficiency or resolution.

It is also desirable that the system should be capable of operation with a range of column sizes.

2.7.1 Design of the All Glass Splitter

The shape of the all-glass splitter was determined by the geometry of the Pye 104 oven (fig. 9), while other design features have a more general application. Dead volume, one of the major causes of loss in resolution, was kept to a minimum by constructing the splitter from 0.5 mm i.d. thick walled capillary tubing fitted with modified glass/metal seals at either end (fig. 10; 1 and 2). Only the dead volume of the glass/metal seal at the detector end of the splitter can adversely affect resolution, and even this can be eliminated by silver-soldering the metal capillary so that it butts directly against the end of the glass capillary. The prevention of

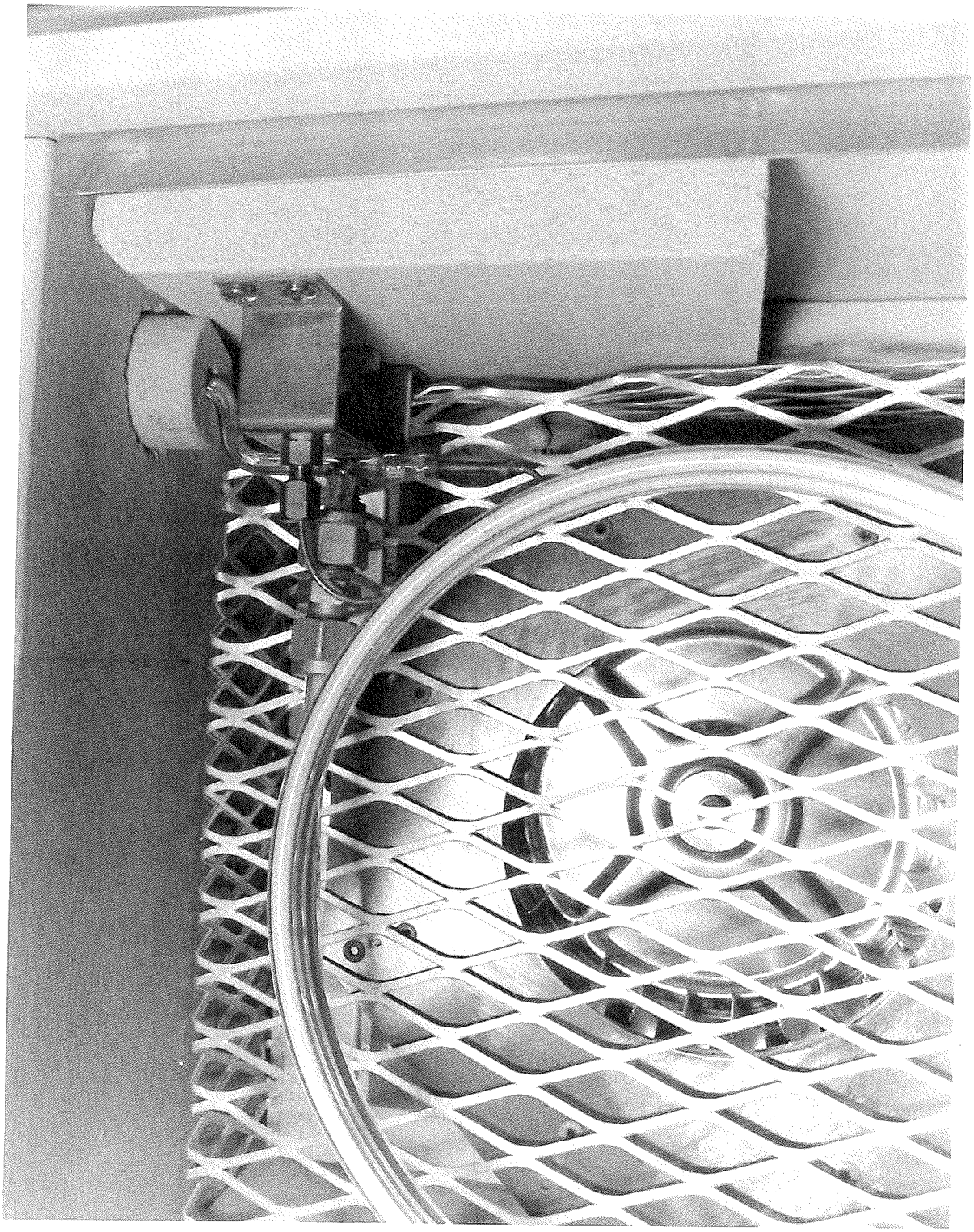


Fig 9. All-glass splitter mounted in the GC oven

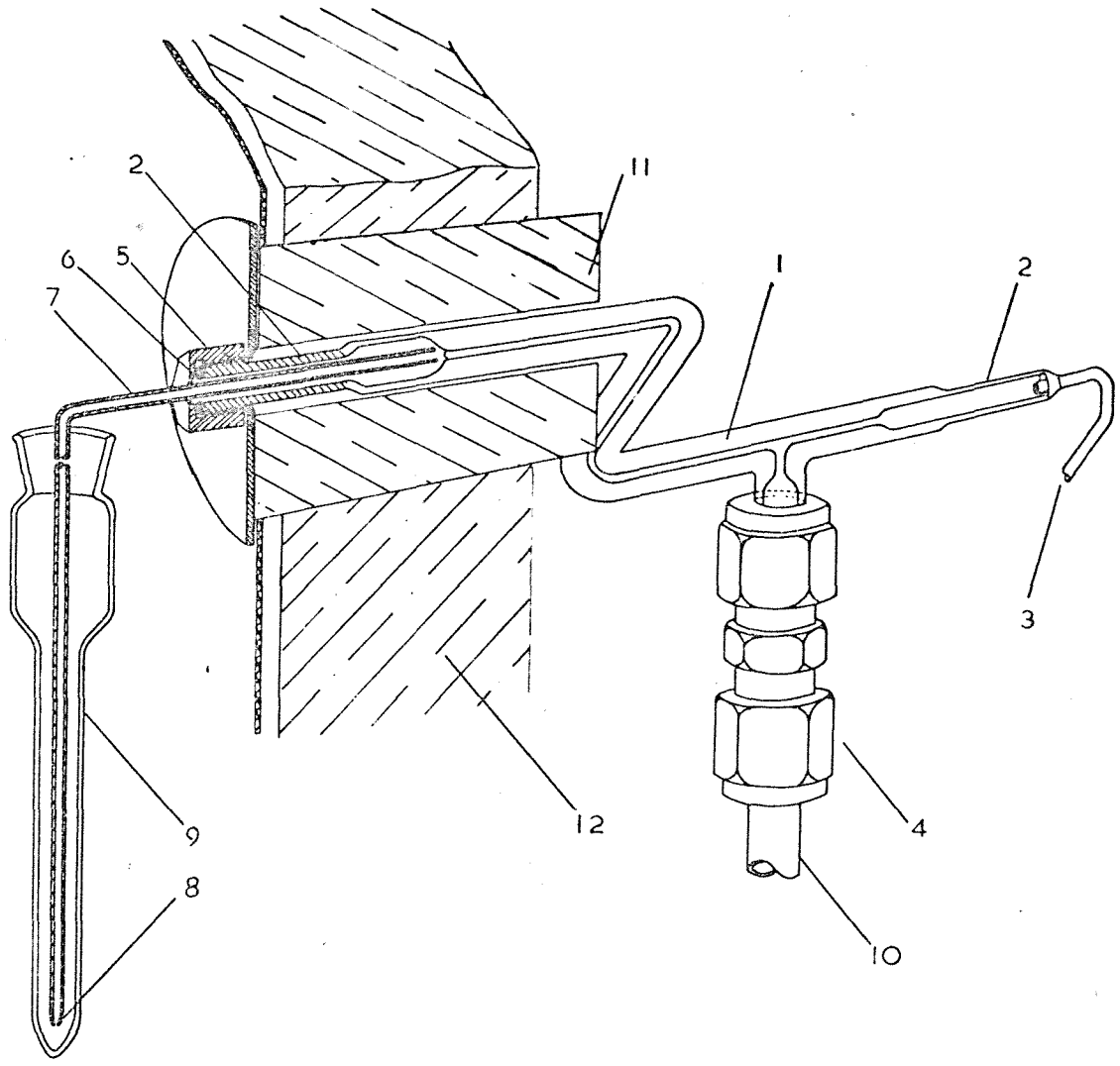


Fig 10. Splitter and trapping system.

1. 0.5 mm i.d. glass capillary
2. Glass/metal seal
3. 0.5 mm i.d. stainless steel tubing
4. 0.25 in stainless steel Swagelok union
5. 0.25 in barrel connector thread
6. 5 mm Viton 'O' ring
7. Transfer line
8. 0.5 mm jet
9. Solvent trap
10. Glass column
11. Injection port heater
12. Oven wall

contamination of consecutive fractions falls into two areas. Firstly the compound must arrive at the detector at the same time as it reaches the exit-port. This was achieved for the Pye 104 with a 100 : 1 split by inserting a 25 x 0.475 mm piece of Nichrome wire into the metal capillary (fig. 10, 3). The split ratio can however be varied between 25 : 1 and 200 : 1 with other sizes of wire. This produced little observable contamination resulting from differences in the time required for the effluent to reach the detector and trap. Secondly, the problem of pre-condensation is overcome by incorporating a removable 1 mm glass capillary transfer line into the trapping system (fig. 10, 7) which is inserted via the glass/metal seal so that it butts directly onto the 0.5 mm bore tubing of the splitter (fig. 10, 1). In this way any compound which pre-condenses in the region of the exit-port heater (fig. 10, 11) is removed each time a trap is changed.

The splitter produces a minimal change in column efficiency: the effect on a mixture of terpenes is shown in fig. 11, there is some peak broadening, resulting in a loss in resolution between neral and α-terpineol (peaks A and B). However, baseline separations are still retained and peak tailing is slight.

2.7.2 Methods of Trapping and Trap Design

Efficient trapping of column effluent has proved a major difficulty in preparative GC and many schemes have been reported in the literature. Most of these methods entail solvent free trapping onto an inert substrate such as silica, alumina or charcoal[73,74],

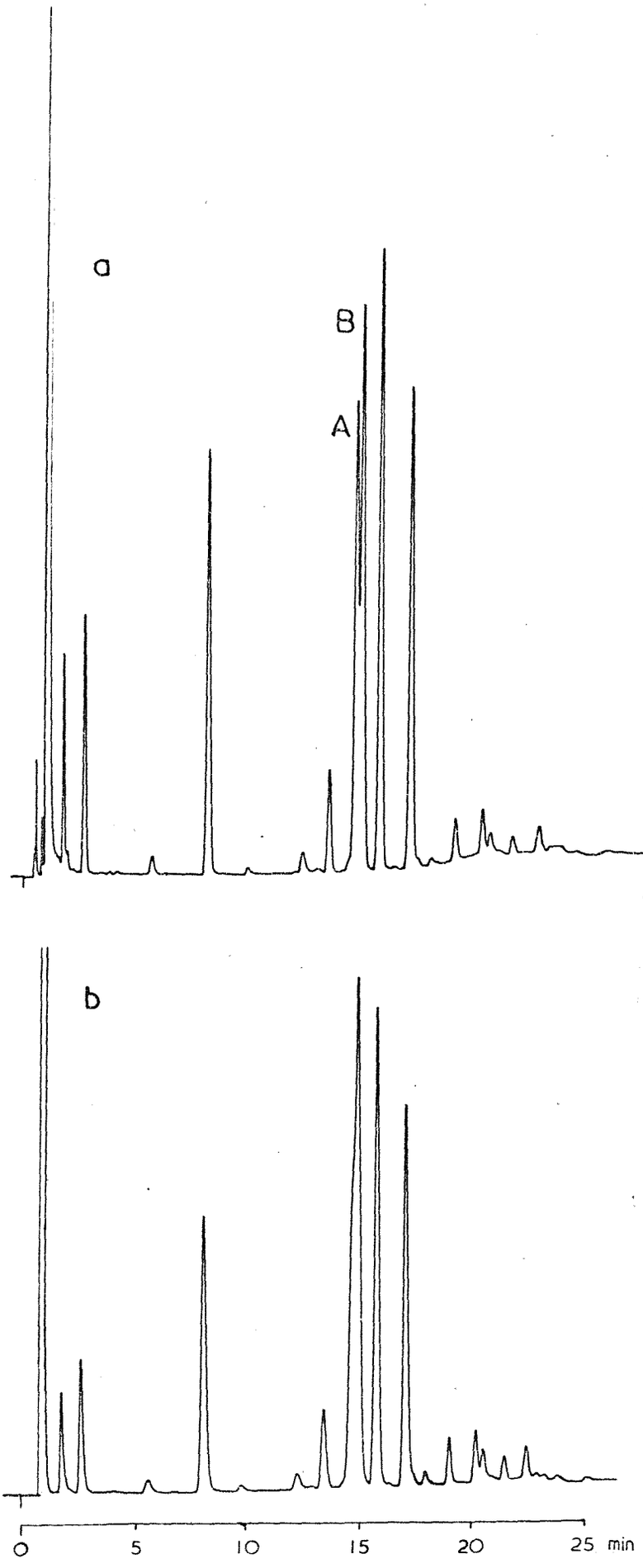


Fig 11. Effect of the splitter on column resolution; (a) without splitter; (b) with splitter.

organic polymers including XAD4[75], Poropak Q and Chromosorb 102[76] have also been used. All these absorbants were used in an attempt to find a method suitable for trapping. Unfortunately they proved too difficult for routine work, requiring storage under an inert atmosphere after pre-treatment at high temperature and low pressure to desorb contaminants. Even when this procedure was followed the trapped samples were found to contain impurities originating from the absorbant. A further disadvantage was the low percentage recovery by solvent desorption due to excessive surface activity of these materials[77]. Thermal recovery was no more successful and the organic polymers were particularly poor in this respect as the high temperatures required produced artifacts from the ensuing bleed.

The trapping method finally devised entailed passing the column effluent through a depth of cooled solvent. The trap consisted of a B10 ground-glass socket (Quickfit, Stone, Staffs) joined to a length of 4 mm i.d. tubing (fig. 10, 7). The trapping efficiency was found to depend upon the depth of solvent through which the sample passes. When filled with 300 µl of solvent, the minimum required for NMR, the trap described enabled samples to be recovered in good yield. Advantages of this design are two-fold, firstly, samples can be sealed for storage without transferring it from the trap, and secondly, the change in diameter prevents the solvent from being blown out of the trap by bubbling.

Trapping efficiencies were usually greater than 90% for a wide range of compounds at flow rates between 25 and 100 ml/min.

Table 3 lists typical recoveries achieved under varying conditions of sample size and solvent. Volatile hydrocarbons and samples

TABLE 3

Trapping Efficiencies

Compound	Total Sample Size[a]	Solvent	Percentage Recovery[b]
Pinocarvone	12 mg (20)	CCl_4	90
Chrysanthanone	15 mg (20)	CCl_4	90
α-Pinene	1 mg	Hexane	82
p-Cymene-8-ol	750 µg (6)	CCl_4	98
Verbenone	600 µg (6)	$CDCl_3$	97
Myrtenol	120 µg (2)	$CDCl_3$	94
Eucarvone	80 µg	EtOH	95[c]
α-Terpineol	75 µg	$CDCl_3$	94
Naphthalene	60 µg	CCl_4	98
α-Phellandrene	50 µg	Hexane	84[c]
Terpinen-4-ol	40 µg	$CDCl_3$	94
Verbenone	10 µg	$CDCl_3$	88[c]

a) Number of injections used to obtain total sample is shown in brackets.

b) Figures do not include sample lost to the detector.

c) Samples collected at room temperature.

prepared by repetitive trapping into the same solvent were generally obtained in slightly reduced yield. Efficiencies were estimated, without correction for material lost to the detector, by measuring peak areas of reinjected samples and by comparison of their Ultra-violet (UV) absorbance with standard solutions.

Samples have been trapped successfully into most solvents commonly used in NMR, IR and UV spectroscopy, with no apparent change in efficiency. For NMR samples of less than 300 µg, solvent purity is critical and cooling the trap to -15°C was found to lead to some condensation of water vapour in the solvent. This was avoided by trapping at room temperature, though there is a small loss in efficiency (Table 3). The system is readily used for solvent-free trapping by cooling the glass transfer line with a dry-ice boat held 1 cm from the exit-port nut (fig. 10, 5)[65]. The efficiencies were much lower by this method and were generally around 50-70%, decreasing still further with high flow rates.

The extraction and purification of volatile materials from insects often results in considerable losses during work-up of the crude extract. Pure compounds have been obtained in high yields directly from insect preparations using solid injection techniques[51,55]; however, fractions obtained by these methods are sometimes contaminated with small quantities of water from the insect tissues, requiring further purification.

The all-glass splitter and trapping system fulfils the criteria laid down for a viable preparative method. Although

primarily designed to meet the requirements of work with natural products, it has proved extremely useful in synthetic work for isolating small quantities of pure product for spectroscopic analysis prior to large scale purification.

3. SPECTROSCOPIC METHODS FOR MICROSCALE STRUCTURAL ELUCIDATION

3.1 Combined Gas Liquid Chromatography-Mass Spectroscopy

Mass spectroscopy which is capable of analysis at the microgram level is one of the most sensitive spectroscopic method available for structural analysis[37]. The combination of gas liquid chromatography with a mass spectrometer provided enormous potential for the direct analysis of complex natural mixtures. However, there is a basic incompatibility between the instruments since the mass spectrometer operates under high vacuum, while large volumes of gas elute from the GC at atmospheric pressure. Several types of interface have been developed to overcome this problem[38], all working on the principle of sample enrichment by selective removal of the carrier gas. The spectrometer available during these studies was an AEI MS 12 to which a Pye 104 oven was interfaced with a single stage Llewellyn silicone membrane separator. Although this system had proved suitable for synthetic work, it was found to be unsatisfactory for microscale natural product work.

Preliminary studies to determine the sensitivity of the combined systems indicated two major drawbacks. Firstly, the interface has a maximum operating temperature of 200°C, above which the membrane softens too much for efficient enrichment, producing a high background bleed in the mass spectrometer. Secondly, a more serious disadvantage was the large sample size required before usable spectra could be determined for compounds other than hydrocarbons.

An average of 50 ug, and as much as 300 µg of acids, had to be injected; below these threshold levels compounds were absorbed on to active sites in the Llewellyn separator and transfer line. Compounds also appeared to dissolve in the membrane, displacing previously absorbed compounds which produced unacceptably high background spectra at high sensitivity. The interface was silanised with 'Silyl 8' in an attempt to decrease the number of active sites with only marginal improvement.

While the Llewellyn separator was satisfactory for studies where sufficient material was available for analysis, its sensitivity was too low for microscale analysis. Consequently in this study a second system was developed which was capable of high sensitivity and which could be used in parallel with the existing system (fig. 12). A commercially available Watson-Biemann porous glass separator was adapted for this purpose. Originally, this system incorporated a metre length of metal capillary, heated resistively, to transfer the column effluent to the separator. This line also acts as a primary restrictor to produce a pressure drop between the column and separator, a second glass restrictor forms an integral part of the Biemann interface. This restrictor is of critical importance in producing viscous gas flow through the separator which is important for effective sample enrichment. A glass restrictor was fitted in place of the interface line in an attempt to eliminate the problems of sample degradation which could arise from contact with metal. The first replacement, which was required to produce the same pressure drop as the metal line, incorporated a capillary with such a small

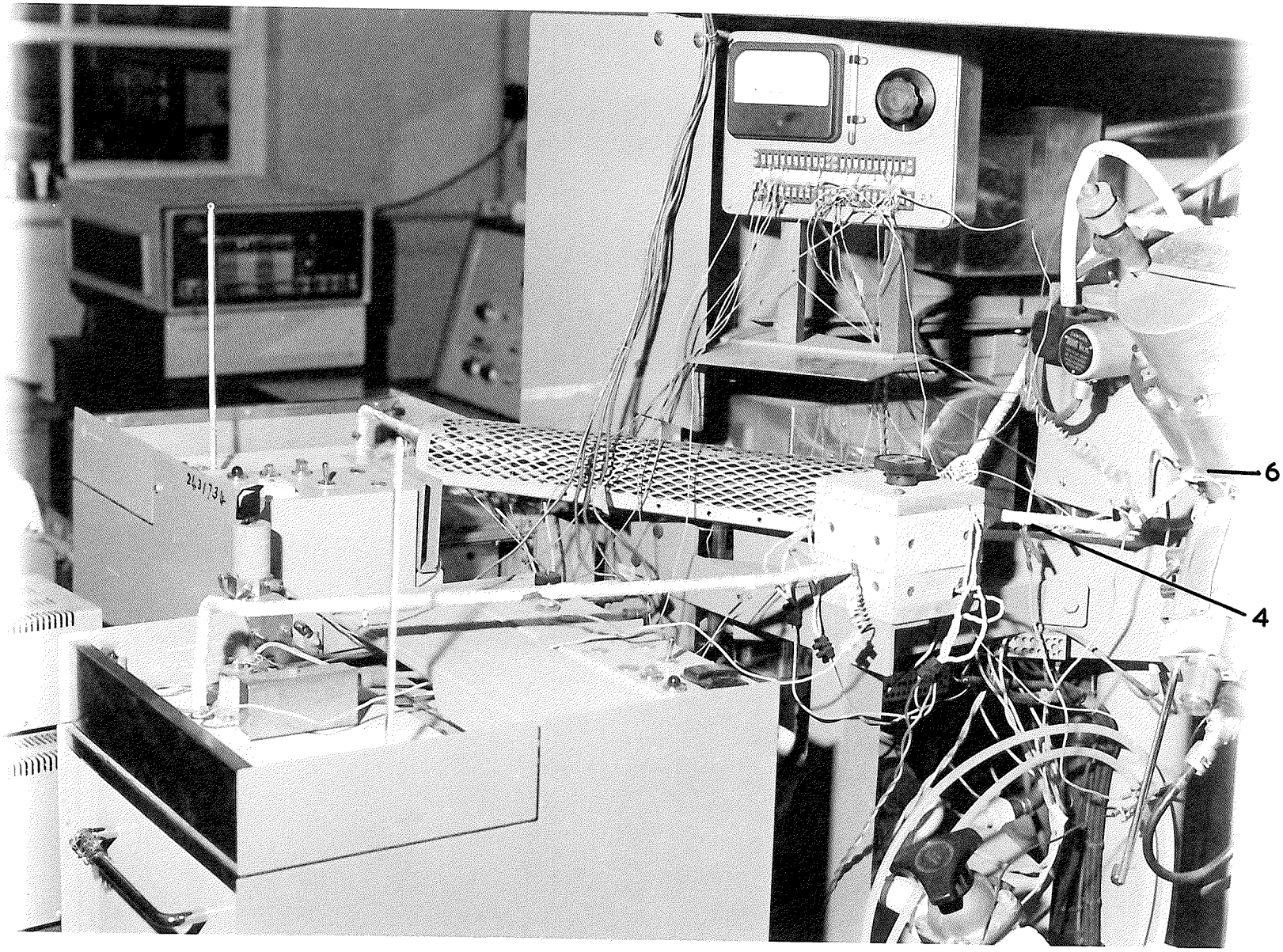


Fig 12. Dual GC–MS system.

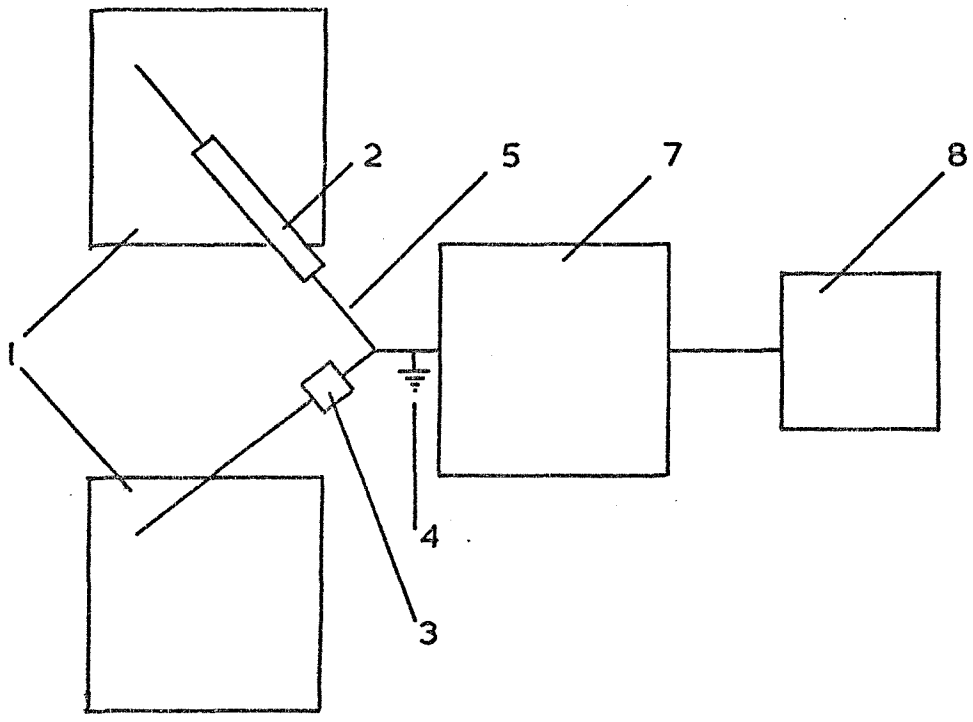


Fig 13. Schematic diagram of the dual GC-MS system

1. Pye 104 GC ovens
2. Watson - Biemann interface
3. Heated valve
4. Earthed tungsten electrode
5. Heated interface line
6. Mass spectrometer source
7. Mass spectrometer
8. Data system

diameter that it frequently blocked. This difficulty was solved using a much longer length of thick walled glass capillary of wider bore, large enough to allow the particles responsible for previous blockages to pass through. The background due to the Llewellyn separator was eliminated by isolating it from the MS source with a heated valve placed in the transfer line between it and the source re-entrant (fig. 13, 3).

The new system was very much more sensitive than the Llewellyn device, but two problems were revealed during operation. There was a tendency for the 8kV source to produce an arc along the transfer line which resulted in cracking on several occasions. This was prevented by sealing an earthed tungsten wire into the transfer line just outside the source re-entrant (fig. 13, 4). The total ion monitor trace of mixtures was not as well resolved as the corresponding GC trace and showed considerable peak-tailing of compounds with long retention times. A closer inspection of the system revealed uneven heating of the transfer line (fig. 13, 5), with temperature differences as large as 30° at some points. Cold-spots have been reported previously as a factor responsible for peak broadening and tailing[78], and rewinding the heater considerably improved the total ion monitor trace. Sensitivity and resolution were further increased by silanising the line and separator with dimethyldichlorosilane (DMCS). It was essential to treat the system while the source was open for repair to prevent contamination of the spectrometer interior, periodic retreatment was necessary to minimise problems caused by active sites.

The sensitivity of this system was now superior to the Llewellyn separator and good quality spectra of polar compounds could be obtained on an average of 300 ng. Highly polar compounds such as acids were still absorbed and spectra could not be recorded on less than 5 µg of sample. Acceptable spectra were obtained at these levels using 4 mm i.d. columns, but the best spectra resulted from 2 mm i.d. columns, particularly with very complex mixtures due to their better resolution and higher efficiencies.

Chlorinated solvents had an undesirable effect in GC-MS which became a problem with the introduction of the data acquisition system. Spectra of compounds dissolved in these solvents had large ions at mass to charge ratio (m/e) 36 and 38, resulting from the formation of hydrogen chloride either on the GC column or in the mass spectrometer. At high sensitivities these ions were the largest present and their persistence in the background for several minutes after the solvent peak meant that most of the spectra taken during this period were incorrectly normalised to base peak m/e 36.

3.1.1 Mass Spectrometer Data System

The data system consisted of a PDP 8E computer with a 16k store coupled to the mass spectrometer by a digital interface. This permitted storage up to forty eight spectra of 230 peaks before output was necessary. The peak times are determined by a centroid calculation and converted to masses using their absolute times with the m/e 28 ion as reference peak. Accuracy was nominally $\pm$ 0.25 amu, although this was dependent upon the reproducibility of the magnetic

scan. Perfluorokerosene was used as the calibrant and gave a working range up to m/e 530.

The input and output threshold levels are controlled by pre-set parameters which are changed by a typed instruction to the computer. Spectra input and output can either be by single or sequential scanning up to a figure determined by the operator.

The data system presented several operational difficulties when first installed, some requiring a considerable amount of time to overcome to meet the requirements of these studies. Although the data system has a nominal accuracy of $\pm$ 0.25 amu, this was only periodically achieved at high mass. The mass spectrometer magnet was found to suffer from hysteresis effects which created a 'new' set start value for the magnetic scan after each spectrum. In turn, this reverted to a stable value over a period of 15 seconds. Therefore, small errors in the expotential magnetic scan from high mass resulted in large errors in calculated mass by the iterative process based on m/e 28. This could be minimised, but not eliminated, by running spectra under identical conditions to those used during calibration.

The input threshold, which is set at a level sufficient to cut off amplifier noise and short term base line drift without loss of signal, posed an operational difficulty at high sensitivity. Multiplier settings during GC runs were critical with very small samples containing components of varying size, under these conditions many ions present have intensities just above the noise level. If the electron multiplier is set at too high a level an effective

threshold is set in the data system at 5-10% of the base peak. On the other hand, too low a setting takes the amplifier noise above the threshold, exceeding the raw data buffer with subsequent loss of the spectrum. Standardisation of sample concentration whenever possible, and determining the multiplier settings required for a given size peak on the total ion monitor trace with a series of standard solutions, minimised losses of spectra by operator error.

Despite the remaining difficulty of magnet hysteresis, the data system presents a considerable improvement in mass spectrometer use, providing a permanent record and reducing the time required to obtain normalised spectra.

3.1.2 Mass Spectra and Mass Spectrometers

It is commonly recognised that different mass spectrometer designs utilising the same method of ionisation produce slightly different spectra with certain classes of compound, while spectra run under the same conditions on the same spectrometer are expected to be almost identical. This was true for the MS 12 with certain exceptions, notably allylic alcohols and aromatic alcohols.

The spectrum of p-cymene-8-ol normally obtained from the MS 12 (fig 14A) is very similar to that reported in the literature[79]. On two separate occasions different spectra of this compound were reproducibly recorded over a period of several weeks (fig. 14B and C). The only common factor to the appearance of spectra B and C was that each began to occur just after repairs to the source. This effect

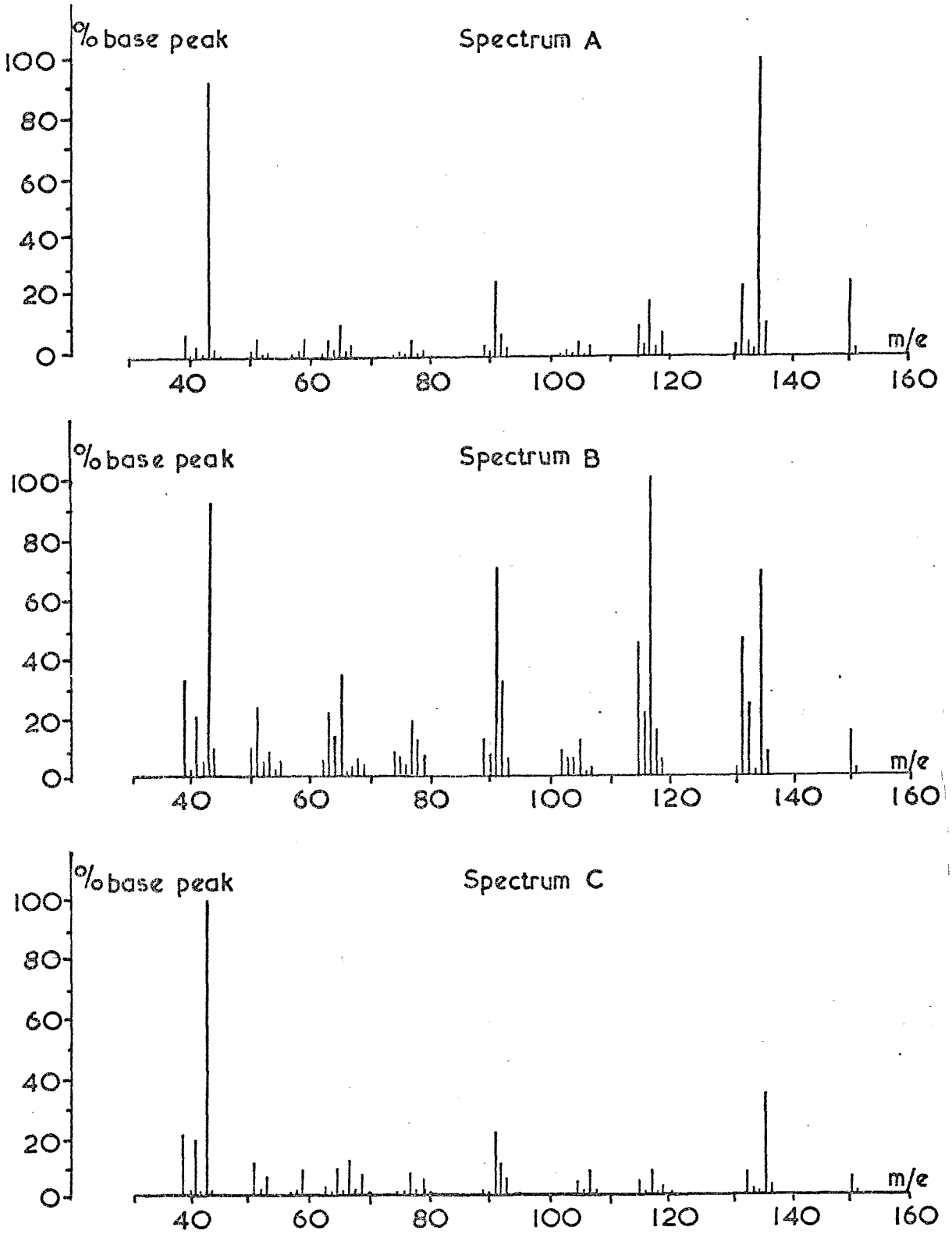


Fig 14. Changes in the mass spectrum of p-cymene-8-ol caused by repairs to the spectrometer source.

was the result of poor re-alignment of the source after cleaning. Similar observations have been made during studies on another allylic monoterpene alcohol[80]. Large differences in the spectrum of a compound can be produced by the same spectrometer, in addition to the variation caused by different instruments. This demonstrates the care with which structural assignments are made by mass spectroscopy. When such identification is made, the spectrum of the unknown should be re-run under the same conditions as the reference compound.

3.2 Ultraviolet and Infrared Spectroscopy

Ultraviolet spectroscopy (UV) requires only micrograms of material for chromophores with extinction coefficients above 1000, making it a true microscale technique. However, it has been universally ignored in microscale structural elucidation studies.

Ultraviolet spectra were determined on a microscale by trapping between 10 and 50 µg of sample, by preparative GC, into either spectroscopic hexane or ethanol and the solution made up in a small volumetric flask. The concentration of the sample was initially determined by comparison of GC peak area with that of a standard solution of a mono-oxygenated monoterpene e.g. verbenone. The FID response is sufficiently similar for mono-oxygenated monoterpenes for extinction coefficients to be determined sufficiently accurately for chromophore identification. Once the structure of a compound was fully established, the value was recalculated by comparison of GC peak areas with a standard solution of a synthetic sample of the compound.

Unlike UV, infrared spectroscopy (IR) has been adapted frequently for microscale work, either by the use of a spectrometer coupled directly to a GC[39,40], or by use of micro-cells or KBr discs[81,82]. Silver chloride micro-cells, designed to utilize the whole of the IR beam, were used in conjunction with a beam attenuator to determine the feasibility of this technique. The sample size of 500 µg needed to record spectra of mono-oxygenated monoterpenes was too large for the quantities available from the natural sources under investigation. Difficulties were also experienced in filling and emptying the cells, particularly the latter operation when the sample was invariably lost.

Effective use of IR spectroscopy on microgram samples requires either a very long cell path length, as used in the systems directly interfaced with a GC system or beam condensers. Since neither of these were available in this study this technique was largely ignored.

3.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) by continuous wave is not a viable technique for microscale applications, even with micro-cells and computer averaging time (CAT) techniques, the Varian HA 100 spectrometer required samples in the order of half a milligram. The extended use of the CAT technique was restricted by magnet instability, after 100 scans yielding a tenfold increase in sensitivity, severe loss in resolution was experienced.

After installation of a Varian XL 100 spectrometer, single

scan continuous wave spectra could be recorded on 400 ug using a 5 mm NMR tube. The Fourier transform (FT) mode of operation presented the possibility of proton NMR on microgram samples.

3.3.1 Solvent Purity in Microscale FT-NMR Spectroscopy

A series of experiments was undertaken before NMR's were run on the natural samples to determine the minimum quantity needed for spectra capable of unambiguous interpretation and the conditions required for this mode of operation. When acquisition of spectra involved a large number of pulses, maximum resolution was attained with an internal deuterium lock. The preferred solvent for this purpose was deutero-chloroform. At these levels of sample concentration, both isotopic purity and general solvent purity are important. Only 'Gold Label' d-chloroform (99.8%) (Diaprop Inc., Milwaukee) of the 99.8% isotopically pure deutero-chloroform solvents tested had sufficiently clean FT-NMR spectrum for microscale work. After 20,000 pulses the only resonance, in addition to the $CHCl_3$ proton was that of residual water. This was present in smaller amounts than in the other chloroform solvents.

The presence of water presents considerable problems with samples in the order of 50-100 μg. At this level of concentration water protons resonate between 8 and 9 τ, with a peak size in the same order of magnitude as those observed for methyl groups at the sample concentrations used. The water content was reduced as much as possible by drying the solvent with activated 3A molecular sieve immediately before use and by trapping samples at room temperature

during preparative GC (see Section 2.9.2). Carbon tetrachloride was far less hygroscopic than deutero-chloroform, but had the disadvantage of requiring the ^{19}F external lock. This is a far less satisfactory procedure, particularly when a large number of transforms are acquired since the lock is more susceptible to drift.

3.3.2 Conditions for FT-NMR with Microgram Samples

Fourier transform conditions were determined for solutions of verbenone, myrtenol and carvone, which were representative of the type of compounds to be investigated. Initial spectra on 50 µg of verbenone in 300 µl of deutero-chloroform, the minimum quantity of solvent required were very encouraging. The methyl resonances were strong with narrow line width, the 2 Hz splitting of the vinyl methyl group clearly showing as did the fine splitting of the vinyl proton. However, the methylene envelope resonances were almost completely absent, this was also observed in the spectra of myrtenol and carvone. The relaxation time of these protons is a possible reason, though no data appears to be available on this subject. Increasing the acquisition and pulse time from the original values of one and four seconds respectively, to two and twelve seconds, resulted in the recording of these resonances with no adverse effect on the rest of the spectrum.

Spectra run under these conditions were readily recognisable and compared very favourably with the corresponding continuous wave spectra (fig. 15). The lower limit at which identifiable spectra could be obtained for mono-oxygenated monoterpenes was 25 µg in 300 µl of solvent (0.165 µmoles).

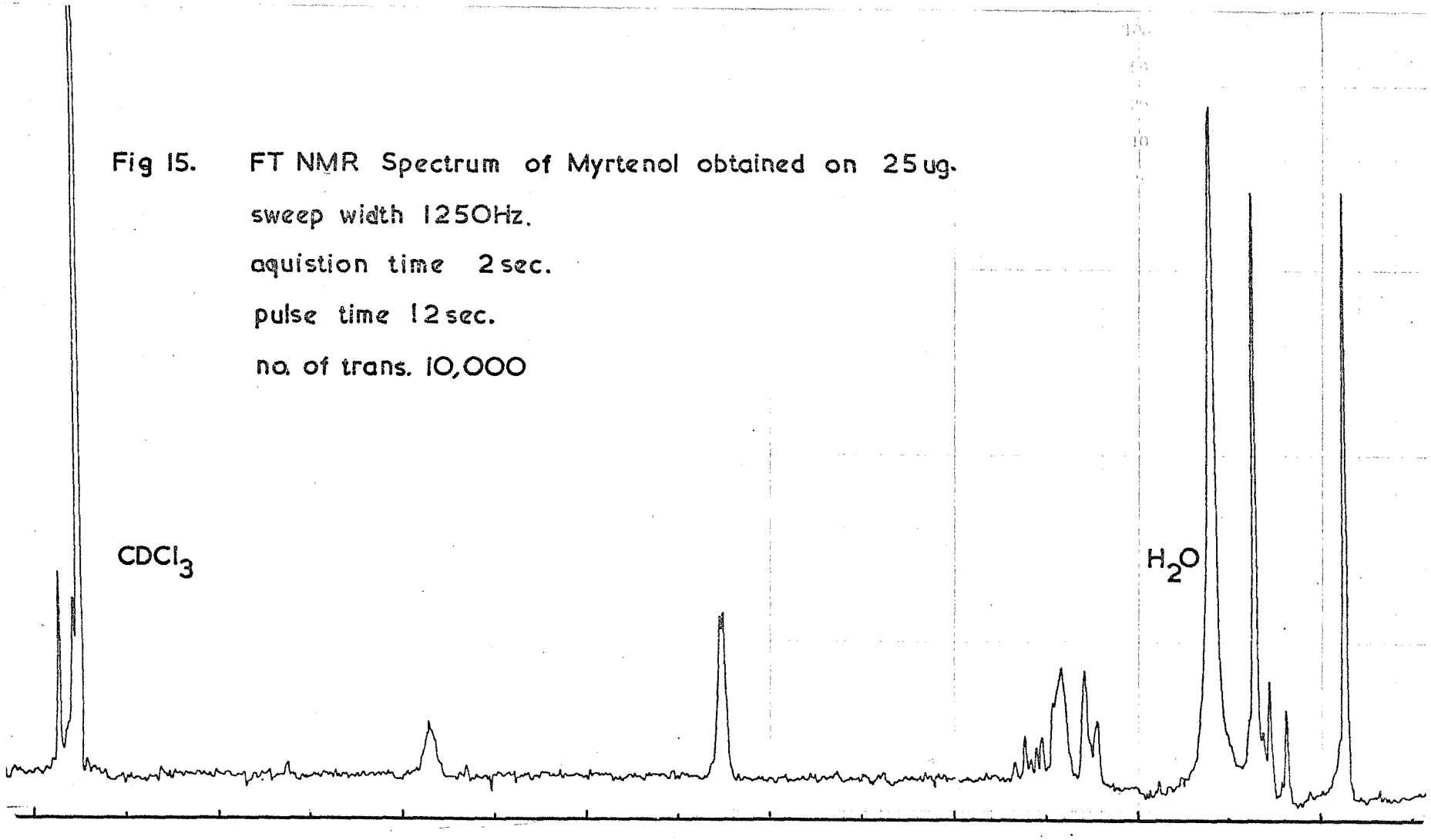


Fig 15. FT NMR Spectrum of Myrtenol obtained on 25 ug.
sweep width 1250Hz.
aquistion time 2 sec.
pulse time 12 sec.
no. of trans. 10,000

3.3.4 FT-NMR Samples by Micropreparative GC

The technique which has commonly been used in recent years for analysis of complex mixtures of natural products has been GC-MS. This technique can be used to great effect for identifying previously known structures and a considerable amount of information of unknown compounds can be gained by studying their fragmentation patterns. With certain types of compounds this may be sufficient for complete identification. Monoterpene hydrocarbons have very similar mass spectra which makes such identification almost impossible. Even when differences are present in the spectra, as with the oxygenated monoterpenes, the fragmentation patterns are usually too complex for complete analysis.

Fourier transform NMR enables a new technique to be applied to the problems encountered in this area of study. When samples are prepared with an efficient GC micropreparative system, structural analysis on micrograms of material can be made with a greater certainty than with GC-MS alone.

Spectra of samples trapped by GC generally suffered from poorer resolution than those recorded using the standard solutions, due probably to small dust particles blown into the solvent from the GC-column. Great care had to be taken to prevent contamination of the sample with impurities arising from small quantities of material bleeding off the column from previous GC runs. It was not possible to eliminate completely column contamination and together with the slightly poorer resolution, the lower limit for samples from

preparative GC was nearer 50 μg, however, useful spectra could still be recorded on smaller amounts. Despite these minor difficulties, FT-NMR on small quantities was so effective that complete structural identification, impossible by the other techniques used, was made on two of the minor components by interpretation of their FT-NMR spectra.

4. BIOLOGICAL STUDIES ON H. bajulus

4.1 Behavioural Studies on the Cerambycidae

In order to establish the role played by chemical factors in the behaviour of Hylotrupes bajulus, various aspects of its life cycle have been studied. The sexual behaviour of the family Cerambycidae has been reviewed by Butovitsch[83] and Linsley[16,84] and a comparative study has been published for the sub-family Lepturinae[85]. A study has also been published on the evolution of tactile stimulatory actions in longhorn beetles[86]. Although no specific studies appear to have been made on H. bajulus, an investigation of the sexual and fighting behaviour has been reported for the sub-family Cerambycinae to which the House longhorn belongs[17].

4.1.1 Sexual Behaviour of Hylotrupes bajulus

Studies on the sexual behaviour of H. bajulus were made under laboratory conditions with insects cultured by the method of Berry[82] (see section 4.4). Field studies were not possible as the beetle is not widespread in this country, the infestations being largely limited to house timbers. Observations of the behaviour of unmated beetles were made using glass tanks fitted with a glass lid. Results of these observations are summarised in fig. 16.

The beetles normally gathered on the blocks of Pinus sylvestris placed inside the tank. The males would remain inactive for five to ten minutes, after which they exhibited increasing signs of activity, particularly vibratory antennal movements. The male

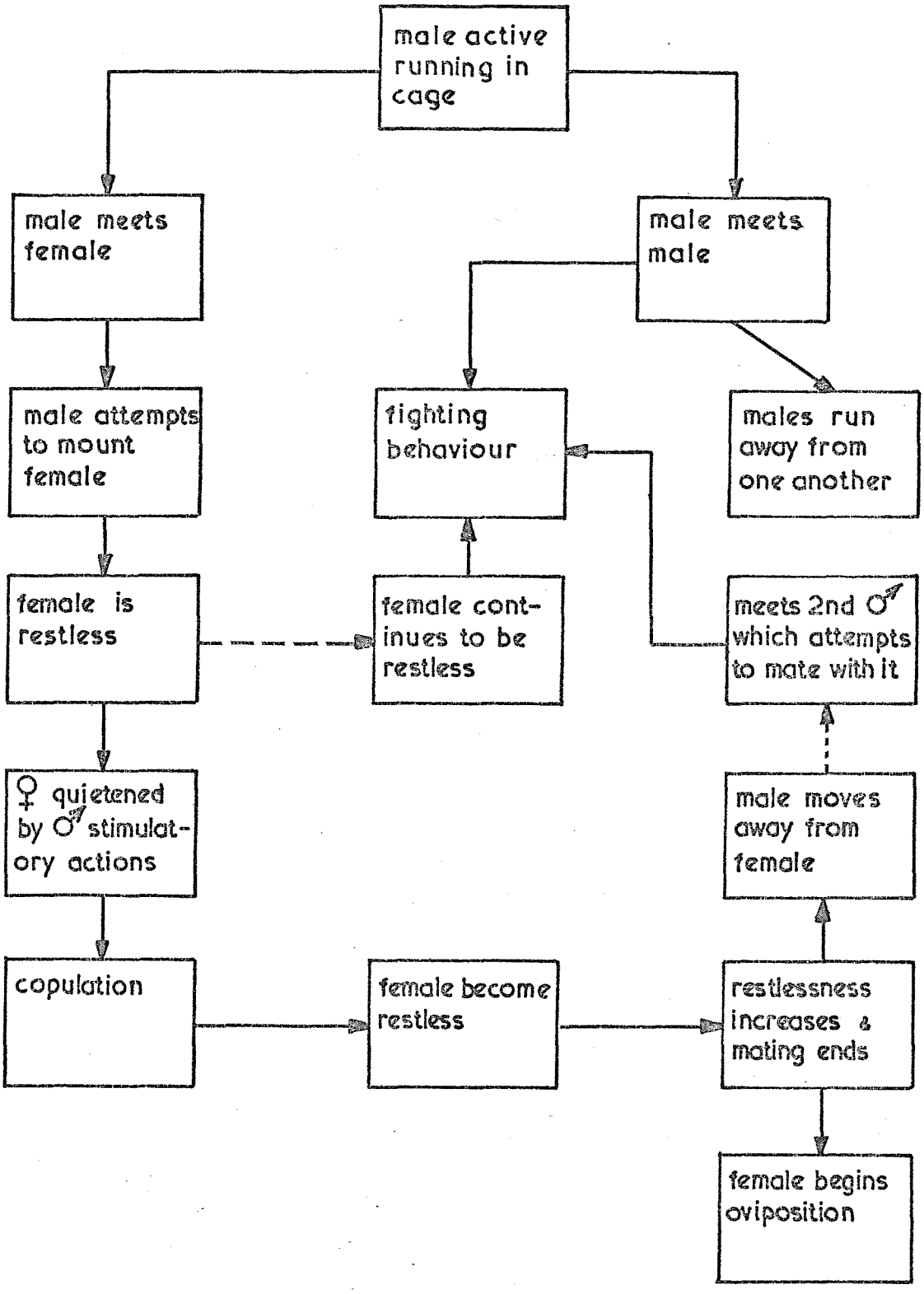


Fig 16. Schematic representation of the behaviour of H. bajulus adults.

then begins moving, antennae still vibrating, in the general direction of a female. At a distance of 1-2 cm from another beetle it usually stops with antennae oscillating rapidly. When the beetle is male they normally run away from one another. If the beetle remains immobile, the first beetle will advance to less than 1 cm, and if the stationary beetle is a male, fighting behaviour usually results. When the second beetle is a female, the male mounts it in an attempt to copulate. Unlike other members of the sub-family Cerambycinae the female H. bajulus does not start running away when approached by another beetle. This suggests that the stimulus provided by a retreating abdomen, used by other species in this sub-family as a sex recognition signal[17], does not play a role in the sexual behaviour of the House longhorn beetle. In contrast to the activity of males, females of this species do not usually react to the close proximity of another female unless they touch, when they will rapidly move off in opposite directions. Other than instances of this type, females of H. bajulus are totally inactive until after copulation.

Although attraction of a male to a female is strongest at distances of 1-5 cm, the presence of a female will elicit a response from a male over distances of 30-50 cm in still air. At these distances the antennae of the male begin to vibrate and the tip of the abdomen is repeatedly bent downwards to the position adopted during copulation. The male then becomes very active until it finds a female.

To establish whether the activity of males at long distances was the result of visual or chemical stimuli a number of experiments were run in which direct visual contact between male and female was removed by placing the female in a small opaque container fitted with a gauze top. Male activity was not affected by the loss of visual contact, suggesting the presence of a chemical stimulus emanating from the female. Additional support is lent to this hypothesis by two other observations; namely (a) other males will sometimes approach and attempt to copulate with a male which has just finished copulating, even though normal male-male interactions had been previously observed for both beetles, and (b) virgin females, which have been isolated in cages open to the atmosphere for several days no longer attract males even at short distances, whereas females kept under the same conditions but in small sealed specimen tubes attract males and are capable of copulation and oviposition.

4.1.2 Copulation and Oviposition

In common with most Cerambycids, the male takes the female without her co-operation and mounts by climbing on her back. The female immediately tries to remove the male from her back but is rapidly quietened by actions performed by the male. Tapping the abdomen of the female appears to be the principal action used by the male. Abdominal movements by the male, including pulling the ovipositor have also been observed, particularly when the male first mounts. A number of actions of this type have been reported for other species of Cerambycidae[17,86]. After the male has mounted the

antennae of the female are held back along the body, while those of the male, which vibrate throughout copulation are held out in front and above the female's head. Stimulatory actions appear to play an important function in the sexual behaviour of the Cerambycidae species studied, this is especially true for species such as Cyltrus mysticus (L) where no sex specific odour appears to be present. However, it is not known if variations in stimulatory behaviour together with the restless behaviour of the females operate as an isolating mechanism at species level.

After approximately five minutes the female again becomes restless and makes efforts to dislodge the male by running movements and extending the ovipositor. Initially quietened by stimulatory actions, the male is finally removed from the back of the female by increasingly vigorous movements. In the species H. bajulus the female normally mates several times over a period of two to three days, at the end of copulation a male will sometimes follow a female during oviposition, mating again at periodic intervals.

The search for suitable egg-laying sites begins immediately after copulation. At close range tactile stimuli predominate and the female probes the wood surface with the ovipositor until a suitable crack is found. Eggs are laid in several fan-shaped batches, of which the first is the largest, subsequent batches containing a decreasing number of eggs. The possibility of chemical stimuli in oviposition was suggested by the observation of egg-laying between infested culture blocks and in old flight-holes

in preference to uninfested pine wood. Attempts to demonstrate this more definitively by bioassay were only partially successful as it was not possible to eliminate variation in results caused by tactile stimuli from this group of tests (see section 7.2). Although a quantitative analysis could not be made of the results from this choice test, it was possible to conclude on a qualitative basis that females laid the majority of their eggs between previously infested blocks. It has long been known that hydrocarbons present in pine wood (α-pinene, β-pinene and car-3-ene) act as oviposition attractants in this species[33]. On the basis of this, together with the observations of oviposition, it seemed reasonable to conclude that chemical constituents of the frass acted as an attractant at long range, while tactile stimuli were more important at close range.

4.1.3 Fighting Behaviour

Fighting behaviour, which is quite marked among the Cerambycids and particularly species in the sub-family Cerambycinae[87-89], is also a feature of the behaviour of adult House Longhorns. Observations on fighting behaviour, which were made as part of the study of the sexual behaviour of H. bajulus, showed that it was restricted mainly to males. Within a group some males showed greater aggression than others and would attack on encountering another male whereas the normal male response was to move off rapidly in opposite directions. Even very brief fights can result in the loss of legs and antennae, at the end of which both beetles are very restless and rapidly move about for several minutes. 'Love play', a term used

by Dürr to describe the fighting behaviour between males and females prior to copulation which resulted in loss of limbs by both beetles, was not observed during these studies[24]. However, males did attack if the female was too restless while being mounted with subsequent loss of legs and antennae to the female.

Fighting behaviour in H. bajulus is generally so intense that at the end of bioassays it was normal to find the majority of beetles had lost at least one limb. Although the frequency of fights is probably related to population density they still occurred when only two males were placed in a bioassay tank.

4.1.4 Sound Production by H. bajulus (L)

In common with other Cerambycids[16,84,90] both males and females of H. bajulus stridulate when disturbed, although a number were found to produce little or no sound. The stridulation is produced by friction between the inner edge of the posterior margin of the prothorax and a specially striated area on the median anterior prolongation of the mesosternum. Dissection of beetles which were unable to produce sound showed these areas to be poorly developed, possibly a result of laboratory culturing. The sound produced by both sexes appears to have the same pitch and was audible to the human ear over a distance of 30-40 cm. It appears to serve a purely defensive mechanism and is not associated with sexual activity.

4.2 Sectioning and Dissection

Many species of Cerambycidae have a pair of scent glands

situated in the metasternum near the hind coxae which serve a function in sex location[16]. Results of these studies suggest that H. bajulus do use volatile chemicals in sex location but dissection of the meso and metathorax revealed no glands in this region and all that could be found were leg and flight muscles, tracea and nerve cord. Other dissection work has confirmed the absence of thoracic glandular material[91]. Labial glands are another source of volatile secretions in this family[20] and although such glands have been found in the larvae of H. bajulus[92], it was not possible to demonstrate their presence in the adult by dissection.

Transverse sections of the thorax showed only flight muscle, tracea and nerve. The toughness of the head capsule prevented good sections being made; those that were produced were too badly damaged to show anatomical detail.

4.3 Stereoscan Electron Microscopy of the Antennae

The antennae of the Cerambycine Tetropium castaneum (L) have been reported as having numerous thin-walled setae, described by Schimitschek as chemoreceptors[93]. The antennae of H. bajulus are also reported to possess such setae[24].

H. bajulus beetles of both sexes had antennae which were externally identical and stereoscan electronmicrographs enabled visual classification into four types of setae. The first four segments from the base of the antenna were sparsely covered with very long hairs covering the medial surface (fig. 17a) while the remaining surface of

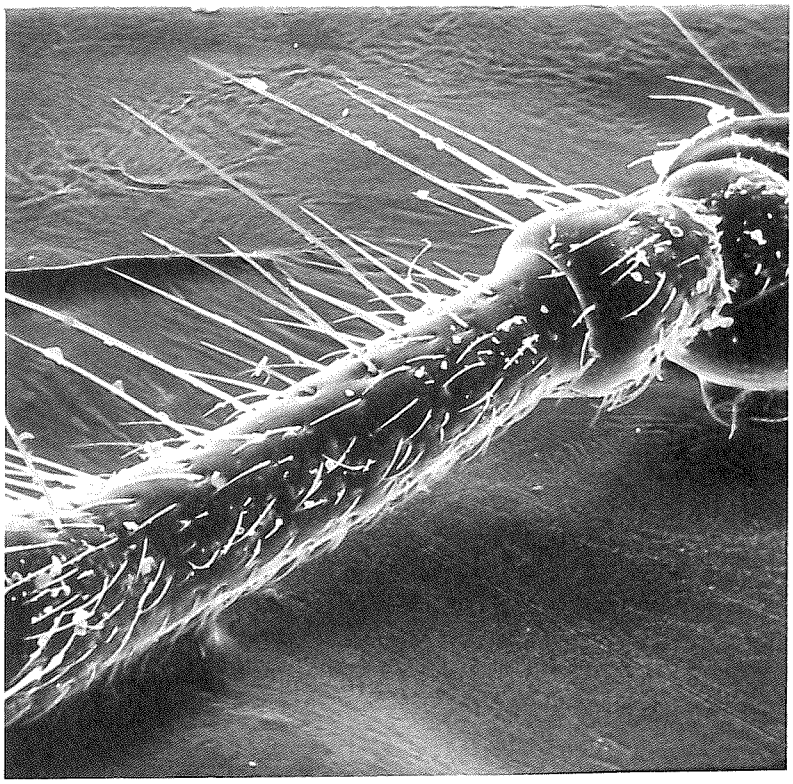
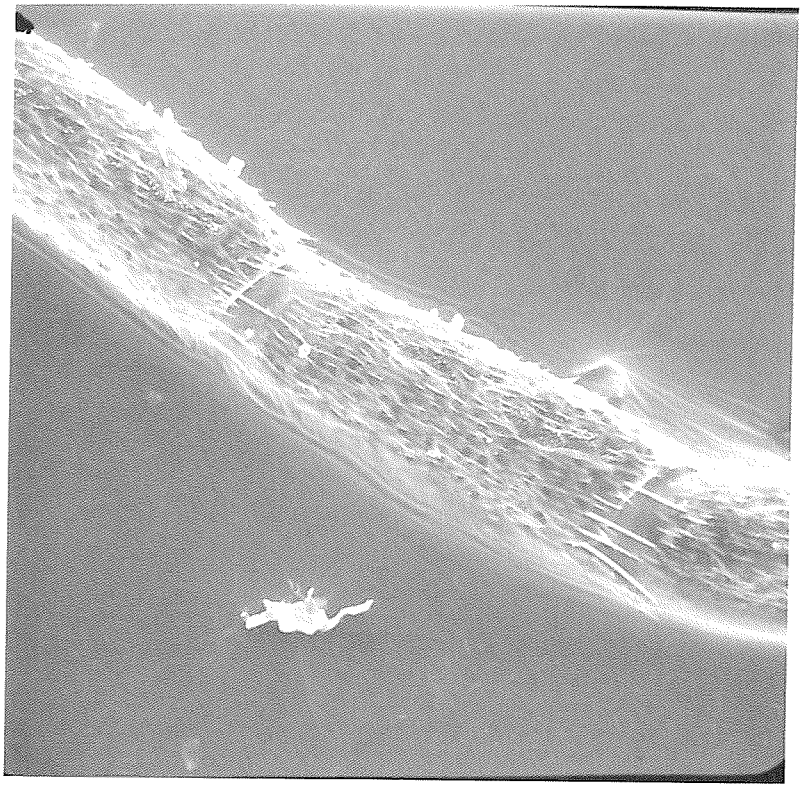
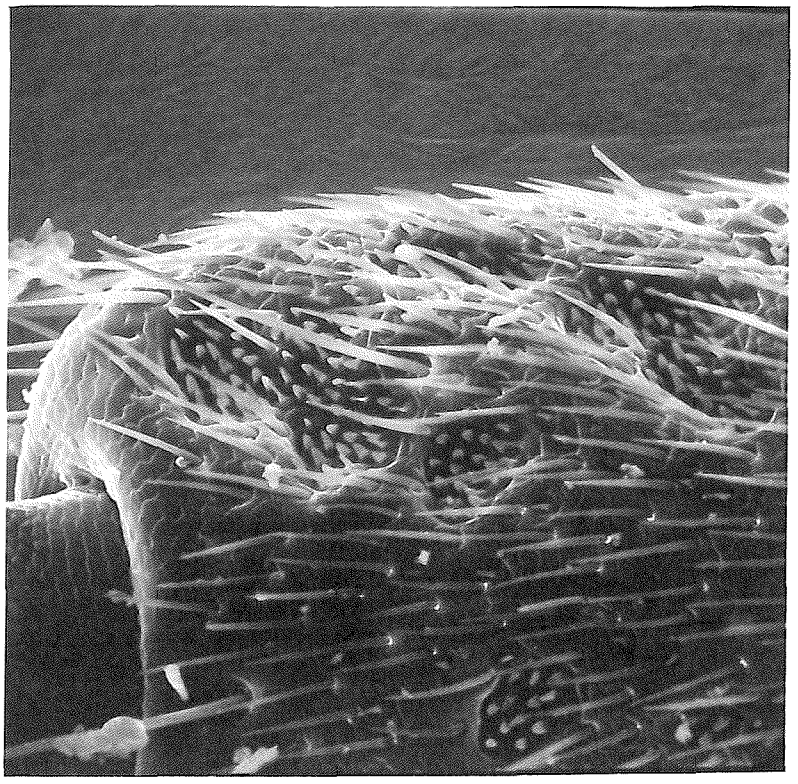


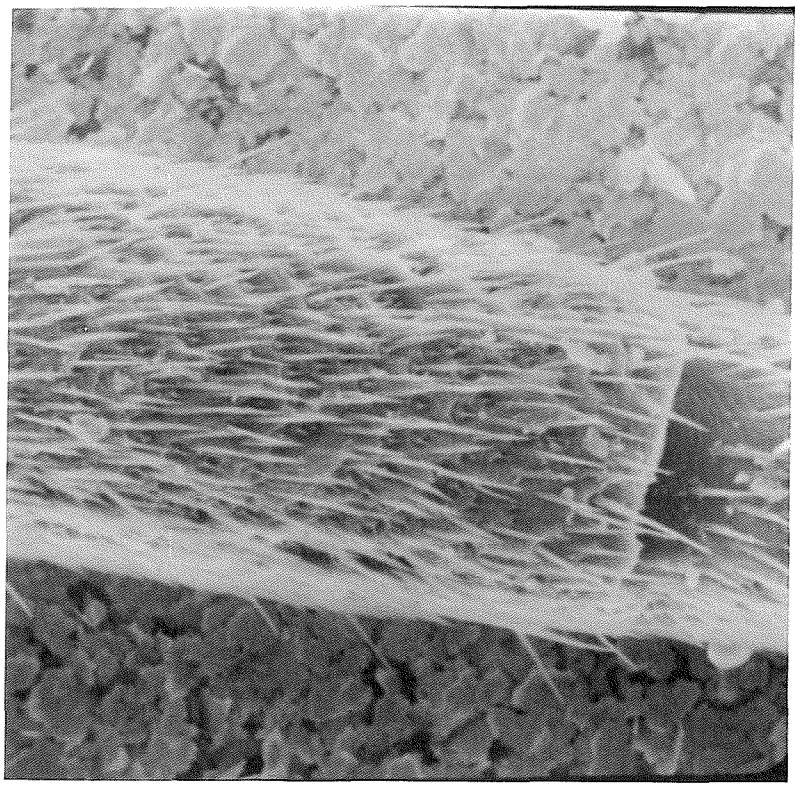
Fig 17a. Base segment of the antenna of H. bajulus x 125



b. 9th and 10th segments showing sencilli in shallow depression along the anterior surface x 125



c. 5th segment showing setae in one of the depressions. x 500



d. Lateral surface of 9th segment showing absence of large depressions containing setae. x 250

of these segments was covered with shorter hairs. These shorter hairs continued, with increasing density, along the remaining segments to the distal tip (fig. 17b). The other two types of sensilla consisted of short setae (fig. 17c), one of which occurred singularly or in groups in shallow depressions, mainly along the anterior surface of the antenna. These setae differed from the other short type as they projected through a small raised ring in the exo-cuticle.

4.4 Laboratory Culturing of H. bajulus

A culture of H. bajulus was established using the method of Berry[94]. The culture used Pinus sylvestris obtained from one source[95] so that variations in chemical composition of the wood were eliminated as far as possible.

The survival rate of larvae was initially very low and many died soon after being placed in the peptone treated blocks used in the first stage of culture. The reason for this low survival rate was thought to be due to dehydration since larvae, particularly when newly hatched, rapidly lose body water[24]. It was not possible to maintain the recommended 85% relative humidity in the culture rooms, therefore eggs and recently emerged larvae were kept in sealed tanks containing trays of cotton-wool soaked in water. Larvae hatched in this way had a survival rate for the first stage of culture of almost 80%.

Beetles from natural infestations were used to supplement the eggs from laboratory culture, this measure was taken to reduce the problems associated with inbreeding[96,97,98].

4.5 Electroantennographic Screening for Physiological Activity

The electroantennograph (EAG) is a technique first used by Schneider for measuring the small electrical potentials generated in the antennal nerve as a result of receptors being triggered by a stimulus[58]. The EAG system developed in these laboratories for allied work on termite repellants (fig. 17)[61] measured the total response of all stimulated chemoreceptors of antennae placed between two glass micro-electrodes.

Although the method was effective as a primary screen for biological activity of extracts and for following this activity during purification, operational difficulties were experienced with the antennae of H. bajulus. It usually required several attempts at fixing the antenna between the electrodes, and more often several antennae before a satisfactory response was obtained. This was probably due to the size of the H. bajulus antennae, since tests with termite antennae produced results almost every time. The antennae of H. bajulus were too big and heavy to be firmly held by surface tension and as a result were frequently dislodged from between the electrodes when a sample was blown across it. Once a good electrical contact was made, results could be obtained for up to thirty minutes and were reproducible from one antennae to another. Antennae of males and females behaved in a similar way, and responses were generally in the order of 50-100 mV for compounds with highest activity at solution concentrations of 100 ng/μl (fig. 19a). The antenna adapted rapidly at these concentrations (fig. 19b) and a

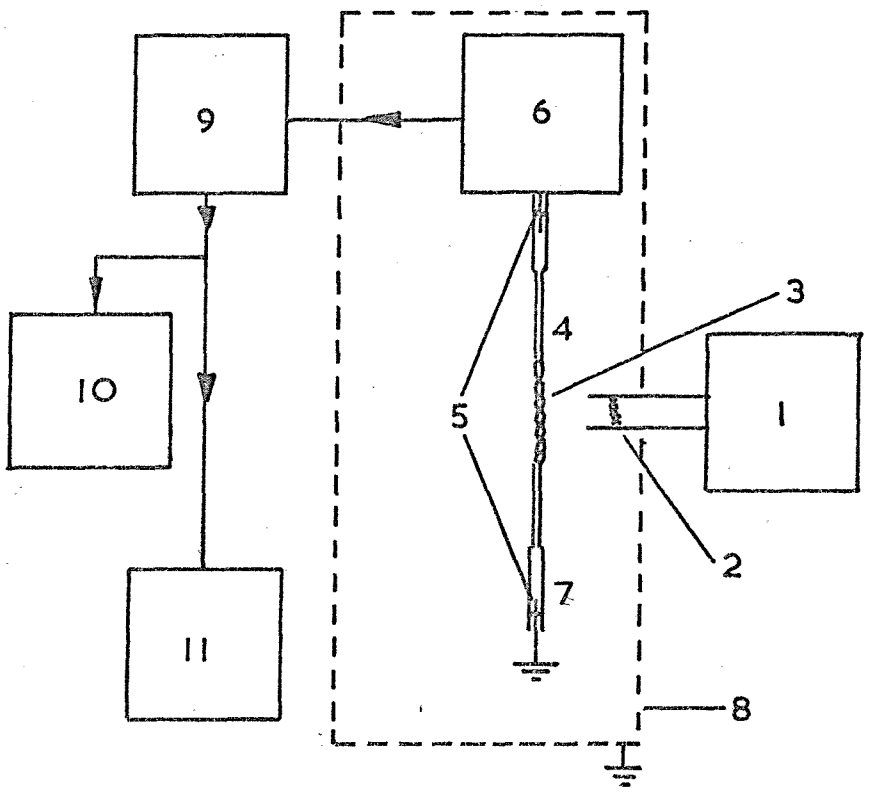


Fig 18. Schematic diagram of the Electroantennograph system.

1. Solenoid controlled gas puffer
2. Sample on filter paper
3. Excised antenna
4. Glass micro-electrodes mounted on micro-manipulators and filled with insect ringer solution
5. Silver wire
6. FET pre-amplifier
7. Floating electrode
8. Earthed cage
9. Amplifier
10. Oscilloscope
11. Spray-pen recorder

twenty second delay between samples was necessary before full response was reattained.

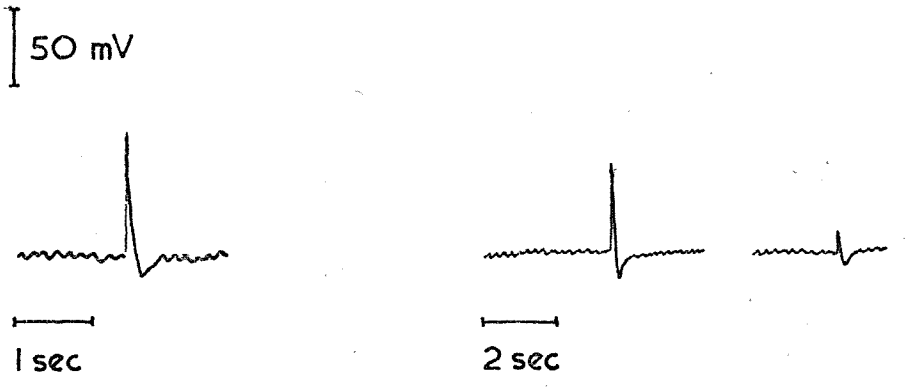


Fig 19a. EAG responce to (-)-verbenone.

b. Effect of antennal adaption on EAG responce at 100 ng/µl.

To establish which parts of the antennae were responsible for chemoreception a series of tests were run using various parts of the antennae. No response to any chemical stimulus could be produced from the first four segments. The fifth and sixth segments produced a small response while the largest signal was obtained with the remaining segments. There appears to be a relationship between the distribution of EAG response with the number of short setae, and it therefore seems likely that these have a function in chemoreception.

5. CHEMICAL STUDIES ON H. bajulus (L)

5.1 Choice of Solvent for Frass Extraction

The criteria applied to the choice of solvent for extraction of organic compounds from a natural source are that it must be efficient at taking the compounds of interest into solution and produce the minimum of artifacts by rearrangement or degradation. Extraction of microgram amounts of volatile compounds imposes further restrictions on the choice of solvent. It is necessary for the solvent to be pure enough to allow extracts to be concentrated without the build-up of solvent impurities. It should also have a boiling point sufficiently low to permit sample concentration with minimal loss of the more volatile compounds.

The hydrocarbon solvents are among the most inert which can be used, but their low polarity often results in inefficient extraction particularly when compounds are contained within the cell wall. Polar solvents can suffer from long term problems of stability, particularly in the presence of traces of acids. Low boiling point polar solvents which are frequently used are diethyl ether and methylene chloride, acetone has also been used in the extraction of pine woods[99]. Peroxide formation from diethyl ether results in oxygenated artifacts, while acetone can undergo condensation reactions with extracted compounds. Although methylene chloride can also lead to the formation of artifacts these would be more easily identified in frass extracts since they

would contain chlorides. However, methylene chloride also produces hydrogen chloride which can lead to less readily detectable artifacts by acid catalysed rearrangements.

5.1.1 Purification of Solvents

Technical grade hexane was bought in bulk and treated with 65% oleum followed by fractional distillation from calcium hydride. The resulting hexane, boiling point 68°C, had a better UV transparency than commercially available spectroscopic hexane. The presence of other impurities was tested by reducing 100 mls to 10 µl on a rotary evaporator followed by injection of a tenth of this onto an OV-1 column programmed from 80° to 325°C. Only the hexane isomers could been seen above the 10 ng level. Methylene chloride was also purified to this level by treating Puriss grade with saturated sodium bicarbonate followed by fractional distillation of the dried solvent using a one metre fractionating column filled with Fenske's Helices..

5.2 Extraction Methods

Cold solvent extraction, particularly with an inert solvent, provides the mildest conditions for extraction. However, this method was slow and inefficient for frass as compounds were still being taken into solution after the third extraction. In spite of the low efficiency this method was used for a preliminary chemical investigation and to produce chromatographic traces against which artifact formation in more efficient methods of extraction could be assessed.

Frass was collected from laboratory cultures of H. bajulus at the Building Research Establishment[100]. Before extraction, the frass was gently sieved to separate the fecal pellets and 'wood flour'. A small quantity of each was extracted for one week by stirring in the dark under nitrogen with hexane, followed by extraction with methylene chloride. From an analysis of the extracts by GC on an OV-1 column it appeared that the chemical composition of fecal pellets and 'wood flour' was identical. This agrees with the findings of Seifert who demonstrated that the lignin, carbohydrate and protein content of the fecal pellets and 'wood flour' were identical[28]. Although the methylene chloride extract contained more material than the hexane extract, both appeared to have a very similar chemical composition in the high molecular weight region when separated on an OV-1 column. The methylene chloride extract contained two groups of low molecular weight compounds which were absent in the hexane extract. Preliminary analysis of these compounds by GC-MS indicated the presence of several oxygenated monoterpenes. However, none of the extracts contained the characteristic pine wood monoterpene hydrocarbons. When these four extracts were screened for biological activity by EAG only the two methylene chloride extracts produced any response. Since the only observable difference between the extracts produced by the two solvents was the presence of the low molecular weight compounds, it seemed reasonable to conclude that these were the most likely sources of EAG activity.

5.2.1 Impurities from External Sources

Two of the major components in the low molecular weight region were identified during the preliminary GC-MS study. The first of these, di-n-butyl phthalate which is commonly used as a plasticiser, was traced to the distilled water used for washing glassware, though the polythene bag in which this batch of frass was collected may also have been a source. The second compound, 2-naphthol was traced to the laboratory culture where it was used as an acarcide and its occurrence in the extract appeared to be the result of accidental contamination of the frass during collection.

5.3 Other Methods of Extraction

The low efficiency of cold extraction together with the fact that EAG activity appeared to be associated with the low molecular weight compounds made it desirable to find an alternative extraction procedure. This method had to be efficient, and if possible isolate the monoterpenes in preference to the high molecular weight compounds which formed the bulk of the material isolated by cold solvent extraction.

As terpenes are readily steam distilled, it was hoped that this might be a suitable method for isolating these compounds directly from the frass. A methylene chloride extract of the steam distillate contained little high molecular weight material but had no EAG activity. Analysis by GC showed the presence of few of the oxygenated compounds found in the cold solvent extract. Although

this method was about 50% more efficient than cold extraction, the large number of artifacts produced ruled this method out.

The third method attempted was Soxhlet extraction with hexane followed by methylene chloride. After four days the hexane extract contained only a small quantity of low molecular weight material and had no EAG activity. In contrast to this, the methylene chloride rapidly extracted the low molecular weight compounds to produce a solution with considerable EAG activity. The method was estimated to be in the order of three times more efficient than cold extraction and produced only a few minor artifacts. The other advantage of this method was that exhaustive extraction was achieved much more rapidly, with a considerably smaller volume of solvent than was required to cold extract the same weight of frass. One drawback of this method was that a large quantity of high molecular weight material was also taken into solution.

5.4 Isolation and Purification of Oxygenated Monoterpenes

Methylene chloride Soxhlet extraction of 300g of frass, collected immediately prior to use, produced 6.84g of a viscous brown oil. Extraction of this oil with vigorously stirring hexane effectively removed the majority of the high molecular weight material. This resulted in a pale yellow, EAG active hexane solution and an EAG inactive brown oil. The removal of this material considerably reduced the broad hump observed in the chromatogram of the crude extract which eluted between 200° and 280°C (fig. 20b).

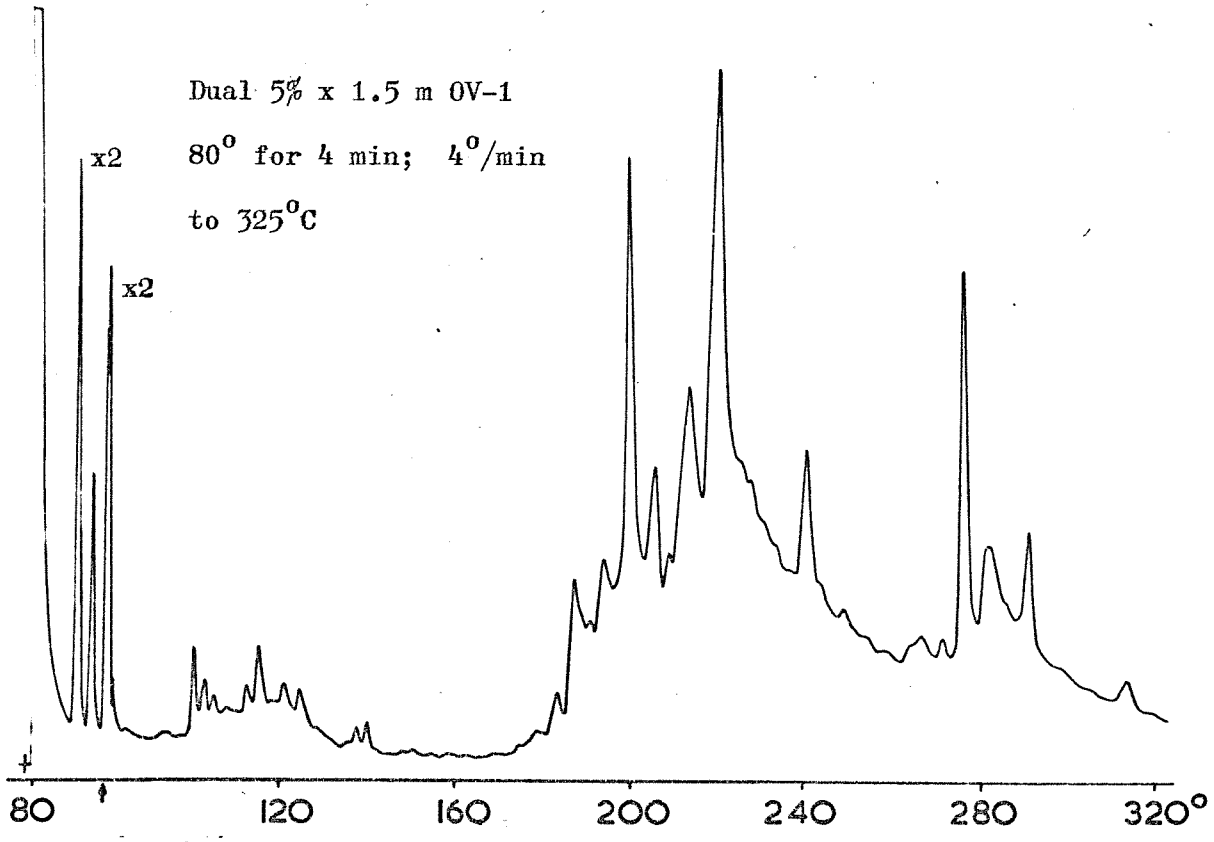


Fig 20a. Hexane soluble fraction of P. sylvestris extract.

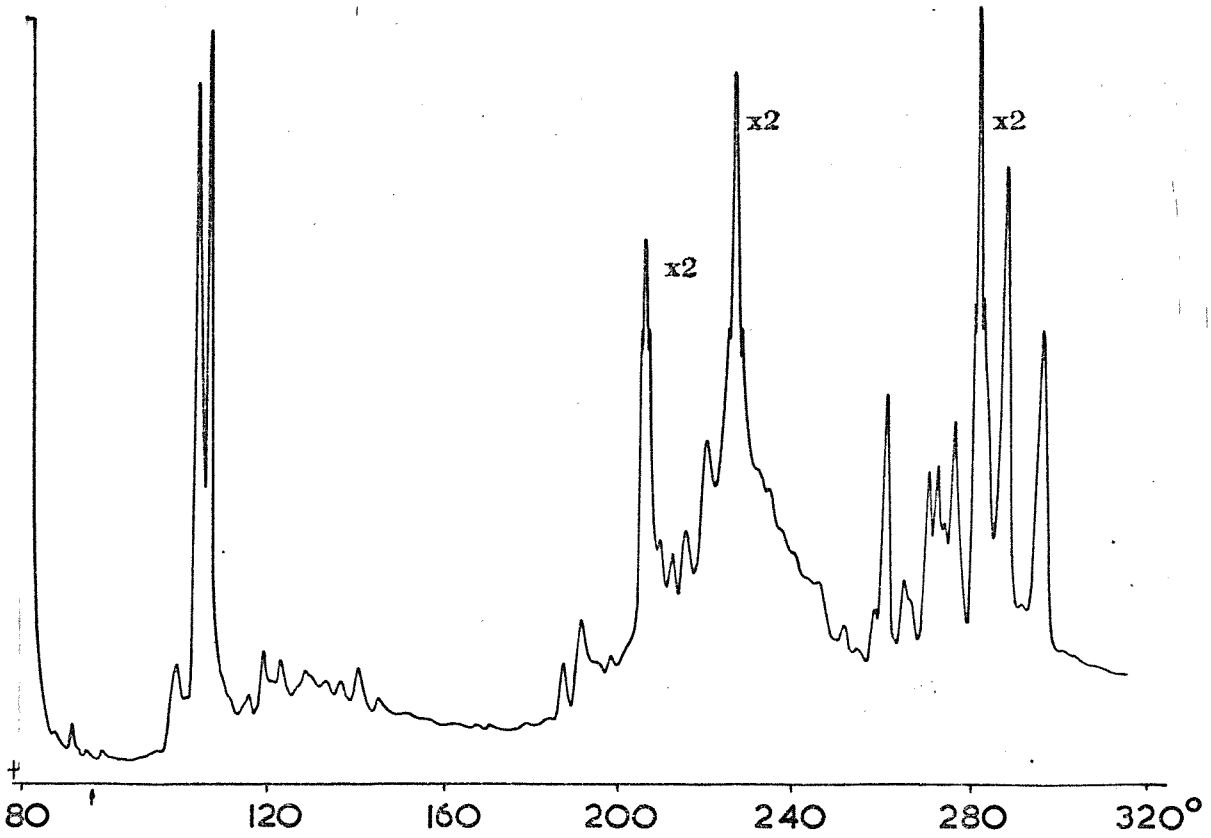
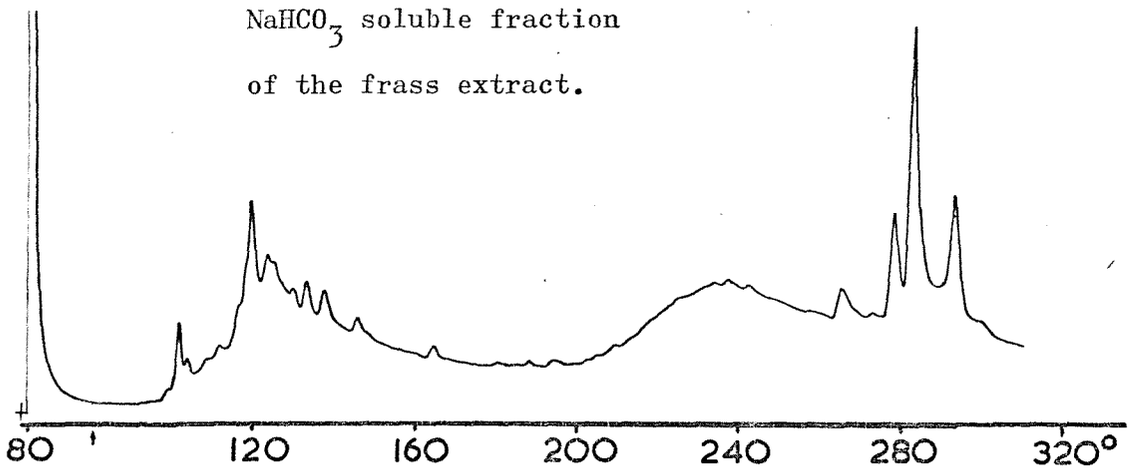
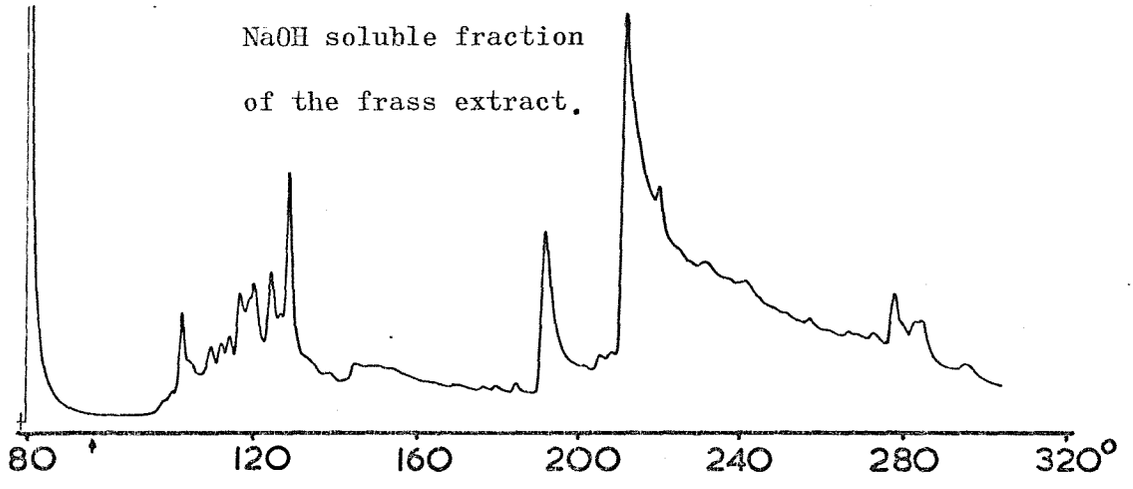


Fig 20b. Hexane soluble fraction of the frass extract.





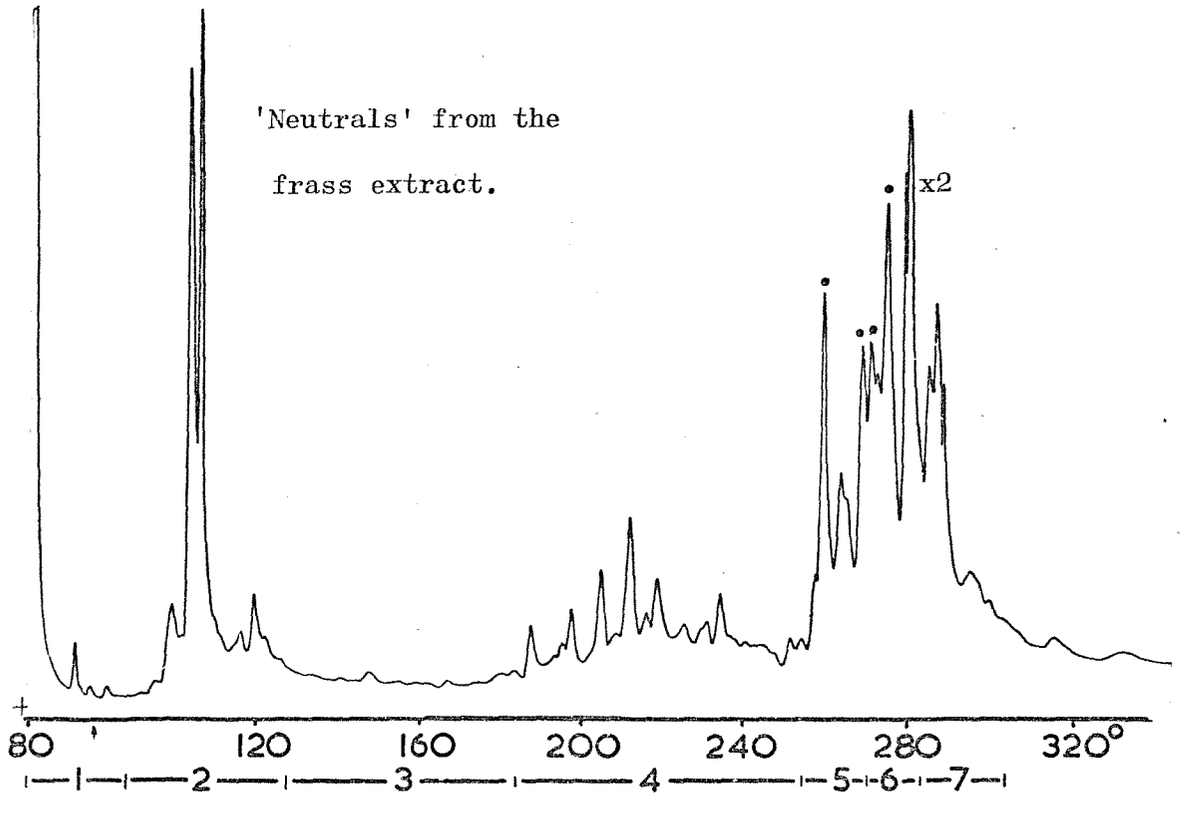
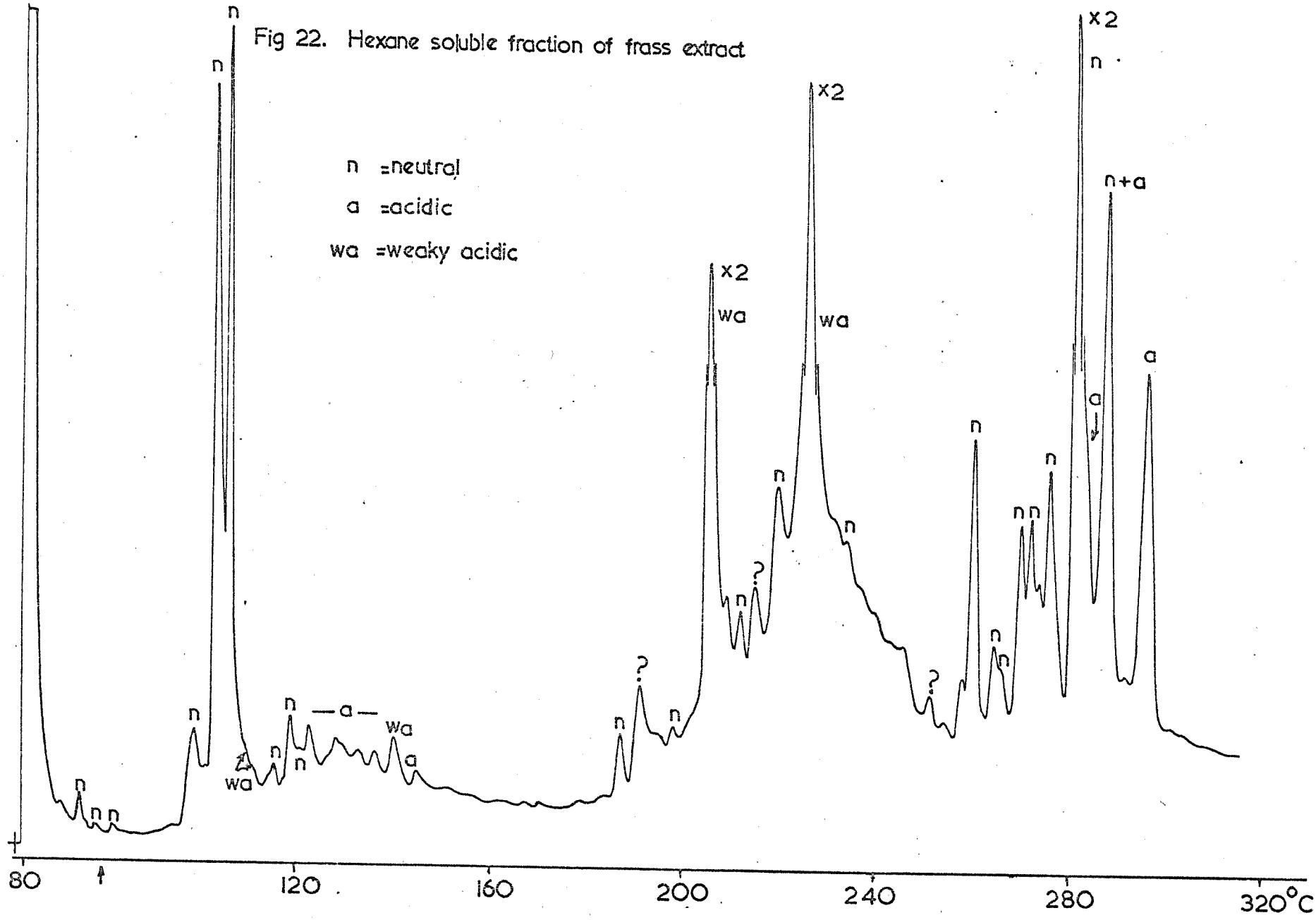


Fig 21 a-c. Base extraction of the frass hexane solubles.

Fig 22. Hexane soluble fraction of frass extract



A further separation with saturated sodium bicarbonate followed by 10% aqueous sodium hydroxide produced three solutions classified as 'strong acids', 'weak acids' and 'neutrals' (fig. 21) of which only the latter showed EAG activity. This simple technique was so efficient and reproducible that it was possible to identify almost all the components of the hexane soluble fraction as belonging to one of the three categories (fig. 22). A similar fractionation was attempted on a small quantity of the hexane solubles with 10% aqueous tartaric acid followed by 1M HCl. Analysis of the three fractions by GC failed to detect any bases at this level.

Preparative GC of the frass 'neutrals' on a crude molecular weight basis with an OV-1 column gave seven fractions, SHN1-7 (fig. 21). Only fraction SHN-2 was EAG active at the nanogram level, although fractions SHN-3 and 5 showed activity at much higher concentrations. The remaining fractions showed no activity even at high concentration.

5.5 Comparison of the Chemical Composition of Frass and Pinus sylvestris

To determine the differences in the chemical composition between frass and host-wood, methylene chloride extractions of identical weight of frass and very fine wood shavings from uninfested culture blocks were run in parallel. The two extracts were quite different in appearance and smell, the wood extract was pale yellow and had the characteristic smell of pine wood, while the frass extract was dark brown with a faintly musty odour. The extracts

were worked-up to afford hexane solutions and analysed by GC using OV-1 columns (fig. 20a and b). The monoterpene hydrocarbons α and β-pinene and car-3-ene were the major low molecular components identified in the wood together with smaller amounts of limonene, p-cymene and several sesquiterpene hydrocarbons. These hydrocarbons were not present in the frass extract which contained only oxygenated monoterpenes in the low molecular weight region. It was interesting to note that the concentration of the oxygenated monoterpenes in the frass extract was close to that of the monoterpene hydrocarbons in the wood extract. The only other difference between wood and frass extracts which could be seen from the chromatograms was a group of high molecular weight compounds (marked with * in fig. 20b).

The acid composition of both frass and wood was identical, with the exception of the group of low molecular weight acid in the frass which eluted just behind the oxygenated monoterpenes on the OV-1 column. Since the pine acids have been well documented[101] and as these fractions were EAG inactivity no further work was performed on them.

5.6 Identification of the Mono-oxygenated Monoterpenes

The compounds in the neutral fraction SHN-2 were first analysed by post-loops and syringe reactions as described in Sections 2.4 and 2.5. The sodium borohydride reaction was initially used to selectively remove carbonyl compounds from the mixture by injecting the reaction products onto the GC without aqueous work-up. The mixture was also analysed by GC-MS using

column G under isothermal conditions this, together with the data obtained by the reaction techniques was sufficient to allow structural identification of several components.

Micropreparative GC yielded fractions T1-10, nine of which contained pure compounds which were analysed by UV and FT-NMR spectroscopy while the tenth contained several minor compounds which appeared to be mainly products of degradation or rearrangement.

5.6.1 Analysis by Post-loops and Syringe Reactions

The chromatogram of SNH-2 run isothermally on column G at 120°C (fig. 23b) showed the presence of two major and seven minor components. The remaining group of minor compounds with retention times shorter than T2 was classified as T1.

Bromination totally removed all compounds except T8 and T10 which were only partially affected, while reaction with sodium borohydride, without aqueous work-up, removed compounds T6 and T7. Since these two compounds were unaffected by the o-dianisidine post-loop their structures were probably unsaturated ketones. With the exception of T8 the remaining compounds appeared to be unsaturated alcohols, T3 and T9 were identified as either primary or secondary alcohols by boric acid post-loop subtraction, while T2, T4 and T10 appeared on the chromatogram as distorted peaks suggesting a tertiary alcohol functionality. Compounds T3 and T9 were converted to their silyl ethers while T2 and T5 were not, confirming them as primary or secondary and tertiary alcohols

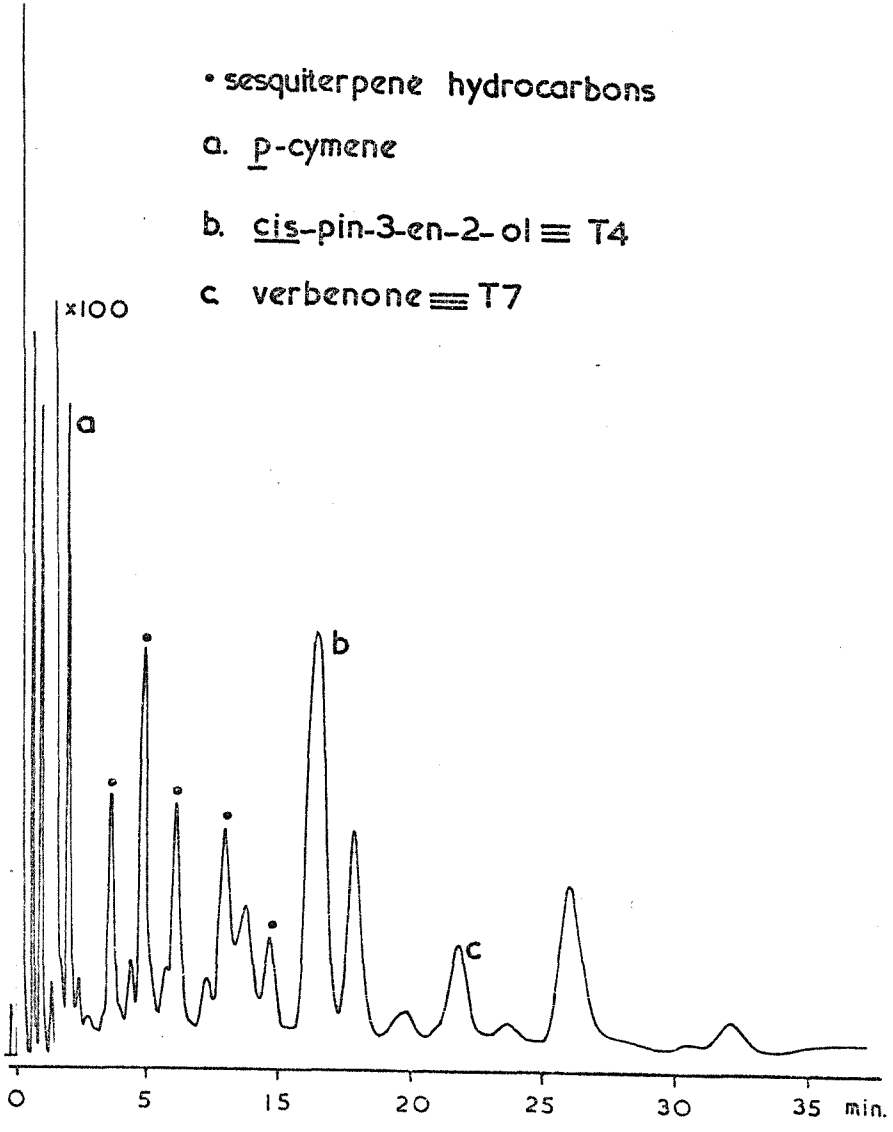
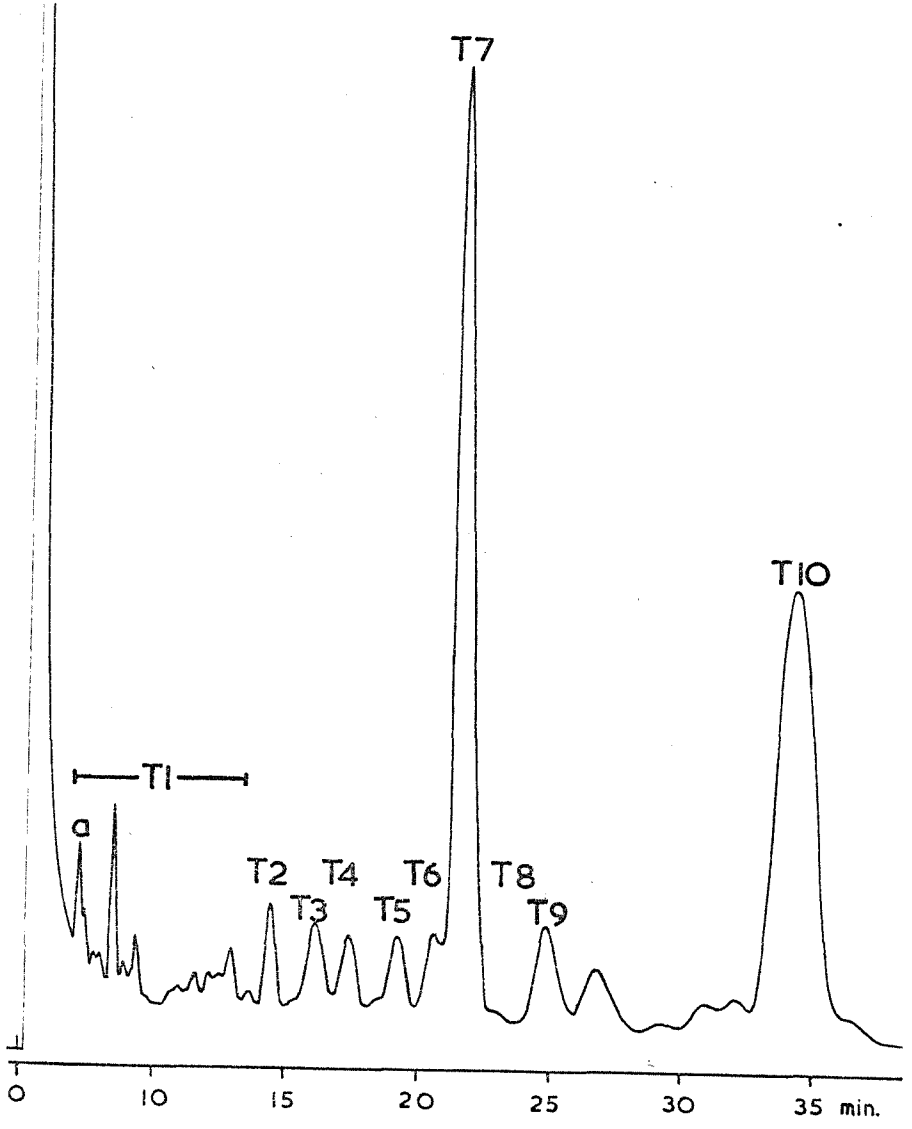


Fig 23a. Low molecular weight compounds from P. sylvestris.



b. Fraction SHN-2 from the frass extract.

respectively. The results for compounds T4 and T10 were more difficult to interpret. Mass spectroscopy revealed that in common

TABLE 4

Results of Analysis of Fraction SHN-1 by Post-Loops and Syringe Reactions

	Post-Loops		Syringe Reactions		
Compound	o-Dianisidine	Boric Acid	Br_2	$NaBH_4$	Silylation
T1	-	-	+	?	?
T2	-	*	+	-	-
T3	-	+	+	-	+
T4	-	*	+	-	≠
T5	-	*	+	-	-
T6	-	-	+	+	-
T7	-	-	+	+	-
T8	-	-	≠	-	-
T9	-	+	+	-	+
T10	-	*	≠	-	≠

+ Reactive; ≠ Partially Reactive; - Unreactive; * GC Peak Shape Changed; ? Could not be determined

with the identified alcohols they both showed a strong loss of water from a small parent ion. However, it was not possible to decide to

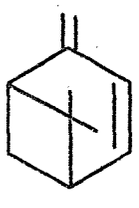
which category of alcohols they belonged, for whereas they appeared as distorted peaks in the chromatograms with the boric acid post-loop they also partially silylated, which had not been observed for any similar test compounds used in earlier trials with this reaction.

5.6.2 Structural Assignment of Monoterpenes

Spectroscopic analysis of pure compounds isolated by micropreparative GC confirmed the structures tentatively assigned to several of the components on the evidence of the work described in the previous section. Furthermore, FT-NMR spectroscopy permitted structural assignment of the remaining compounds. In each case the structural assignment was confirmed by comparison with synthetic or commercially available samples. The syntheses of samples required for the purpose of comparison are discussed in chapter 6.

T1, Minor Low Polarity Compounds

The small quantities of compound present in this fraction limited analysis to GC-MS. Four of these compounds were readily identified by their mass spectra; verbenene (16), 4,α-dimethylstyrene (17), chrysanthenone (18), and p-cymene (19). The remaining compounds



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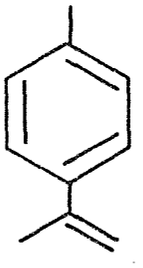

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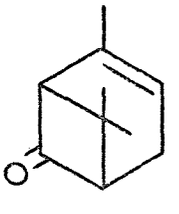

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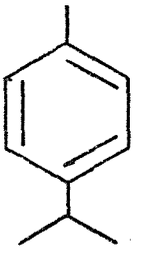

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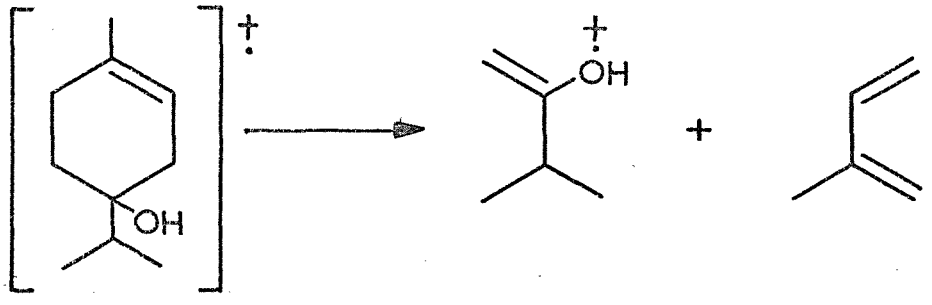
all appeared from their mass spectra to be monocyclic trienes. With the exception of p-cymene the compounds in this fraction were probably artifacts caused by thermal degradation during preparative GC.

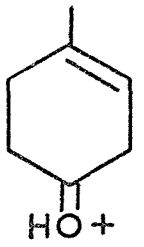
T2, Terpinen-4-ol, 30 µg

Demonstrated by the boric acid post-loop, bromination and silylation to be an unsaturated tertiary alcohol, this compound was identified as terpinen-4-ol (20) by its mass spectrum (m/e 71 (100%), 93 (45), 111 (42), 43 (42), 86 (32), 69 (31), 55 (31), 154 (30), 68 (12), 136 (12)) which has the characteristic ions at m/e 111, 86 and 71. The major fragmentation pathway of this alcohol, which has a large parent ion for a tertiary alcohol (30% base peak), is a retro-Diels Alder type reaction.



20 m/e 86

$-C_3H_7$ $-CH_3$



$[C_4H_7O]^+$

m/e 111 m/e 71

Insufficient material was available to run an FT-NMR spectrum, and the structure was confirmed by comparison of the mass spectra of T2 and an authentic sample, together with co-injection on GC columns .

T3, trans-Pinocarveol, 60 µg

From reaction data this compound appeared to be a primary or secondary unsaturated alcohol. The complex mass spectrum (m/e 41 (100%), 55 (95), 92 (93), 70 (70), 91 (67), 83 (63), 39 (53), 69 (44), 81 (40), 134 (33)) was one of those found to be very dependent on the condition of the MS source (section 3.1.2). The spectrum contained two major rearrangement ions at m/e 92 and 70 but despite the presence of several metastable peaks it proved impossible to determine the fragmentation patterns which led to the formation of these ions. The only real information which could be deduced was that the compound was an alcohol of mass 152 which possibly had an iso-propenyl group. This would require the molecule to be an unconjugated monocyclic dienol since no UV absorbance could be detected.

The FT-NMR spectrum (fig. 24) contained resonances which on first analysis were assigned as two methyl groups, an exo-methylene group and a vinyl proton which suggested structures such as cis and trans carveol (21) and cis and trans iso-piperitenol (22). However, the vinyl methyl groups of both these compounds would be expected to resonate between 8.1τ and 8.4τ, while those in the NMR spectrum of T3 resonated at 9.34 τ and 8.71τ. These values are characteristic of

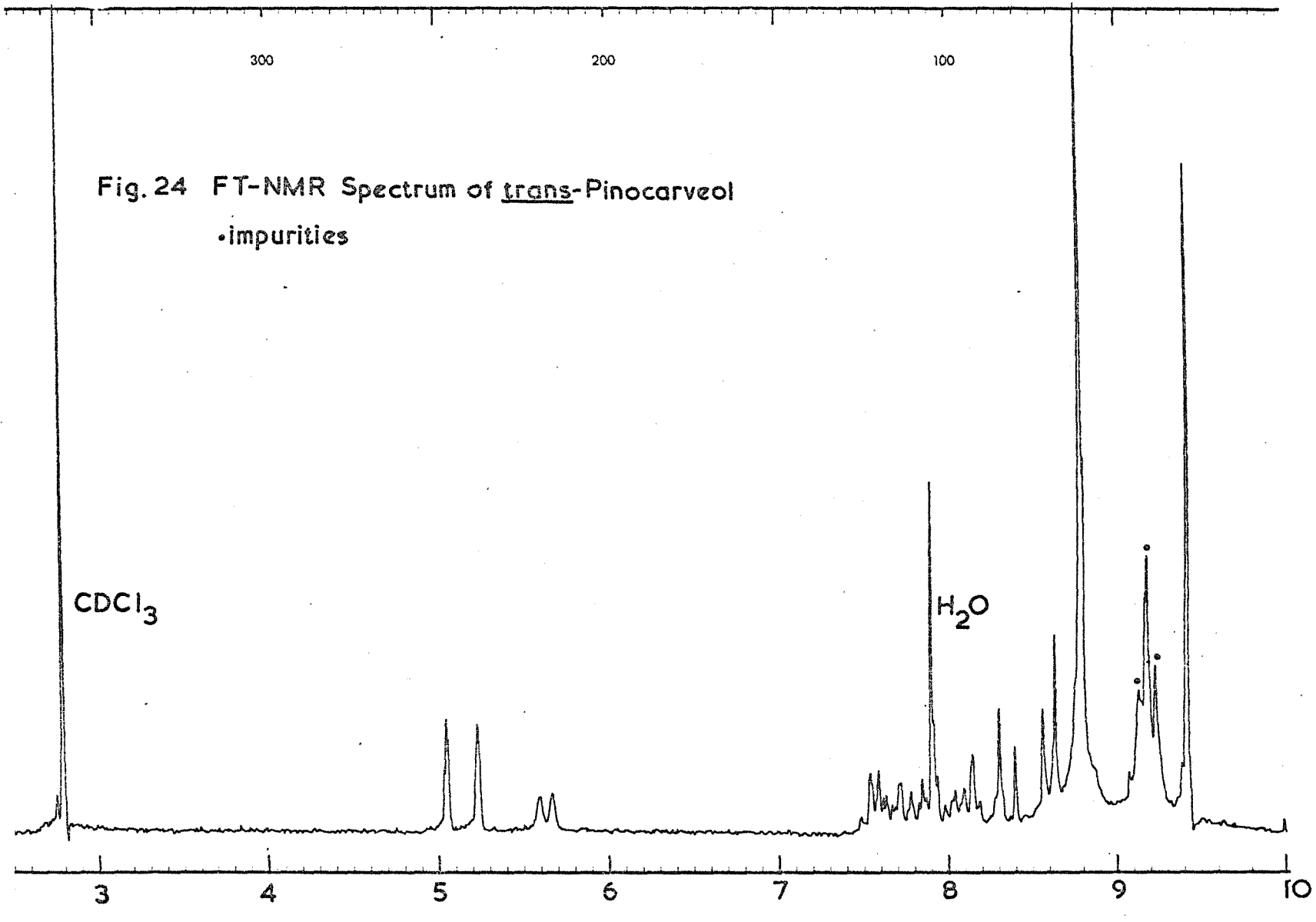
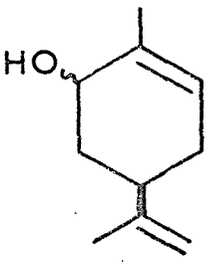


Fig. 24 FT-NMR Spectrum of trans-Pinocarveol
•impurities

bicyclic compounds with a pinane skeleton, which in this case would have to be based on β-pinene (23) since the NMR spectrum showed the presence of an exo-methylene rather than a third methyl group.



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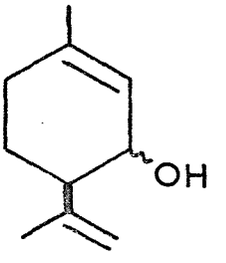

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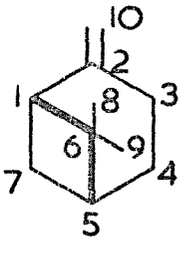
This was supported by the broad methylene envelope at 6.41-8.32τ which is characteristic of the methylene group resonances in β-pinene.



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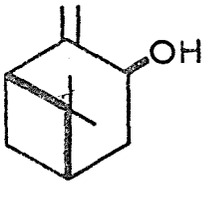

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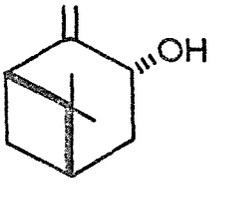

25

The resolution of the exo-methylene group, a broad singlet in β-pinene, into two finely split resonances at 5.17τ and 4.97τ pointed to the structure of T3 being either cis or trans pinocarveol, (24) and (25) respectively. Although a value of 5.56τ is low for an allylic proton of an allyl alcohol, this could be the result of deshielding by the exo-cyclic double bond. A study of models of cis and trans pinocarveol showed that both these compounds would result in a splitting of similar magnitude to the value of 8Hz observed in the NMR spectrum of T3. The angles made by the C_3-H_a bond with C_4-H_a and C_4-H_b bonds are close to 30° and 90° respectively, which would be expected to give rise to coupling constants $J_{3a4a} \simeq$ 8Hz and $J_{3a4b} \simeq$ 1Hz. In the case of cis-pinocarveol the dihedral angles made by C_3-H_b bond with C_4-H_a and C_4-H_b bonds are approximately 25° and 45° respectively and couplings of $J_{3b4a} \simeq$ 7Hz and $J_{3b4b} \simeq$ 2Hz would be expected. It was therefore impossible to distinguish between the two isomers on the basis of first order interpretation of the NMR spectra. Unambiguous synthesis of both isomers identified compound T3 as trans-pinocarveol.

T4, Pin-3-en-2-ol, 20 µg

The amount of this compound collected was insufficient for an FT-NMR spectrum to be obtained and it was necessary to assign the structure by chemical methods. The boric acid post-loop results suggested that T4 was a tertiary alcohol, but the compound was nevertheless converted in 100% yield to its silyl ether with BSA. The mass spectrum which indicated a molecular weight of 152 was

characteristic of a cyclic monoterpene alcohol (m/e 91 (100%), 41 (69), 109 (59), 119 (55), 39 (51), 81 (41), 92 (35), 94 (37), 77 (32), 79 (31))with ions at m/e 134 and 119 resulting from the loss of water followed by a methyl radical. Despite the apparent simplicity of the mass spectrum little else could be deduced concerning the structure of T4. Dehydration of 10 µg of this alcohol with a few grains of phosphorous pentoxide resulted in a 98% conversion to a diene with a UV maximum at 244 nm. This value was consistent with a diene in which one of the double bonds was exo-cyclic. The mass spectrum which identified the compound as verbenene (16) confirmed the diene configuration (m/e 119 (100%), 134 (31), 91 (18), 120 (11), 117 (9), 65 (7), 41 (7), 115 (6), 51 (6), 77 (5)).

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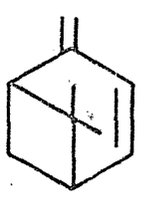

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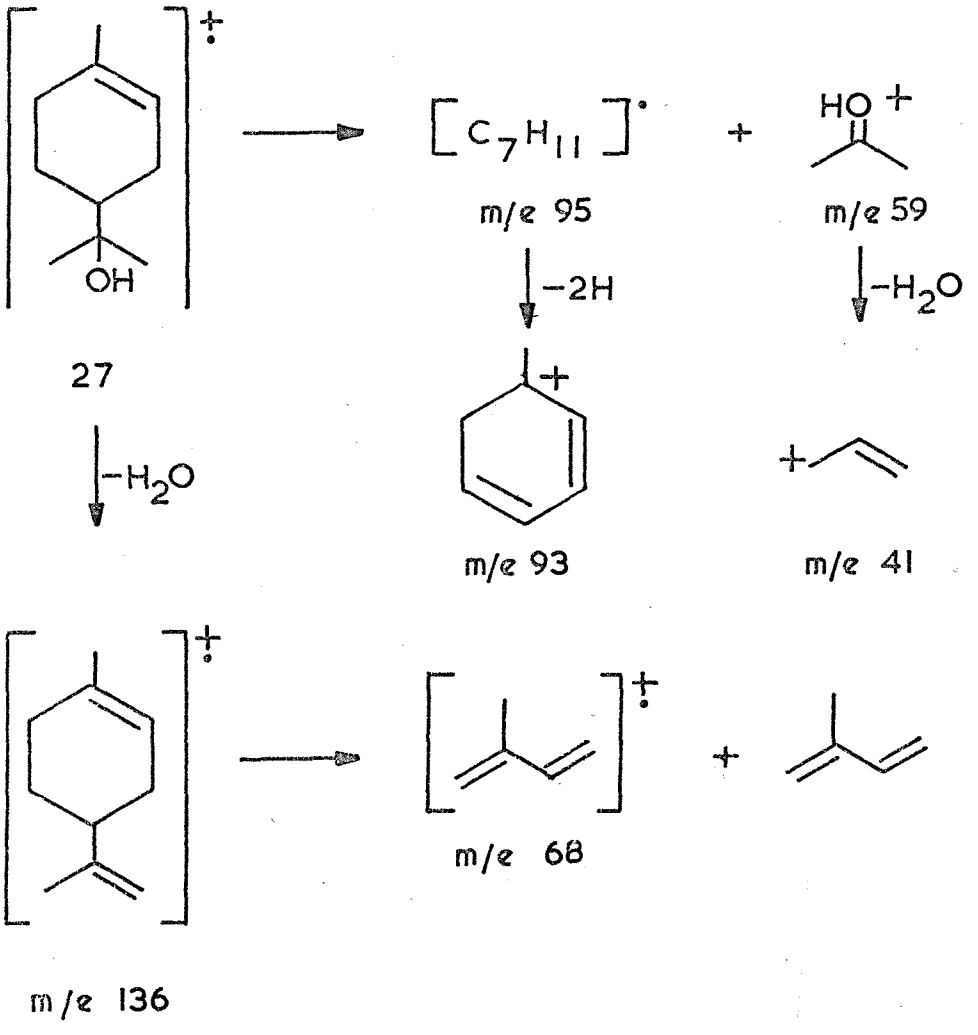
* The structures 24 and 26 show relative stereo-chemistry of compounds T3 and T4.

Dehydration of trans-pinocarveol (25) under the same conditions was considerably slower than the dehydration of T4, requiring 20 minutes to proceed to completion. Verbenene was only produced in 55% yield, the remaining products consisting of 4,α-dimethystyrene (17), p-cymene (19) and two other hydrocarbons of molecular weight 134. This suggested that T4 could rapidly dehydrate by an anti-periplanar mechanism, and if it is assumed that no skeletal rearrangement occurred during this process, then a possible structure for T4 was cis-pin-3-en-2-ol (26). Synthesis of (26) produced a compound with identical reaction and spectroscopic properties to T4.

T5, α-Terpineol, 70 μg

The structure of this compound was assigned on the basis of post-loop, syringe analysis and mass spectroscopy alone. Shown to be a tertiary unsaturated alcohol by the first two techniques, this compound had a mass spectrum which was readily assigned to α-terpineol (27), (m/e 93 (100%), 59 (94), 136 (77), 121 (71), 43 (51), 81 (43), 41 (42), 79 (31), 55 (25)). The base peak at m/e 59 arising from cleavage of the iso-propanol group and the ion at m/e 68 probably arises by retro-Diels Alder from the dehydration product limonene, produced either thermally or by electron impact. Thermal dehydration in the GC-MS interfact was a problem with this compound and spectra had to be run at the lowest possible temperature to avoid forming limonene in the separator. The FT-NMR spectrum with resonances at 8.87 τ, 8.36τ and 4.67τ for the iso-propanol methyl



groups, vinyl methyl group and vinyl proton confirmed this structure. The spectroscopic and GC properties of the natural compound were identical to those of a commercial sample.

T6, 3,6,6-Trimethylcylohepta-2,4-dienone, 55 µg

The mass and UV spectra of T6 were identical to those of eucarvone (28) (m/e 107 (100%), 150 (50), 91 (47), 135 (28), 79 (26),

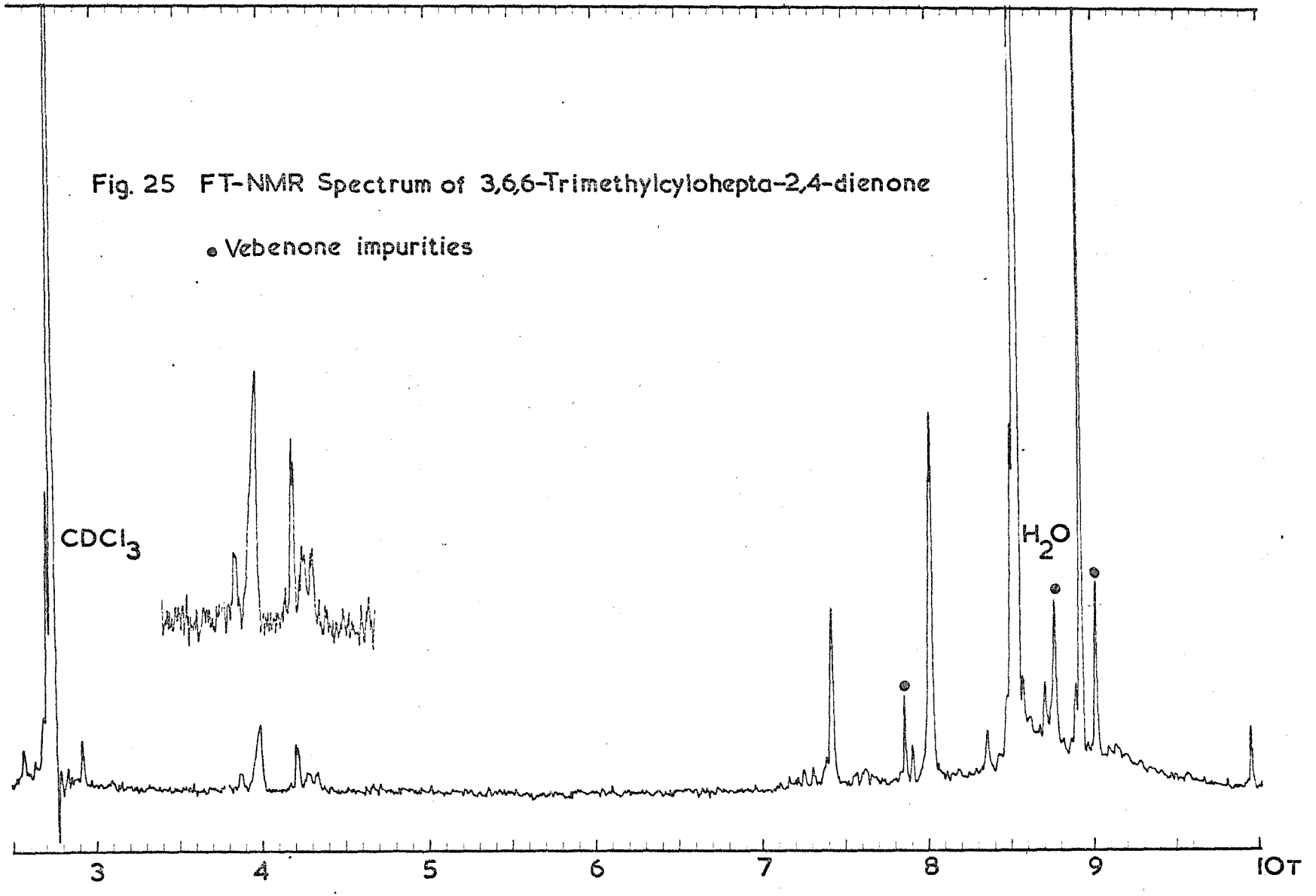
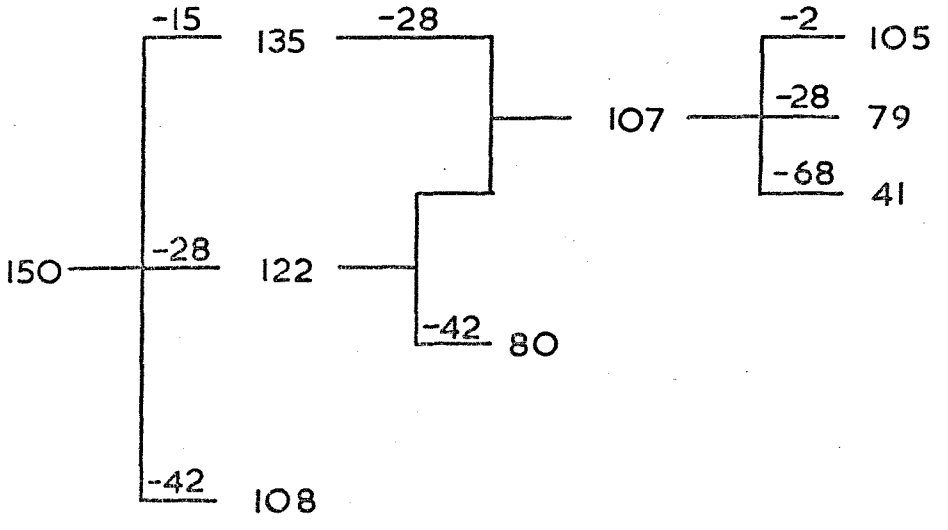


Fig. 25 FT-NMR Spectrum of 3,6,6-Trimethylcyclohepta-2,4-dienone

• Vebenone impurities

39 (25), 108 (25), 41 (18), 77 (16 80 (15)), λ_{max}^{EtOH} = 294 nm, ε = 7,400; sodium borohydride reduction, which produced a single



product and bromination, were also consistent with this structure. However, the retention times of T6 on column D at 100° (16.0 min) and column G at 120° (15.2 min) were almost double those of eucarvone (28)(8.1 min and 7.6 min respectively).

It proved impossible to isolate T6 completely free from T7 because of the similarities in their retention times on all columns used. The FT-NMR spectrum therefore showed small peaks corresponding to resonances of T7 (fig. 25). However, it clearly showed a gem dimethyl group at 8.90τ a vinyl methyl group at 8.00 and a single methylene group at 7.40τ, all of which were singlets indicating a molecular structure for T6 very close to that of (28) (cf. eurcarvone with singlet resonances at 8.93τ, 8.07τ and 7.34τ respectively).

From the splitting pattern of the vinyl protons it was possible to deduce that compound T6 and (28) differed only in the substitution of the vinyl methyl group. The vinyl region of T6 consisted of two

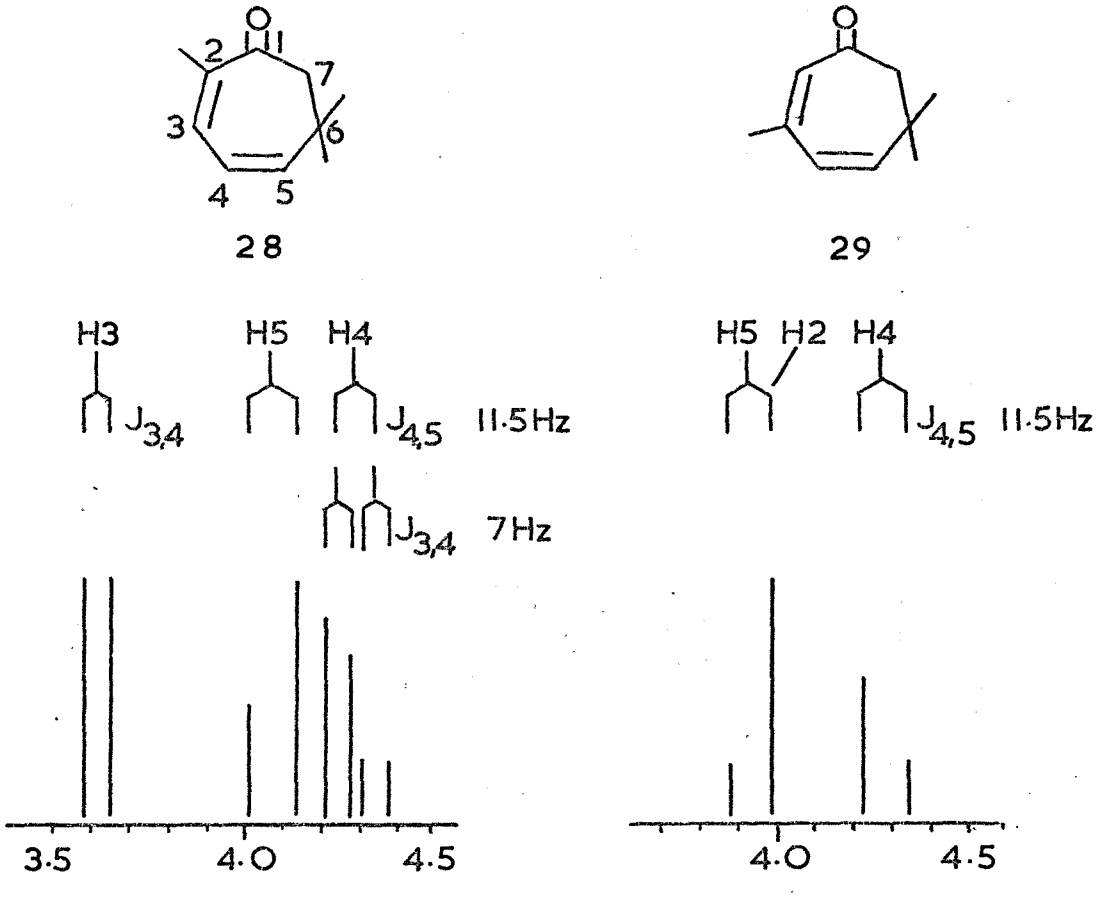


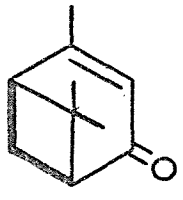
Fig 25. Vinyl splitting pattern of compounds (28) and (29)

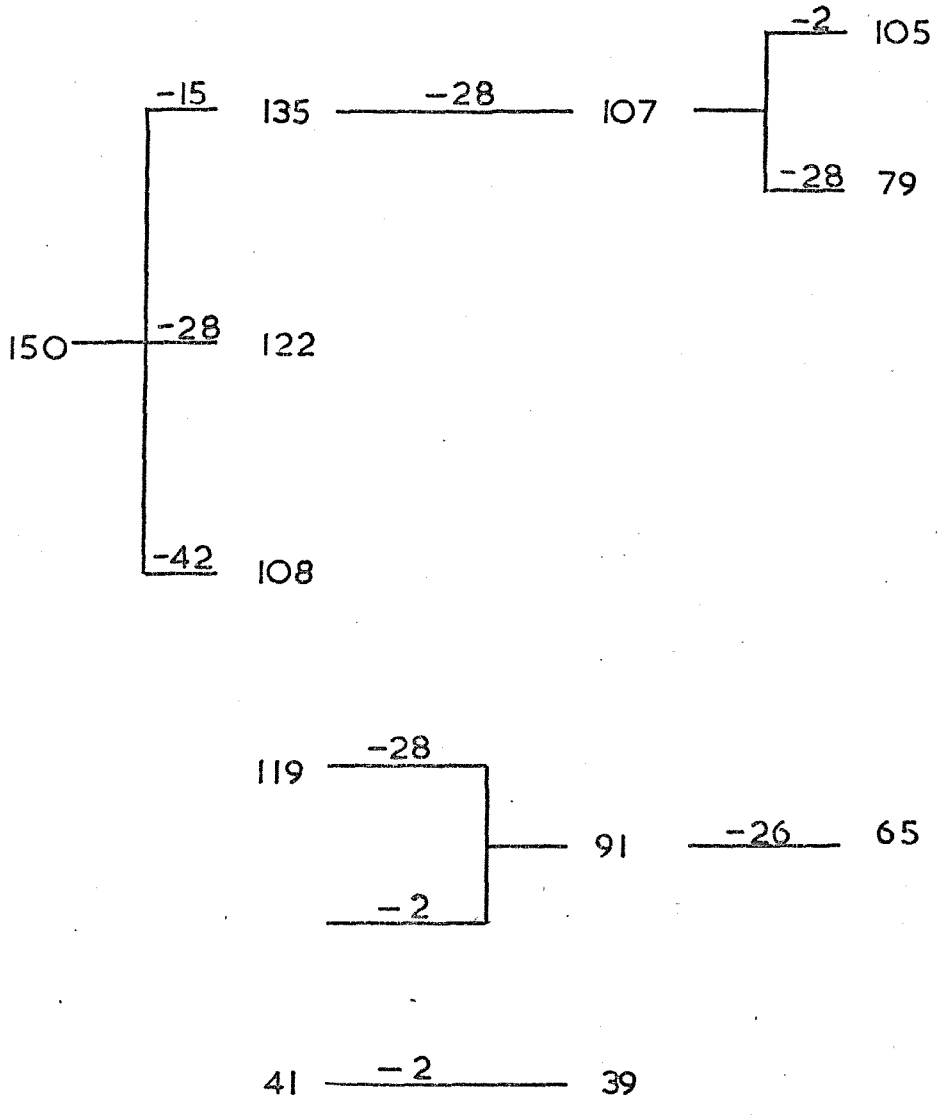
vinyl proton doublets at 4.54 τ and 3.92 τ with a cis vinyl coupling of J = 11.5 Hz and a single uncoupled vinyl proton at 3.98 τ. This pattern would be produced by the 3-methyl and 4-methyl isomers. Decoupling experiments on eucarvone allowed the resonances to be assigned to H_3, H_4 and H_5, as shown in fig. 25. The vinyl resonances of T6 are very close to the values for H_4 and H_5 in (28), with no resonance as low as the value of 3.48 τ observed for the H_3 of (28).

Since the effect of the vinyl methyl group would be expected to be slight it was concluded that T6 was the 4-methyl isomer (29). A synthetic sample prepared by the aerial oxidation of car-3-ene had identical spectroscopic properties to the natural compound. Further confirmatory evidence was obtained by comparison of the 2,4-DNP's of both synthetic and natural compounds by TLC. The two derivatives had identical R_F values in both solvent systems used. This is the first time that (29) has been isolated as a natural product, although it has been previously synthesised[102,103].

T7, Verbenone, 450 µg

This compound which had a molecular weight of 150, was one of the major compounds isolated from the frass. Post-loop and syringe analysis indicated an unsaturated ketone, and reduction with sodium borohydride followed by aqueous work-up produced two products in a ratio of 3 : 1, suggesting a bicyclic structure. The compound which had a UV absorption at λ_{max}^{EtOH} = 254 nm (ε = 6,450), consistent with an α,β -unsaturated ketone, was finally identified as verbenone (30) by its mass spectrum (m/e 107 (100%), 135 (75), 39 (61), 91 (58), 80 (54), 79 (39), 41 (51), 108, (28), 77 (24)).





Continuous wave NMR spectra of T7 and an authentic sample of verbenone were identical with resonances at 8.97 τ and 8.47 τ resulting from the geminal methyl group, and a vinyl methyl group at 7.97 τ , coupled to the vinyl proton (J = 2Hz). The vinyl proton at 5.31 is a finely split multiplet as a result of coupling with the vinyl methyl and W-coupling with the two bridge-head protons. Verbenone has a large molecular rotation and a sufficient quantity

was isolated from the frass to determine the optical activity of T7. The optical rotation was found to be -248°, calculated as an average of six readings because of low concentration which could be used. Although the dextrorotory form has been reported to occur naturally ($[\alpha]_D^{20}$ = + 249°)[104], it has not been reported to occur naturally as the levorotory isomer.

Final confirmation was made by comparison with a synthetic sample[105] including TLC of the 2,4-DNP derivatives.

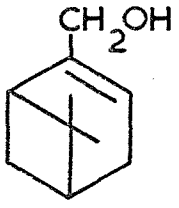
T8, Naphthalene, 60 µg

This compound was unaffected by both post-loops and silylation and only partially reacted with bromine. Isolated by micropreparative GC as a white solid with a camphorous smell, it was identified by its mass spectrum as naphthalene (m/e 128 (100%), 129 (11), 127 (10), 102 (8), 63 (7), 51 (6), 124 (5), 75 (4), 74 (3)). The only resonance in the expanded FT-NMR spectrum was a finely split AB quartet at 2.44τ which proved to be identical to an authentic sample.

T9, Myrtenol 90 µg

The third most abundant component in SHN-1, this compound was demonstrated to be a primary or secondary unsaturated alcohol by bromination and silylation. The mass spectrum tentatively identified it as myrtenol (31) (m/e 79 (100%), 91 (41), 41 (37), 108 (15), 39 (24), 77 (20), 93 (18), 95 (17), 67 (14)), the ion at m/e 79 being characteristic of both the alcohol and the related

aldehyde. The gem dimethyl groups resonated at 9.08 τ and 8.83 τ , while the broad singlets at 6.06 τ and 4.53 τ were consistent with



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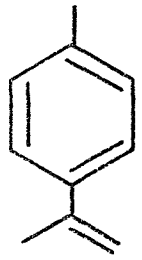
an allylic hydroxymethyl group and a vinyl proton respectively. The structure was confirmed by comparison with a synthetic sample, which showed spectroscopic and GC properties to be identical.

T10, p-Cymene-8-ol 570 µg

This alcohol was the major component of the mono-oxygenated monoterpene fraction from the frass. It was not subtracted by the boric acid post-loop and only partially reacted with bromine and BSA. The molecular weight of 150 determined by mass spectroscopy suggested a molecular formula $C_{10}H_{14}$ which requires this molecule to have four degrees of unsaturation. An acyclic structure was ruled out on the basis of the bromination reaction and the mass spectrum. A more likely possibility was an aromatic structure which would explain

this data and further evidence for this came from the presence of several doubly charged ions in the mass spectrum (m/e 43 (100%), 135 (52), 91 (19), 132 (14), 150 (11), 107 (10), 65 (9), 39 (8), 105 (6), 92 (6); doubly charged ions m/e 67.5, 64.5, 63.5, 57.5). The major ions at m/e 135, 132 and 107 result from losses of a methyl radical, water and a C_3H_7 radical from the parent ion, the latter most likely being an iso-propyl group if the skeleton is terpenoid.

The NMR spectrum showed an AB quartet at 2.90 τ (J = 8Hz) which indicated a 1,4 aromatic substitution. The spectrum contained two other resonances at 8.48 τ (6H) and 7.96 τ (3H) which were both singlets, which could only be produced by the tertiary alcohol p-cymene-8-ol (32).



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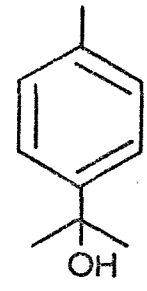

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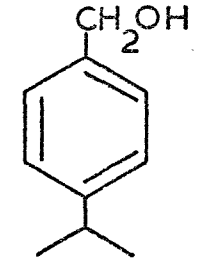

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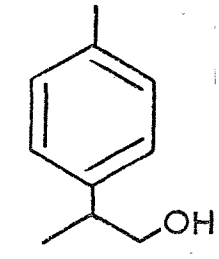

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Further evidence was supplied by the thermal dehydration of (32) which produced a compound identified by GC-MS as 4,4-dimethylstyrene (35) (m/e 132 (100%), 119 (84), 91 (41), 105 (41), 92 (31), 39 (24), 65 (21), 131 (16), 51 (16), 63 (14)). Synthesis of all three possible isomers (32), (33) and (34) confirmed the structure of T10.

The preliminary results of these studies on the monooxygenated terpenes from the frass have been published in two papers[106,107].

5.7 Investigation of the Volatiles from H. bajulus Adults

Solid sample GC of both male and female adults by the method of Bergström (section 2.2) showed that volatile compounds were present in both sexes in the order of 1-10 µg per beetle. From chromatograms of the volatiles from whole beetles (fig. 26) it can be seen that while only two major compounds were present in the males, five were present in the females. The chromatograms were quite reproducible and it appears that the compounds are sex specific.

An attempt to obtain spectra by solid sample GC-MS was unsuccessful because the large amount of water also volatilised from the beetles tissues masked all other responses. The water problem was minimised by using only those parts of the beetle's tissue which contained volatiles. These areas were located by freeze killing four unmated females, dissecting them into head and prothorax, meso and metasternum and abdomen and solid sampling the three sections separately.

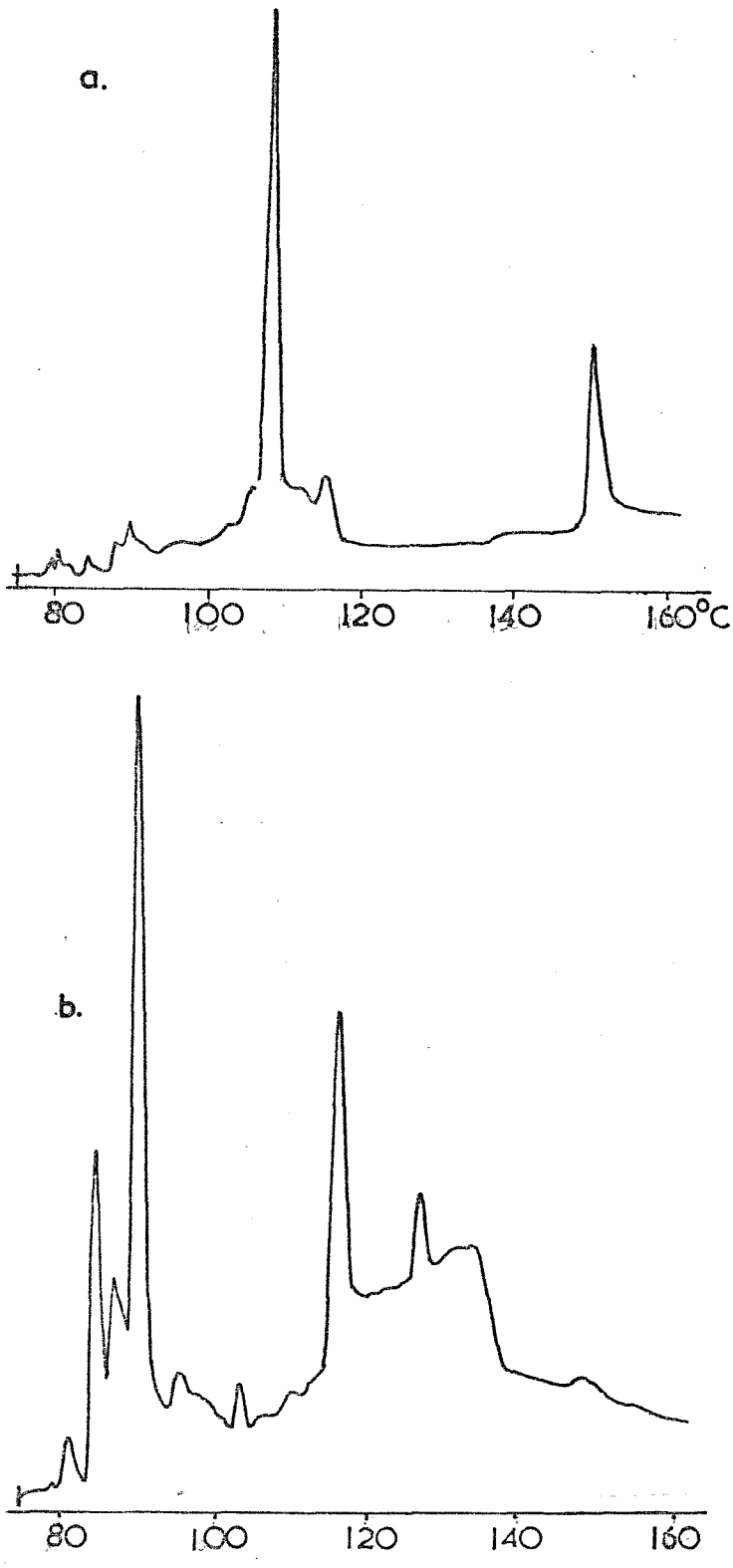


Fig 26. Solid sample GC on whole H. bajulus adults

a. 3 males; 3 females;

The abdomen contained little or no volatile material and could therefore be discarded, the major concentration of the compounds from the female were located in the meso and metathorax which contained approximately five times more material than the head and prothorax. The two volatile compounds from the males were found to be almost equally distributed between the head and prothorax, and meso and metathorax with no volatile material again found in the abdomen.

These preliminary studies demonstrate a marked difference between the volatile constituents of male and female H. bajulus adults and these may well play a role in controlling the behaviour of these insects described in sections 4.1.1 and 4.1.3.

6. SYNTHESIS OF MONO-OXYGENATED MONOTERPENES

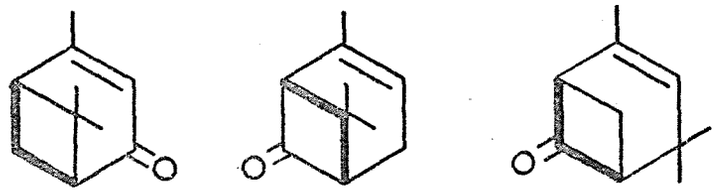
Structural identification of an unknown compound can be made on the evidence of spectroscopic data. However, the final confirmation of the assignment can only be made by comparison with a synthetic or authentic sample. The compounds described below have been synthesised for this purpose and no attempt has been made to optimise yields for those methods which have not been described in the literature. All the diagrams show the correct absolute stereochemical relationship between reactants and products.

6.1 Chrysanthenone

The photolytic rearrangement of verbenone (30) to chrysanthenone (18) involving a 1,3 shift of the gem-dimethyl bridge has previously been reported[108,109]. Since the formation of chrysanthenone (18) in the frass extract was observed to accompany a decrease in the amount of verbenone present, an experiment was set up to see if this could be occurring while the extracts were left on the bench during purification processes.

A 1% solution of (30) ($[\alpha]_D^{20}$ = -245° in hexane was left on the laboratory bench for one week, after which the solution was examined by GC. A product with a shorter retention time than verbenone was formed in 31% yield which corresponded with a 43% decrease in the size of (30) the 12% difference was presumably converted to polymeric material since no other peaks were observed

in the chromatogram. Preparative GC produced optically active



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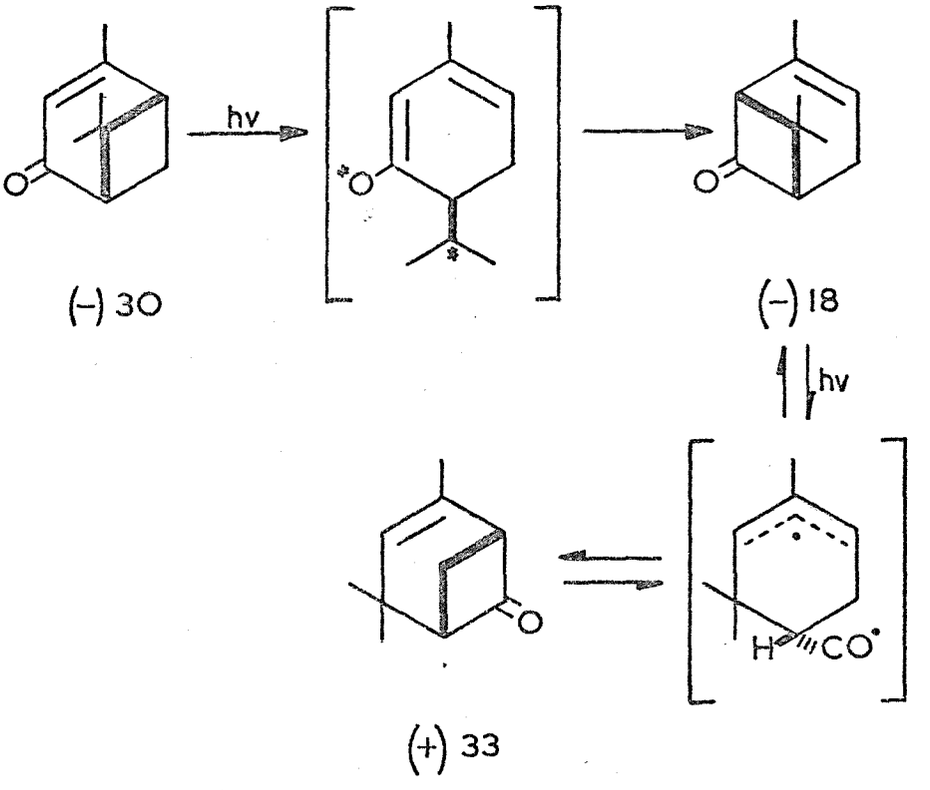
chrysanthenone ($[\alpha]_D^{20} = -60°$) which in keeping with the findings of Whitham and Hurst[108], indicated a loss of optical purity accompanying the rearrangement process. In a study of this process by Erman[109], chrysanthenone itself was found to undergo further photolytic rearrangement under certain conditions to afford (33). The IR spectra of both (18) and (33) are very similar with bands at 1785 1655 and 840 cm^{-1} ($R_2C = CHR$). The UV maximum of (18) at 295 nm ($\varepsilon = 206$) is also similar to the absorption of (33). The NMR spectra, however, serve to distinguish these two compounds and the spectrum of the sample obtained during this study was identical to the previously published spectrum for chrysanthenone[109]. The

relationship between the optical rotation of (30) and the



photolytic products confirmed the assignment of structure. Levorotatory verbenone leads to levorotatory chrysanthenone, while levorotatory (30) produces dextrorotatory (33).

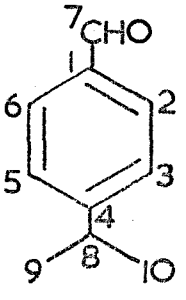
6.2 p-Cymene-7-ol, p-Cymene-8-ol, p-Cymene-9-ol

Reduction of cuminic aldehyde (34) with ethanolic sodium borohydride gave p-cymene-7-ol (35) as a single product. The IR spectrum consists of a strong sharp band at 3610 cm^{-1} (OH), with other bands at 1460, 1380, 1060 and 830 cm^{-1}. The mass spectrum gave the molecular weight as 150 and showed ions at m/e 117, 91, 79 and 77 which are characteristic of aromatic systems of this type. The NMR resonances at 8.78 and 7.15 τ (J = 7Hz) were consistent

with an aromatic iso-propyl group as was the hydroxymethyl group at 5.57 τ. Treatment of p-methyl acetophenone (36) with methyl magnesium bromide in dry diethyl ether gave a white solid magnesium alkoxide intermediate which, on work-up with saturated aqueous ammonium chloride, gave p-cymene-8-ol (32). The product was purified on a silica column to remove the small quantity of unreacted (36) and a hydrocarbon product also formed. The IR spectrum was again consistent with a 1,4 disubstituted aromatic alcohol with a molecular weight given by mass spectroscopy as 150. The NMR spectrum of (32) was easily recognisable by the aromatic AB quartet at 2.99 and 2.73 τ split by an eight Hz ortho coupling. The spectrum showed two other resonances at 7.69 and 8.48 τ corresponding to the aromatic methyl and iso-propyl groups respectively.

The third isomer, p-cymene-9-ol was synthesised by hydroboration of 4,α-dimethylstyrene (17). Dehydration of (32) in good yield presented certain difficulties due to the speed with which (17) underwent polymerisation. The first method attempted used p-toluene sulphonic acid in refluxing benzene as the dehydrating agent[110]. However, only polymeric material could be identified in the product. Overberger and Saunders[111] prepared meta-chlorostyrene by heating the alcohol in the presence of potassium bisulphate. This method again led to polymeric material when applied to (32). A sample of (17) was finally obtained in 33% yield by refluxing the alcohol at 20 mm Hg followed by slow distillation from a crystal of iodine[112]. The product was then separated on a neutral alumina column with hexane eluant to remove polymeric material and unreacted (32). The mass spectrum, which had a parent ion at m/e 132 (100%),

showed the product to be monomeric. Confirmation of the structure was obtained by examination of the NMR spectrum which showed vinyl and aromatic methyl groups at 7.92 and 7.74 τ respectively. The exo-methylene protons which had a 2Hz geminal coupling were observed at 5.01 and 4.71 τ.



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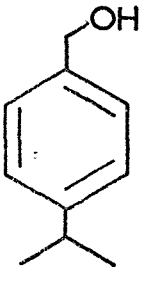

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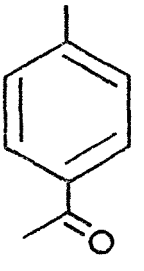

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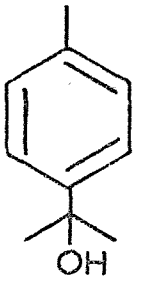

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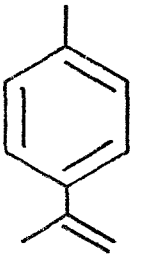

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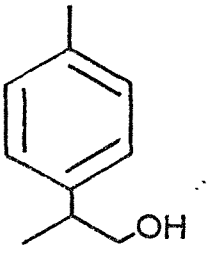

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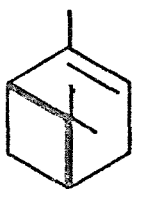
Hydroboration of (17) followed by oxidation of the borane intermediate with alkaline hydrogen peroxide[113] on work-up and column chromatography yielded (37). As would be expected, the NMR spectrum of (37) is more complex than those of (32) and (35) resulting in the splitting of the 2-substituted propanol. The C10 methyl group at 8.81 τ is coupled to the C8 methine proton at 7.25 with a coupling constant of 7Hz, which is in turn coupled to the C9 hydroxymethyl group at 6.53 τ with a splitting of 6.9 Hz resulting in sextet splitting pattern. Since the aromatic protons resonate as a singlet at 2.99 τ confirmation of the 1,4 aromatic substitution pattern was obtained from the IR bands at 1606, 1515 and 830 cm^{-1}.

6.3 Myrtenal and Myrtenol

Selenium dioxide oxidation of (+)-α-pinene (38) in refluxing ethanol, followed by distillation at reduced pressure (95-97°C/10 mm Hg), gave a pale yellow oil containing 95% myrtenal (39) which readily polymerised on standing in air. The product was confirmed as (39) by GC-MS with the characteristic base peak at m/e 79. Reduction of this crude product by ethanolic sodium borohydride gave an alcohol with a molecular formula $C_{10}H_{16}O$, indicated by the parent ion in the mass spectrum at m/e 152, which had bands in the IR spectrum at 3320 and 1060 (OH), 3032, 1650 and 800 cm^{-1} ($R_2C = CHR$). Analysis of the NMR spectrum indicated geminal methyl groups at 9.08 and 8.83 τ and a hydroxymethyl group at 6.06 τ. The methylene and bridgehead protons resonate as a broad envelope at 7.65 τ with the exception of the C7 endo-proton which was observed as a doublet with

8Hz splitting at 8.75τ. Although this value is small for a geminal



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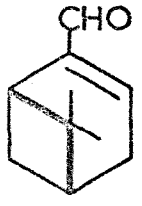

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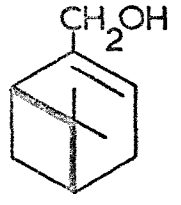

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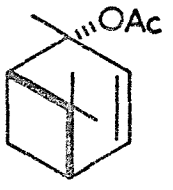
methylene coupling it is consistent with those observed for other compounds with the pinene skeleton[114].

6.4 cis-Pin-3-en-2-ol

Lead tetracetate oxidation of (+)-α-pinene in dry benzene, buffered with calcium carbonate, gave an oil containing predominantly cis-pin-3-en-2-yl acetate (40)[115]. Analysis of a small quantity of (40) purified by preparative GC enabled the structure of this compound to be assigned. The IR showed bands associated with the acetate at 1780 cm^{-1} and the cis disubstituted olefin at 730 cm^{-1}. In the presence of acetic acid the primary product (40) can rearrange to

trans-verbenyl acetate (41) by an acid catalysed process[116]. However, the absence of strong bands at 970 and 733 cm^{-1}, found in the IR spectrum of (41), confirmed the product to be (40). The NMR spectrum was also consistent with the structure (40) with methyl



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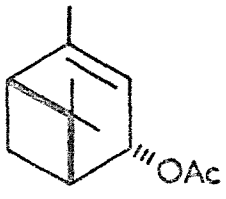

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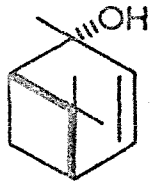

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groups at 9.10, 8.69 (gem dimethyls), 8.58 τ while the acetate methyl group was observed at 8.00 .

Hydrolysis of the crude acetate product for 24 hours in aqueous methanolic potassium hydroxide yielded the alcohol cis-pin-3-en-2-ol (26) and a hydrocarbon identified as verbenene (17). Purification of the crude product by preparative GC gave an optically active oil ($[\alpha]_D^{20} = +47^\circ$). Analysis of the NMR showed the geminal methyl

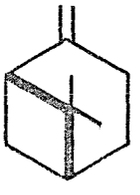
groups at 9.13 and 8.66τ, while the allyl methyl group had moved downfield from 8.58 to 8.28 τ. The vinyl protons in (40) occur as a broad singlet at 4.86 τ, whereas in the alcohol (26) they are less deshielded and separate into two resonances at 6.72 and 5.64 τ.

6.5 Pinocarvone, cis and trans pinocarveol

Selenium dioxide oxidation (+)-β-pinene in refluxing carbon tetrachloride gave a brown oil which on distillation gave pinocarvone (42)[117]. The distillate was further purified by preparative GC to give an optically active colourless oil ($[\alpha]_D^{20} = -65°$) with a molecular weight of 150. The IR spectrum contained bands at 1710 and 1627 cm^{-1} identified by the UV maximum at 234 nm (ε = 11,700) as arising from an exo-cyclic α,β-unsaturated ketone. The NMR spectrum exhibited two vinyl protons at 5.10 and 5.07 τ, split by a 2Hz geminal coupling, and methyl groups at 9.10 and 8.57 τ.

Reaction of (42) with bromine followed by zinc metal reduction produced crude cis-pinocarveol (43) which was purified by preparative GC. The unsaturated alcohol with bands in the IR spectrum at 3280 (OH), 1642, 893 cm^{-1} ($R_2C = RCH_2$), showed a parent ion at m/e 152 in its mass spectrum. The NMR spectrum was broadly similar to that of (43), although the exo-methylene protons at 5.25 and 4.95 τ are more finely split as a result of additional coupling with the C3 proton. The position of this allyl proton at 5.56 τ is lower than normally expected, possibly the result of deshielding by the olefin, while the methyl groups have shifted up-field to 9.27 τ and 8.75 τ due to the loss of the carbonyl group deshielding.

Lead tetraacetate oxidation of (+)-β-pinene (23) in refluxing dry benzene, buffered with calcium carbonate, by the method of Hartshorn and Wallis[117], gave a colourless oil consisting predominantly of two mono-acetates. Distillation under reduced pressure produced two fractions containing trans-pinocarvyl acetate



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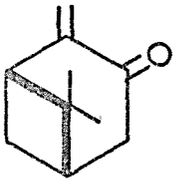

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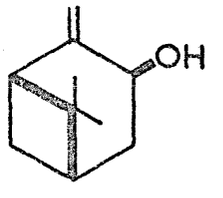

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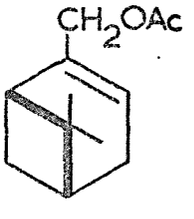

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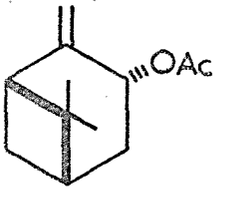

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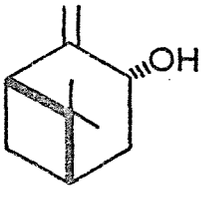

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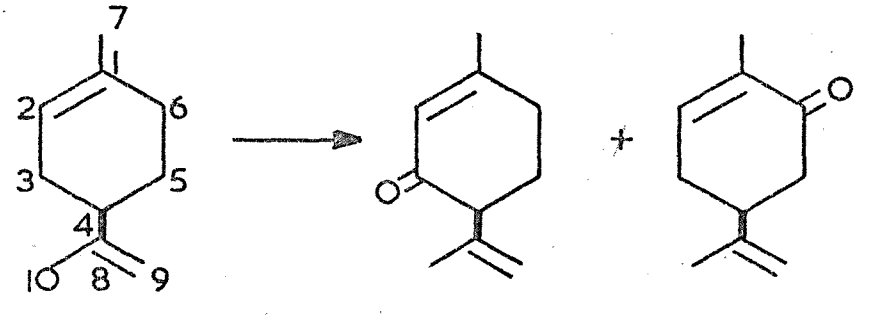
(44) (85%) and myrtenyl acetate (45) (15%). Preparative GC of a small quantity of (44) for spectroscopic analysis gave an optically active colourless oil ($[\alpha]_D^2 = -67^o$) which had bands in the IR spectrum at 3070, 1680 and 865 ($R_2C = CH_2$), 1775 and 1280 cm^{-1} (OAc). The NMR spectrum showed characteristic resonances at 9.27 and 8.90 τ (gem-dimethyl groups) and 7.94 τ for the acetate methyl group. The exo-methylene group at 5.95 and 4.93 τ shows fine splitting resulting from coupling with the C1 and C3 protons while the latter, which is strongly deshielded by the acetate carbonyl group, resonates as a doublet with a 7.5 Hz splitting at 4.40 τ.

The crude acetate distillate was treated as previously described to yield an alcohol which, on purification by preparative GC, gave dextrorotory trans-pinocarveol (25) ($[\alpha]_D^{20} = +78^o$). The NMR spectrum was almost identical to that of the acetate (44) showing only minor shifts in the positions of the geminal methyl groups (9.33 and 8.69 τ) and the exo-methylene group (5.15 and 4.97 τ). The greatest change was observed with the C3 proton which moved up-field by 1.16 τ to 5.5 τ, presumably as a result of the removal of deshielding by the acetate group.

Myrtenol (31), also obtained by the cleavage of (45) had identical spectroscopic properties to the sample produced by the method discussed in section 6.3. It is interesting to note that both samples were dextrorotory ($[\alpha]_D^{20} = +49^o$ and 53^o) which is consistent with the absolute stereochemistry of (+)-α-pinene and (+)-β-pinene.

6.6 iso-Piperitenone

Oxidation of limonene (46) with chromium trioxide pyridine complex results in the formation of carvone (47) and iso-piperitenone (48)[118]. The work-up procedure described in this paper proved ineffective at removing all the pyridine from the product, which remained a dark brown colour. Repeating the work-up with 10% sodium hydroxide instead of saturated sodium bicarbonate, and 10% instead of 5% hydrochloric acid, resulted in a pale yellow oil which contained three products by GC in the ratio of 1 : 2 : 4. The smallest component proved to be unreacted limonene while the other two compounds had parent ions at m/e 150 in their mass spectra giving their molecular formulae as $C_{10}H_{14}O$. The minor oxygenated product



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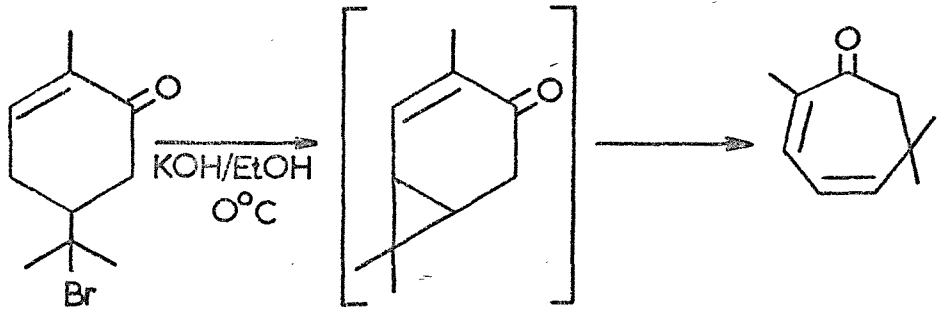
was identified as carvone by comparison with an authentic sample. The major product, obtained in 34% overall yield, had an NMR spectrum consistent with the structure of (48) with two vinyl methyl groups at 8.26 and 8.06 τ. The exo-methylene protons at 5.24 and 5.04 τ were distinguished from the C3 vinyl proton by the 2Hz exo-methylene coupling. The C4 methine proton resonates as a triplet at 7.06 τ with a splitting of 8Hz.

The UV spectrum maximum at 233 nm (ε = 9,450) is also consistent with an endo-cyclic α,β -unsaturated ketone bearing one alkyl substituent.

6.7 3,6,6-Trimethylcyclohepta-2,4-dienone

Eucarvone (28) can be formed from carvone hydrobromide (48) in base via a carenone intermediate[119].



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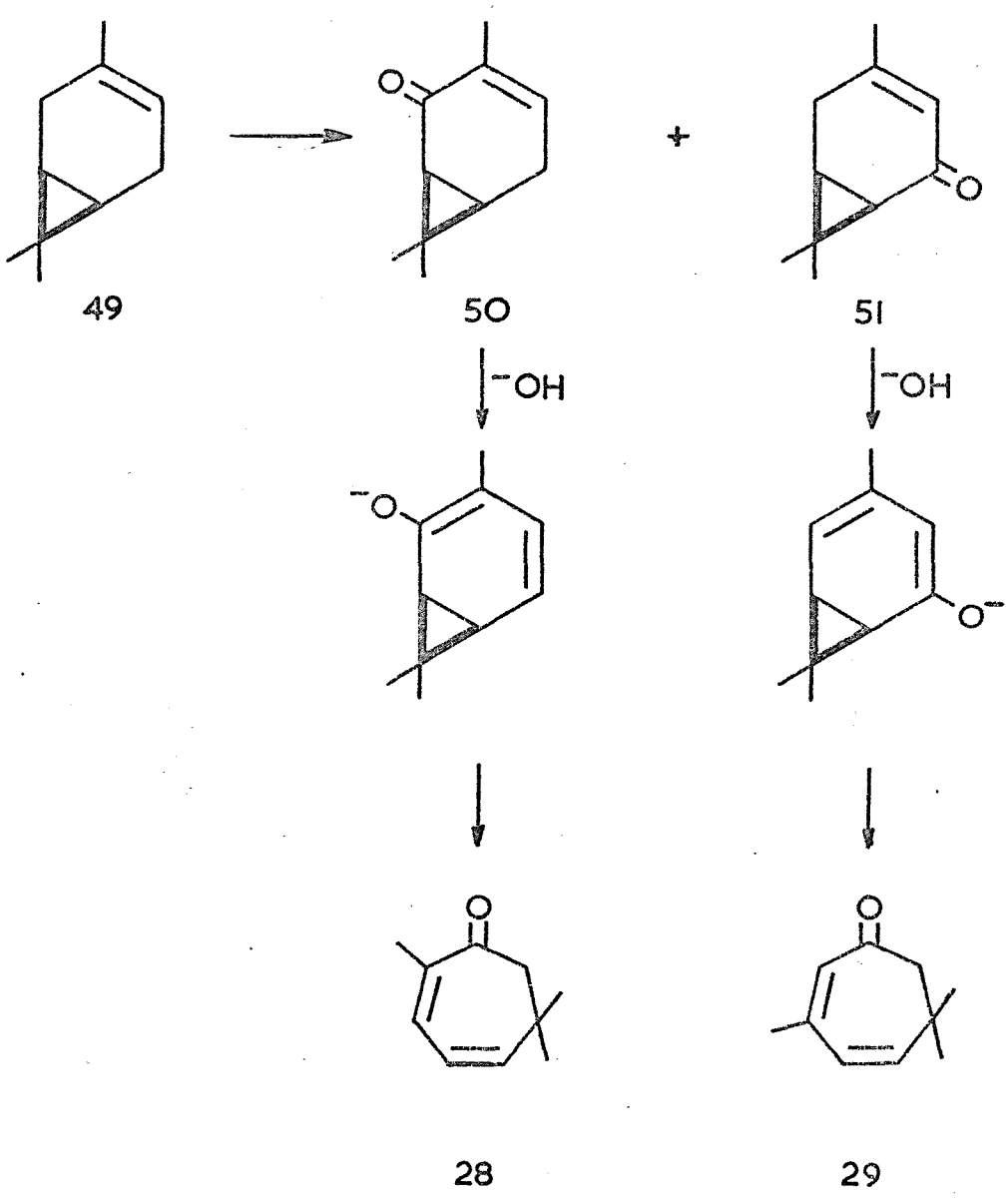
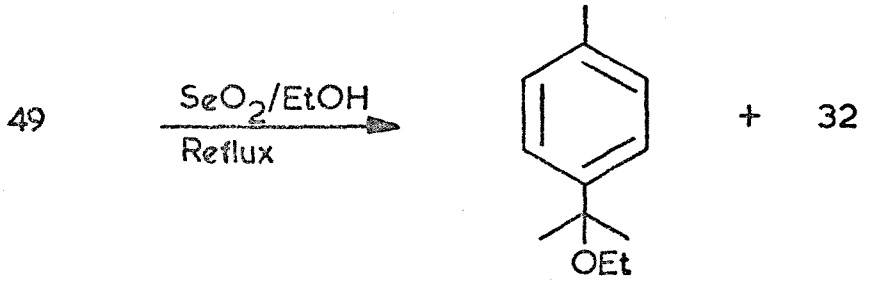
If car-3-ene could be oxidised to give the two cyclic ketones car-3-en-2-one (50) and car-3-en-5-one (51) then these should also be capable of undergoing enolisation to give (28) and (29) respectively.



Selenium dioxide in refluxing ethanol oxidised (49) to two products which on work-up were identified as p-cymene-8-ol (32) and its ethyl ether (52) in 6 and 22% overall yields. A further 10%



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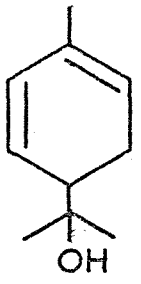
consisted of several minor unidentified products.

A second selenium dioxide oxidation in refluxing methylene chloride, using the same procedure as that adopted for the oxidation in ethanol, gave an oil which on distillation at 1 mm Hg gave a hydrocarbon fraction consisting of predominantly unreacted car-3-ene (48% of total product) and an oxygenated fraction containing several compounds of which the five most abundant were identified. The major component was again identified as (32) while the second most abundant was assigned the structure p-mentha-1,5-dienol (53) on the

evidence of UV, NMR and mass spectra. The latter showed a small parent ion at m/e 152 and a base peak at m/e 59 which is characteristic of an iso-propanol substituent. The UV spectrum maximum at 263 nm (ε = 12,600) was consistent with a monocyclic diene in which both olefinic bonds are endo-cyclic which would give the molecular formula as $C_{10}H_{16}O$. The NMR spectrum showed a singlet at 8.40 τ (iso-propanol methyl groups) and a vinyl methyl group at 8.30 τ. Three vinyl protons were observed at 4.64 (1H, broad) and 4.27 τ (2H singlet), which ruled out the 1,4-diene structure, but was consistent with the 1,5-diene structure. This was supported by the IR spectrum with bands at 800 and 720 cm^{-1}, suggesting the presence of both cis-disubstituted and trisubstituted olefins.

Two minor products had identical mass and UV spectra, but different retention times on GC columns. The first of these was identified as eucarvone (28) by comparison of its IR and NMR spectra with an authentic sample. The NMR spectrum of the second compound differed from (28) only in the vinyl region, which showed a two proton AB quartet at 4.54 and 3.92 τ split by 11.5 Hz and a singlet vinyl proton at 3.98 τ. This vinyl splitting pattern could arise from isomers (29) and (54) which could be distinguished on the evidence of the NMR spectrum. The C3 proton of eucarvone resonates at 3.48 τ and since the methyl group substitution would be expected to have little effect on chemical shift of the vinyl protons, the absence of a resonance below 3.92 τ indicated (29) to be the structure. The melting point of the 2,4-DNP derivative of (54) is quoted as 143°C[120], while that of the compound isolated was

177°C, identical to the literature value for (29)[121].



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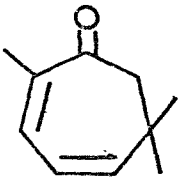

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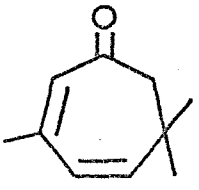

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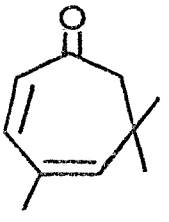

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The third minor optically active product ($[\alpha]_D$ = + 212°) was tentatively assigned the structure of (50) or (51) on the basis of the IR and NMR spectra, the latter showing a gem-dimethyl group (8.90 and 8.78 τ) and a vinyl methyl group at 8.28 τ. A series of resonances equivalent to two protons between 8.43 to 8.70 τ were assigned to the cyclopropyl methine protons. The vinyl proton at 3.74 τ was a broad finely split singlet indicating the structure (50) rather than (51), where the isolated vinyl proton would be expected to be uncoupled. Final proof of the structure came from reaction with ethanolic potassium hydroxide which yielded a compound identical in spectroscopic properties to eucarvone, thus giving the structure as (50).

Aerial oxidation of car-3-ene followed by reductive work-up with sodium sulphite gave a product containing ten major products plus a series of minor products representing 9% of the material separated by GC. Distillation at 1 mm Hg gave a hydrocarbon fraction containing (17), (19) and (49) in 4, 1.5 and 13% overall yield, while the second distillate fraction contained five mono-oxygenated compounds including (28), (50), (29), (32) in 6%, 2%, 14% and 17% overall yield. The fifth product isolated in 3% yield had an NMR, IR and UV spectra very similar to those of (50). However, reaction with alcoholic potassium hydroxide yielded (29) and, on the evidence of this data, the compound was assigned the structure car-3-en-5-one (51).

7. BIOASSAYS ON THE FRASS MONOTERPENES AND THEIR RELATIONSHIP WITH HOST-WOOD MONOTERPENE HYDROCARBONS

7.1 Bioassay for Oviposition Attraction

The two major mono-oxygenated monoterpenes, verbenone (30) and p-cymene-8-ol (32), isolated from the frass, were both EAG active and threshold responses were obtained with sample concentrations of 100 ng/µl for (30) and 20 ng/µl for (32). This result, together with the oviposition observations (section 4.1) indicated (30) and (32) as possible oviposition attractants.

A simple choice test was devised in an attempt to prove this hypothesis. The bioassay which tested host-wood attraction against host-wood plus sample used two groups of blocks, each group consisting of two blocks placed one on top of the other and separated by a filter paper. The sample to be tested was then placed on the filter paper and bioassay initiated by placing two unmated males and two unmated females in the bioassay tank. Tactile stimuli were not eliminated in these tests and inconsistent results were obtained, apparently due to variations in the surface texture of the wood. The bioassay used by Becker for oviposition experiments with pine essential oils was also a two choice test[33]. Tactile stimuli were minimised in this method by smoothing all rough surfaces and edges to produce a smooth surface. The method involved placing two groups of five blocks in a bioassay tank 20 cm apart, one of which contained three chemically treated and two untreated blocks while those in the other group were all untreated. The blocks in each group were separated by spacers

to produce a 0.3-0.6 mm gap and held together by two elastic bands.

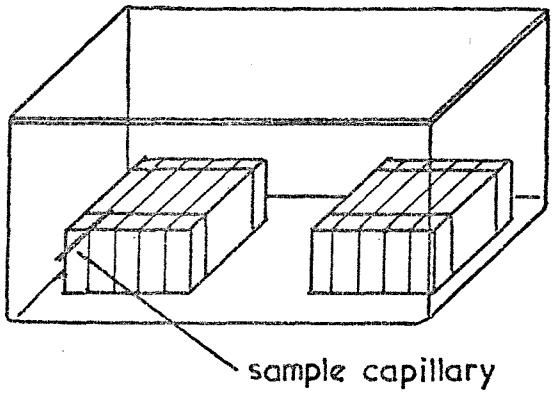

Fig 27. Bioassay tank set-up for testing compounds for oviposition attraction.

The Becker bioassay was set up in these studies to test verbenone and p-cymene-8-ol, however, the chemical treatment of blocks by coating with 2-3 gms of test compound was not used. The EAG results suggested that H. bajulus females would respond to an atmospheric concentration of a physiologically active compound considerably lower than would be generated by the chemical treatment described. A diffusion model technique devised by Wilson, Bossert and Regnier, for estimating behavioural threshold concentrations of active compounds for formicine ants, used liquid samples volatilised

from a capillary tube[122]. After an initial high rate of diffusion the sample volatilises at an almost constant rate over a period of several days, with a value for a given sample dependent upon the internal diameter of the capillary. The bioassay was therefore set-up according to the method described, but with the sample diffusing from a capillary placed between two blocks as shown in fig. 27.

Although the effect of tactile stimuli had been minimised in this procedure, one other factor was found to have an effect on the distribution of eggs laid. The presence of this factor was established by running bioassays in groups of four (fig. 28) with position of sample and blank blocks being changed alternately. The results obtained when this procedure was adopted showed variations which appeared to be associated with uneven lighting of the bioassay tanks. H. bajulus adults, which appear to show positive photo-orientation, were affected by the directional lighting initially used, since even lighting from above eliminated the variations previously observed. After each test the tanks were wiped with cotton wool soaked with acetone and the blocks cleaned by glass-papering to remove all traces of sample compound.

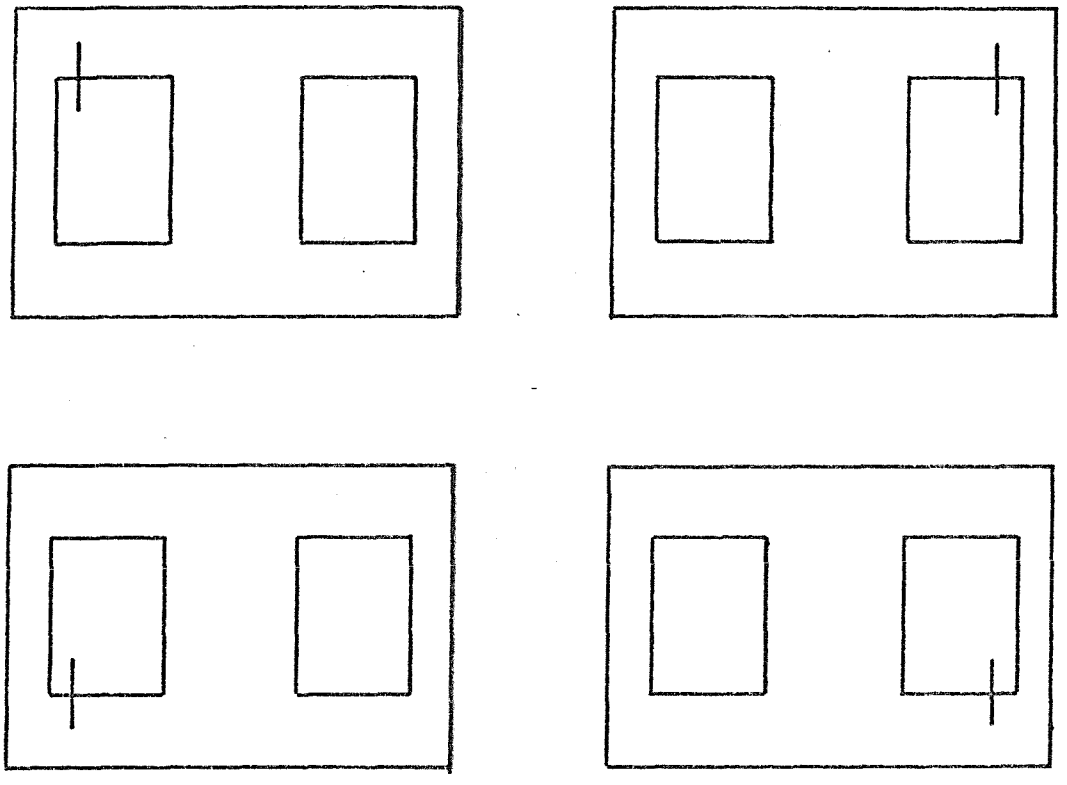

Fig 28. Arrangement of bioassay tanks and samples.
The position of sample and control blocks alternated.

7.2 Bioassay Results

The first of the four series of bioassays compared the oviposition attraction of infested and uninfested culture blocks. The fragile state of the surface of infested blocks made it impossible to remove the rough surfaces and blocks had to be chosen visually for similarity of surface. Since tactile stimuli could not be removed in this series of tests it was only possible to conclude on a qualitative basis that the presence of frass acted as an oviposition attractant. The samples used in the remaining three series were

(-)-verbenone and p-cymene-8-ol against host-wood and a 1 : 1 mixture, the ratio (30) and (32) are present in the frass, against (-)-verbenone. Ten replicates were run in each series using two unmated males and two unmated females per test to allow for statistical analysis of the results.

The size of females, and to a certain extent therefore the number of eggs laid, is dependent upon the length of time larvae are left in the untreated culture blocks before cold treatment (section 4.4). Although little variation in size of emerging adults was observed within a particular batch, differences in size did occur between batches. No assumptions were therefore made about the shape of the background distribution of eggs laid per female to which the oviposition experiment samples belonged. In the absence of such information results can be statistically analysed by non-parametric tests which do not employ the mean and variance directly. These tests generally depend on ranking, i.e. arranging a set of observations in order of size rather than using their actual numerical values[123a]. The bioassay used is a two sample case in which the samples are related, each subject serving as its own control. The Sign test and Wilcoxon matched-pairs signed-ranks test are two non-parametric tests which can be applied to these results. The first test measures differences between two samples on a qualitative basis as a plus or minus, while the second takes both magnitude and sign into account. Since the difference in the number of eggs laid on sample control blocks could be measured quantitatively the results were analysed by the Wilcoxon test[123b]. The analysis of bioassay

results (Table 4) shows verbenone to be an oviposition attractant (Wilcoxon matched-pairs signed-ranks test; $N = 10$, $\bar{T} = 1$, $p < 0.5\%$,

TABLE 4

Bioassay results for oviposition attraction; sample size = 10

Samples	Mean No. eggs laid/♀/test	Significance level
1. Verbenone	152	N = 10, $\bar{T}$ = 1, $p < 0.05\%$
2. Control	42	
3. p-cymene-8-ol	110	N = 10, $\bar{T}$ = 28 $p > 5\%$
4. Control	94	
5. Verbenone/p-cymene-8-ol	140	N = 10, $\bar{T}$ = 0 $p < 0.5\%$
6. Verbenone	80	

N = number of non-identical samples

$\bar{T}$ = sum of ranks with the least sign.

p = probability of acceptance of the null hypothesis.

one tailed test), with respect to the host-wood while p-cymene-8-ol has no activity on its own ($N = 10$, $\bar{T} = 28$, $p > 5\%$, one tailed test). The third series of tests show enhancement of verbenone activity by addition of p-cymene-8-ol ($N = 10$, $\bar{T} = 0$, $p < 0.5\%$, one tailed test). Further tests with different ratios might indicate a greater enhancement than with the 1 : 1 mixture. These results do not agree with the results by Becker[33] who on the evidence of a very

small number of tests concluded that in general the oxygenated monoterpenes, including verbenone, were either neutral or repellent in effect. This could possibly be explained by the large quantity of sample used producing an extremely high atmospheric concentration. This was supported by the evidence of early bioassays run in this study when diffusion from the sample in the sealed tube was not allowed to equilibriate. Under these conditions the majority of eggs were laid on the control blocks suggesting verbenone can function as an attractant at low concentrations and a repellent at higher concentrations, however, this needs to be substantiated by further bioassays. This dual function of a compound depending on concentration has recently been observed for the bark boring beetle Dendroctonus frontalis Zimm[9,10], and in the case of H. bajulus may act as a mechanism for controlling selection of a host which is not too heavily infested. In his studies Becker also used trans-pinocarveol and a mixture of myrtenol and trans-verbenol, the first two of which have been isolated from the frass during these studies. Trans-pinocarveol was concluded to be repellent while the mixture was mildly attractive. In view of this, further bioassays with the minor components isolated from the frass may implicate some of these as oviposition attractants or synergists.

7.3 Relationship between the Frass and Host-wood Monoterpenes

The extractions of P. sylvestris culture wood and frass reported in section 5.5, demonstrated that the mono-oxygenated monoterpenes isolated from the frass are genuine products of

metabolic fate and not artifacts. This was further confirmed by thorough analysis of the low molecular weight GC fractions of both wood and frass extracts on column G (fig. 23a and b). The previous studies of mono-oxygenated monoterpenes from pine have dealt with treated oleo resins used as turpentine and flotation oils[124,125]. Normalisation of the concentrations of these fractions showed p-cymene to be present at the same level in both wood and frass, cis-pin-3-en-2-ol (26) was the only other compound identified at comparable levels in both frass and wood. Although terpinen-4-ol, α-terpineol and verbenone were also identified in the two fractions, they were present at very much lower levels in the wood than in the frass, the remaining compounds identified in the frass could not be detected in the wood. The presence of naphthalene in all the frass extracts is difficult to explain since extraction and purification procedures run as blanks failed to detect it, which suggests it is not an artifact. Naphthalene has recently been isolated from the head of the termite Macrotermes bellicosus[126].

All the compounds isolated from the frass, with the exception of cis-pin-3-en-2-ol and naphthalene, are products of an allylic-type oxidation of the terpene hydrocarbons present in the wood. Although these could be the result of autoxidation, this has been ruled out by a blank experiment as storage of a wood extract at room temperature for several months did not lead to appreciable oxidation. Examination of structural types isolated suggests a number of possible inter-relationships and these are denoted by the broken lines in fig. 29. It is likely that verbenone (30) is derived by

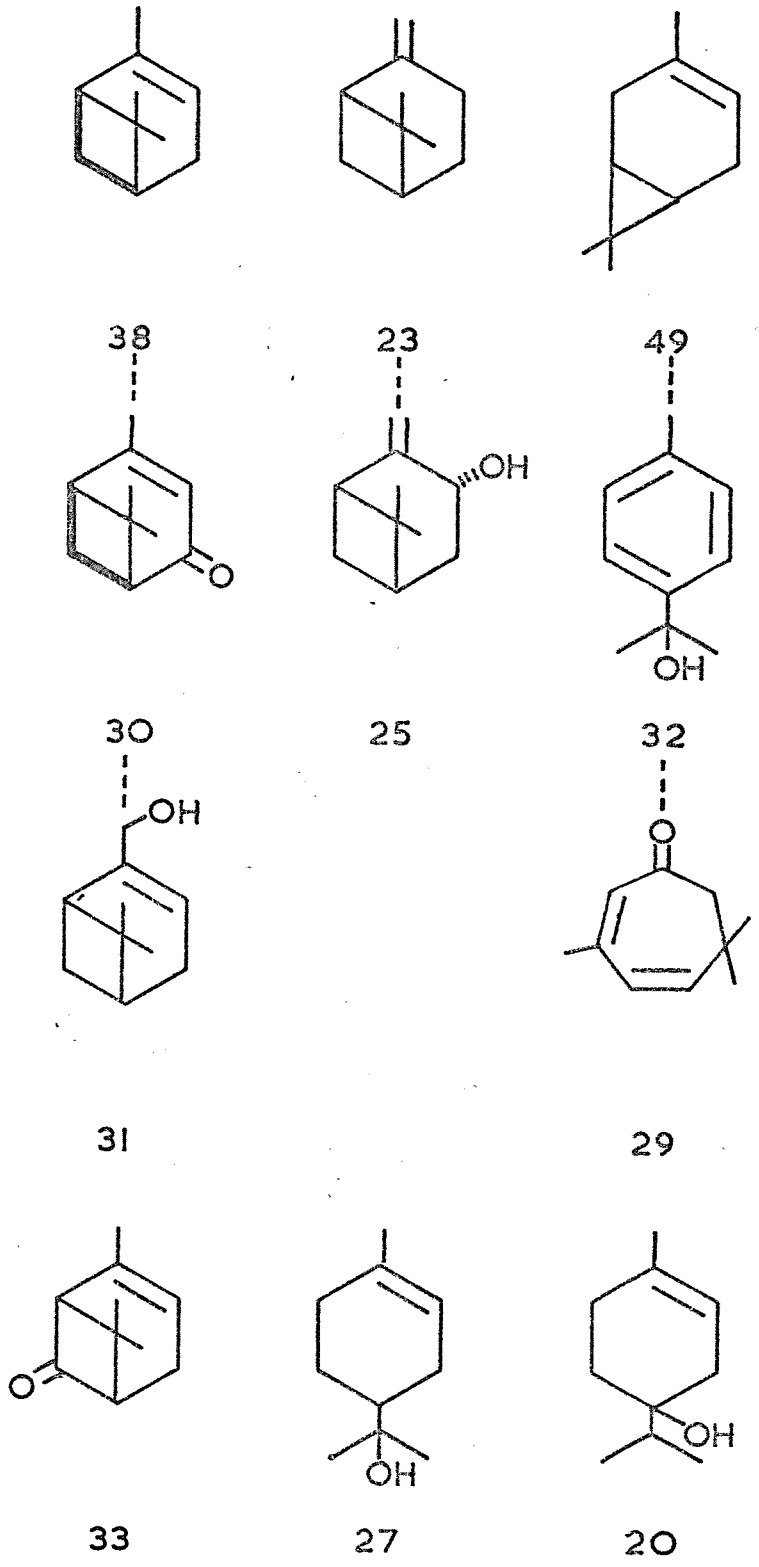

Fig 29. Possible relationships between frass components and host-wood monoterpene hydrocarbons.

allylic oxidation mechanism of α-pinene (38). This is supported by the presence of predominantly (-)-α-pinene ($[\alpha]_D^{20} = -35^o$) in the culture wood which on oxidation leads to (-)-verbenone, the optical isomer isolated from the frass. Since it is reported that British P. sylvestris contains predominantly (-)-α-pinene[127] it would be interesting to observe the behavioural response of beetles cultured in this wood to (+)-verbenone. Although no relationships between (49) and (29) or between (49) and (32) are immediately obvious, it was possible to produce (29) and (32) by aerial oxidation in the ratio of 1 : 1. The ratio of (29) and (32) from the frass was 1 : 10 suggesting again that the products isolated from the frass were not the result of autoxidation.

The method by which these compounds are synthesised in the gut of H. bajulus larvae is not known. Antibiotics do not appear to affect the rate of growth of the larvae indicating the absence of symbiotic bacteria in the gut[31]. Many species of the family Cerambycidae have been reported to harbour symbiotic yeasts in the mycetome, however, H. bajulus is reported to be free of all symbiots[18,19]. In the bark boring Scolytidae beetles two theories have been proposed for the synthesis of pheromones from the host-wood terpene hydrocarbons. From the results of work on several Dendroctonus and Ips species, Hughes has suggested the host-wood terpenes diffuse from the gut into the haemolymph where they are oxidised by a general oxidase as a detoxification mechanism. The products then diffuse back into the mid-gut[128]. More recent work by Brand et al[129] has identified bacteria of the species Bacillus

cereus in the mid-gut of Ips paraconfusus, one of the species studied by Hughes. This bacterium was found to be capable of converting α-pinene in vitro into cis and trans-verbenol and myrtenol. These appear to be the two most likely mechanisms by which the larvae of H. bajulus convert the monoterpene hydrocarbons, with the evidence favouring an enzymatic process.

The other question of interest concerning the relationship between frass components and host-wood hydrocarbons concerns the variation in the concentration of the three major hydrocarbons α- and β -pinene and car-3-ene. The ratio of α-pinene (38) and car-3-ene (49) in particular vary dramatically. The southern European varieties contain a considerable amount of (38) but no (49), while northern European varieties contain up to 65% of (49) and as little as 5% of (38)[130]. This poses the question of the effect this variation would have on the mono-oxygenated monoterpenes present in the frass and therefore the effect on oviposition attraction. If the same compounds are produced with differing ratios, depending on geographical location, then is verbenone still utilised as an oviposition attraction enhanced by p-cymene-8-ol or has a different mechanism been evolved? If the former is true then bioassay tests with laboratory cultured beetles should show the loss of oviposition attraction caused by a lower concentration of verbenone in the frass, compensated by the increased enhancement resulting from the higher concentration of p-cymene-8-ol.

8. EXPERIMENTAL

8.1 Gas Liquid Chromatography

A Pye Unicam Series 104 gas chromatograph fitted with a dual FID detector was used in all chromatographic studies. The system was fitted with injection-port heaters and a detector oven, temperatures were recorded by thermocouple temperature read-out. The all-glass columns were fitted with 6 mm o.d. ground glass ends and connected to the detector with a column connector (Pye Unicam, Cambridge), modified by replacing the thread with one cut from a 0.25 in. stainless steel Swagelok union. A gas-tight seal was achieved with a 0.25 in. graphite ferrule (S.G.E. Melbourne, Australia). Stationary phases were coated on 100-120 mesh Diatomite CLQ acid/alkali washed, DMCS treated (J.J's (Chromatography) Ltd., Kings Lynn), columns used were: 7 mm i.d. x 1.5 m: A, 5% OV-1; B, 5% Carbowax 20M; C, 15% PPGA; 4 mm i.d. x 1.5 m: D, 5% XE 60; E, 5% OV-1; F, 5% Carbowax 20M; G, 10% PPGA; H, 10% FFAP; 2 mm i.d. x 1.5 m: I, 5% Carbowax 20M; 'Bergstrom' column, 10% Carbowax 20M. The nitrogen carrier gas flow rates for the 7 mm, 4 mm and 2 mm i.d. columns were 80, 50 and 25 ml/min respectively, the same flow rates were used for the helium carrier gas in GC-MS. Columns D, E, F, G, H and I were run isothermally at 110°, 100°, 130°, 120°, 120° and 130°C respectively. Column E was also used in a dual FID mode programmed at 80°C for 4 min then 4°/min to 325°C. Standard analytical temperature conditions were used for preparative GC with both 7 mm and 4 mm i.d. columns.

8.2 Solid Sample GC of H. bajulus Adults

The pre-column tube injection device described by Bergström[62] was modified for use in a Pye 104 gas chromatograph. The glass pre-column (fig. 3; 1) consisted of a glass tube (150 mm x 7 mm i.d.) fitted with a B10 cone at one end (Quickfit, Stone, Staffs.) and a ground glass tube at the other (20 mm x 6 mm o.d.), by which it was connected to the column using a 0.25 in stainless steel Swagelok union fitted with graphite ferrules. The 'Bergström' column was mounted and coupled to the detector with the modified column connector as shown in Fig. 4. The material to be degassed was placed in the pre-column while chilled on dry-ice. The pre-column was then connected to the column, the 'U'-shaped part of which was cooled in dry-ice, and sealed with a B10 socket (Quickfit) (fig. 3; 2) fitted with a length of 6 mm o.d. ground-glass tubing by which the column was connected to the injection head (fig. 3; 3). The heater (fig. 3; 4) was heated to 120° over a five minute period, after which the pre-column and heater were replaced by a 150 mm x 6 mm o.d. glass tube. The carrier gas flow rate was allowed to settle for 4 min, the dry-ice removed from the column 'U' and the oven heated ballistically to 80°C. The column was held isothermally for two minutes 80°C, then programmed at 4°/min to 180°C.

8.3 Biological Studies

The oviposition bioassay tests and behavioural studies were set up in a 30 x 30 x 60 cm glass tank fitted with a glass top in a room at 27°C and 70% relative humidity. The tanks were placed in a

group of four and lit from directly overhead by fluorescent strip lights on a 12 hour on-12 hour off cycle. Freshly emerged beetles were placed in the room for two to four days before use. Ten blocks of wood 15 x 5 x 2 cm smoothed with glass paper, including edges and end grain, to a consistent surface, were made up into two groups of five. The blocks in each group, held together by two elastic bands, were separated by two spacers to produce a 0.4 mm gap and placed in the tank 20 cm apart. The sample to be tested (5µl) was placed in a capillary tube (1.4 mm i.d.), sealed at one end and left for 24 hours. The tube was then placed between two blocks with the open end 1 cm clear of the end of the block fig. 27. Each test was started at 10 a.m., 4 hours after the light period had started, by placing two unmated male and two unmated female adults between the two groups of blocks. The tanks were left for five days after which the number of eggs laid on the sample and control blocks were counted. Ten replicates were obtained for verbenone, p-cymene-8-ol, against host-wood, and a 1 : 1 mixture of verbenone/p-cymene-8-ol against verbenone and the results analysed statistically with the non-parametric Wilcoxon matched-pairs signed-ranks test[122a].

Stereoscan micrographs were obtained with a gold coated sample on a Cambridge Stereoscan Type 97113/2A with antennae from both H. bajulus male and female adults.

Electrophysiological studies of frass extracts were made on the Electroantennograph system of Floyd[61]. Excised antennae were placed between glass micro-electrodes fitted with insect ringer solution. The amplified signal was recorded on a high speed spray

pen recorder. Samples on a 1 cm filter paper circle were placed in a Pasteur pipette after the solvent had evaporated and attached to the "puffer". The sample vapour was blown across the antennae with air from a cylinder purified by activated charcoal with a minimum of 20 seconds between tests.

8.4 Microscale Chemical Analysis and Post-loops

Columns D and E were used for on-column silylation using BSA at 130° and 120°C respectively. The columns and syringe were conditioned with reagent until all material which could be silylated had been removed. The sample (approximately 0.1-0.5%) in methylene chloride was injected, followed 15 seconds later by 0.1 µl of BSA. Column E was used for GC-MS analysis of the silyl ethers.

Bromination and sodium borohydride reduction on a microscale were performed in a 10 µl syringe (SGE Melbourne, Australia) with the following reagents:

1. 1 µl of a 1% aqueous bromine solution.

2. 1 µl of saturated ethanolic sodium borohydride (2 min).

The reagent was mixed with the sample by barrel action and expelled from the syringe before injection of the sample onto the column. The reaction with bromine was allowed to proceed until the aqueous reagent had just become colourless. The sodium borohydride reduction of terpene carbonyl compounds required work-up with 1 µl of water mixed again with the sample by barrel action.

Abstractor post-loops were constructed from a single turn 0.2 mm i.d. glass tube, diameter 60 mm, fitted with short lengths of 6 mm o.d. ground glass tube at each end and placed between the column end and the detector, using the Swagelok union and column connector described previously. The following reagents were packed into the loops, the first and last 25 mm of which were filled with untreated Diatomite CLQ.

1. Boric acid. Powdered boric acid (50 mg) was thoroughly mixed with Diatomite CLQ (450 mg).

2. FFAP. FFAP (100 mg) (J.J's (Chromatography) Ltd.) was dissolved in chloroform (10 ml), Diatomite CLQ (400 mg) added and the solvent removed in vacuo.

3. o-Dianisidine. o-Dianisidine (25 mg) was dissolved in chloroform (10 ml), Diatomite CLQ added and the solvent removed in vacuo.

4. Zinc Oxide. Phosphoric acid (2% of 85%) was added to Diatomite CLQ (500 mg) suspended in water (10 ml), and the solvent removed in vacuo. The powder (450 mg) was mixed thoroughly with powdered zinc oxide (50 mg) .

5. Sodium Metabisulphite. Sodium metabisulphite (100 mg) was dissolved in water (10 ml), Diatomite CLQ (400 mg) added and the solvent removed in vacuo.

The micro-ozoniser (fig. 6) consisted of the barrel of a 5 ml glass syringe (1), surrounded in turn by a layer of aluminium

foil (2), 25 turns of 22 SWG copper wire (3) and an insulating polythene sheath (4). A 180 mm length of 2 mm o.d. stainless steel tube (5) was passed through the cork (6), into the centre of the barrel. Ozone was generated by passing oxygen (BOC, Research Grade) at 10 ml/min into the barrel and applying a discharge, produced by earthing the wire (7) and placing a Tesla coil vacuum tester on the lower end of the stainless steel tube (5). Samples for ozonolysis, in methylene chloride, were placed in glass tubes (50 mm x 7 mm o.d.) fitted with a polythene cap through which the tube (5) and an elbowed glass tube outlet (8) (1 mm i.d.) were passed.

Ozone was passed through the sample (1-100 μg) until a starch/KI indicator paper, moistened with dilute hydrochloric acid, was just turned blue by the gases venting from the outlet tube (8). The sample was then purged with nitrogen to remove excess ozone and the carbonyl compounds produced by adding a small crystal of triphenylphosphine. The products were analysed by GC as carbonyl compounds, or as their 2,4-DNP derivatives, formed using a dilute aqueous reagent developed for water soluble carbonyl compounds[131].

8.5 Micropreparative Splitter and Trapping System

The splitter shown in fig. 9 consists of an 'S' shaped length of 0.5 mm i.d. thick-walled glass capillary tubing (1), with a 25 mm length of 6 mm o.d. ground-glass tubing joined at right angles to one arm approximately 15 mm from the end. The geometry of the splitter is such that when the GC column (10) is connected to the 6 mm tubing, the other arm of the capillary passes centrally through the exit port

of the oven (fig. 10). Both ends were fitted with glass/metal seals (2) (Quadrant Glass Co. Ltd., Essex, U.K.) with dead volume kept to a minimum. A length of thread cut from a 0.25 in barrel connector (Pye Unicam, Cambridge, U.K.) and bored out to 2 mm i.d. was silver-soldered to the exit-port glass/metal seal. The other end of the splitter was connected to the FID detector by a length of 0.5 mm i.d. stainless steel tubing (3), silver-soldered to the glass/metal seal and incorporating a 25 x 0.485 mm Nichrome wire. This arrangement gave a split ratio of 100 : 1, with simultaneous arrival of components at the detector and trapping system. Variation in the split ratio was achieved by changing the length and diameter of the wire. No significant change in the time of flow to either end of the wire. No significant change in the time of flow to either end of the splitter was observed for ratios within the range 25 : 1 to 200 : 1. The solumns were connected to the splitter with a 0.25 in stainless steel Swagelok union (4) bored out to 7 mm i.d. and fitted with 0.25 in graphite 'O' rings (S.G.E. Pty., Melbourne, Australia), enabling the system to be used up to 400°C.

The trapping system (fig. 10) consists of a removable transfer line (7) and solvent trap (9). The transfer line was constructed from a 150 x 1.0 mm i.d. glass capillary (Jencons (Scientific) Ltd. Herts U.K.) bent at right angles 50 mm from one end and drawn to a slight jet (8) at the other. The capillary is inserted so that it butts directly onto the 0.5 mm tubing of the splitter and is held in position with a 0.25 in nut (5) and sealed with a 2 mm Viton 'O'-ring (6), (Hewlett Packard, Slough, U.K.).

A standard Pye injection-port heater (11) is used to maintain the outlet at 5-10°C above oven temperature. Samples are collected by passing the effluent through a minimum of 300 µl of solvent cooled to -15°C (acetone, water, solid CO_2): a fresh line and trap is substituted for each fraction.

8.6 Spectroscopic Methods

Mass spectra were obtained by GC-MS using an A.E.I. MS 12 spectrometer interfaced to a Pye Unicam 104 chromatograph with a Watson-Biemann glass frit separator. The spectrometer was tuned at 70 eV to a maximum on the electron multiplier for m/e 28, final adjustment to the tuning was made to obtain maximum response on the total ion monitor at 20 eV. Spectra were run at 70 eV, recorded on a UV galvanometer and processed by a Digispec 16 data system (VG Data Systems, Altringham).

UV spectra were run on ethanol solutions with a SP.800 spectrophotometer (Pye Unicam) and IR spectra were obtained on liquid films using a 157G grating spectrometer (Perkin Elmer). Optical rotations were determined on an ETL-NPL Auto Polarimeter using chloroform solutions.

Continuous wave and Fourier Transform NMR spectra were obtained on solutions in deutero-chloroform (99.8 atom %, Diaprop Inc. Milwaukee), unless stated otherwise and tetramethylsilane was used as the internal standard for continuous wave spectra. Fourier transform spectra were run with an internal deuterium lock using an acquisition time of 2 sec and a pulse time of 12 sec. Samples were

collected in 300 µl of deutro-chloroform at room temperature immediately before spectra were run. The solvent was dried by refluxing over activated 3A molecular sieve and then stored over activated 3A molecular sieve

8.7 Culture Method for H. bajulus

H. bajulus larvae were cultured by the method of Berry[94] using untreated P. sylvestris obtained directly from the Forestry Commission[95]. Newly hatched larvae were put into blocks of wood impregnated with yeast extract and peptone for six months, after which they were transferred to untreated blocks for at least three months. Cold treatment of these blocks for four weeks at 5°C resulted in the emergence of adult beetles 5-7 weeks later.

8.8 Purification of Solvents

Technical grade hexane (5 l) was stirred for six hours with 65% oleum (500 ml), the two layers were separated at the end of this period and the hexane washed with concentrated sulphuric acid (100 ml) until the washings were colourless. The hexane was then washed with water (2 x 100 ml) and saturated sodium bicarbonate (6 x 50 mls) and dried overnight (calcium hydride). Distillation from calcium hydride through a 1 m x 5 cm fractionating column filled with Fenske's helices gave a hexane fraction boiling at 67-68°C (4.5 l).

Methylene chloride (2.5 l) was washed first with saturated aqueous sodium bicarbonate (3 x 100 ml), water (2 x 50 ml) dried overnight (magnesium sulphate) and distilled through 1 m x 5 cm

fractionation column to give a fraction boiling between 40-41°C.

8.9 Isolation and Identification of Frass and Host-wood Volatiles

8.9.1 Extraction Procedure for Frass and Host-wood

Frass (300 g) collected from culture blocks immediately before required was Soxhlet extracted with methylene chloride (1 l) for 3 days. The extract was reduced to low bulk at room temperature in vacuo to yield a viscous brown oil (2.6 g) which showed EAG activity. The oil was added slowly to vigorously stirring hexane (100 ml) over a half hour period and stirred for a further three hours. The hexane solution (approximately 30 mg) was decanted from the insoluble material, stored overnight at -78°C and the resulting white precipitate removed by filtration. The hexane soluble fraction was the only one of the three to show EAG activity.

Frass (40 g) was cold extracted by continuously stirring with methylene chloride in the dark, under nitrogen, for one week. The extract obtained in this manner was worked-up as described above to 40 gms of a viscous brown oil. This cold extraction procedure was repeated with 40 gm of shavings produced from uninfested culture wood from which the surface layer had been removed. In addition to this, an extraction was made of sound wood from infested blocks from which all the frass had been removed.

8.9.2 Purification and Chemical Analysis

The hexane soluble fraction of the frass extract, SH was

shaken with saturated sodium bicarbonate (4 x 20 ml) followed by 10% aqueous sodium hydroxide (4 x 20 ml) and the residual organic layer washed with water and dried (magnesium sulphate) to yield 'neutral' fraction, SHN. The two alkali fractions were neutralised with 2N HCl, extracted with methylene chloride (3 x 10 ml) and dried (magnesium sulphate) to give a 'strong' acid fraction SHS, and a 'weak' acid fraction SHW. Physiological activity, demonstrated by EAG, was associated entirely with the 'neutral' fraction SHN.

A crude molecular weight separation of fraction SHN by preparative GC using column A gave seven fractions SHN1-7, of which only SHN-2 showed EAG activity at the nanogram level of concentration. This fraction was separated into ten components (T1-10) on column G and checked for purity on columns D and H, further purification, where necessary, was achieved with column D.

T1, Non-Polar Low Molecular Weight Volatiles

The majority of these very minor compounds were artifacts of which only three were identified by MS:

Chrysanthenone; m/e 107 (100%), 80 (58), 91 (48), 79 (31), 122 (28) 39 (27), 41 (24), 150 ($M^{+\cdot}$, 17), 77 (16), 70 (16).

4,α-dimethylstyrene; 132 ($M^{+\cdot}$, 100%), 119 (84), 91 (41), 105 (41), 92 (31) 39 (24), 65 (21), 131 (16), 51 (16), 63 (14).

p-cymene; m/e 119 (100%), 134 ($M^{+\cdot}$, 24), 91 (19), 120 (9), 117 (9), 77 (7), 41 (7), 65 (6), 115 (6), 105 (5).

T2, Terpinen-4-ol (20)

This compound was unaffected by BSA but appeared with a changed peak shape after the boric acid post-loop, bromination removed the compound from the chromatogram m/e 71 (100%) 93 (45), 111 (42), 43 (42), 86 (32), 69 (31), 55 (31), 154 ($M^{+\cdot}$, 30) 68 (12), 136 (12).

T3, trans-Pinocarveol (25)

This compound was subtracted by the boric acid post-loop, formed its silyl ether and was removed by bromination m/e 41 (100%), 55 (95), 92 (93), 70 (70), 91 (67), 83 (63), 39 (53), 69 (44), 81 (40), 134 (33) ($M^{+\cdot}$, m/e 152, 1%); τ 9.33 (s, 3H), 8.69 (s, 3H), 8.42 (d, C7 endo proton J = 9Hz), 8.32-6.41 (br.m., 5H), 5.56 (d, C3 H J = 8Hz), 5.15 and 4.97 (br.s. $R_2C = CH_2$).

T4, cis-Pin-3-en-2-ol (26)

The compound in this fraction was eluted from the boric acid post-loop with a changed peak shape, formed a silyl ether and brominated readily; m/e 91 (100%), 41 (69), 109 (64), 119 (55), 39 (51), 81 (41), 92 (38), 94 (37), 77 (32) 79 (31), ($M^{+\cdot}$, m/e 152, 2%). The compound dehydrated to give verbenene; λ max = 244 nm (ε = 11,250), m/e 119 (100%), 134 (31), 91 (18), 120 (11), 117 (9), 65 (7), 41 (7), 115 (6), 51 (6), 77 (5).

T5, α-Terpineol (27)

This compound brominated but was unaffected by boric acid,

o-dianisidine or BSA; m/e 59 (100%), 93 (84), 136 (64), 121 (57), 68 (44), 43 (42), 81 (40), 79 (33), 91 (31), 55 (30); τ 8.87 (s, 6H), 8.37 (br.s., vinyl CH_3), 8.25-7.60 (br.m., 6H), 4.76 (br.s. 1H vinyl).

T6. 3,6,6-Trimethylcyclohepta-2,4-dienone (29)

Reduction of this compound with sodium borohydride by syringe action without aqueous work-up removed this compound from the GC trace, with aqueous work-up a single product was formed. The compound which readily brominated was unaffected by the o-dianisidine post-loop. λ max = 294 nm (ε = 7,400); m/e 107 (100%), 150 ($M^{\cdot+}$, 50) 91 (47), 135 (28), 79 (26), 39 (25), 108 (25), 41 (18), 77 (16), 80 (15); τ 8.90 (s, 6H), 8.00 (s, vinyl CH_3), 7.20 (s, 2H), 4.54 (d, C4 vinyl proton, J = 11.5 Hz), 3.98 (s, C2 vinyl proton), 3.92 (d, C5 vinyl proton, J = 11.5 Hz).

T7, Verbenone (30)

Reduction with sodium borohydride and o-dianisidine post-loop results were the same as for T6, two products in the ratio 1 : 3 being formed. The compound also readily brominated; $[\alpha]_D^{20}$ = -248°; λ max = 254 nm (ε = 6,450); m/e 107 (100%), 91 (88), 39 (77), 135 (70), 41 (66), 80 (59), 150 ($M^{\cdot+}$, 57), 79 (50), 55 (33), 108 (31); τ 8.97 (s, 3H), 8.47 (s, 3H), 7.97 (d, vinyl CH_3 J = 2Hz), 7.95 (d, endo C7 proton, 8Hz), 7.52 (m, 2H) 7.20 (m, 1H), 5.31 (br.s., vinyl proton).

T8, Naphthalene

The only reaction this compound underwent was partial bromination; m/e 128 ($M^{\ddagger}$, 100%), 129 (11), 127 (10), 102 (8), 63 (7), 51 (6), 124 (5), 61 (5), 75 (4), 74 (3); τ 2.44 (m, 8H).

T9, Myrtenol (31)

This compound which formed its silyl ether and was subtracted by the boric acid post-loop was also removed by bromination; m/e 79 (100%), 91 (41), 41 (37), 108 (15), 39 (24), 77 (20), 93 (18), 95 (17), 67 (14), 55 (13); τ 9.08 (s, 3H), 8.83 (s, 3H), 8.75 (d, endo-C7 proton, J = 8Hz), 7.65 (m, 5H), 6.06 (br.s., 2H, hydroxymethyl), 4.53 (br.s., vinyl proton).

T10, p-Cymene-8-ol (32)

Partially affected by bromination, this compound was partially silyated and eluted as from the boric acid with a changed peak shape; m/e 43 (100%), 135 (75), 91 (24), 132 (13), 107 (12), 150 ($M^{\ddagger}$, 10), 65 (9), 39 (7), 105 (6), 92 (5); τ 8.48 (s, 6H), 7.69 (s, Ar-CH_3), 2.90 (AB qt, 4H, J = 8Hz). This compound dehydrated to give 4,α-dimethylstyrene; m/e 132 ($M^{\ddagger}$, 100%), 119 (84), 105 (41), 92 (31), 39 (24), 65 (21), 131 (16), 51 (16), 63 (14).

All compounds isolated were confirmed by comparison of data with synthetic samples and by co-injection on columns D, E, G and H.

8.10 Syntheses

All the starting materials used below were commercial materials with a purity of at least 95% and no further purification was attempted before use. The reduction of carbonyl compounds with sodium borohydride was performed using the method of Johnson and Rickman[70].

Chrysanthenone (18)

A 1% solution of (-)-verbenone ($[\alpha]_D^{20}$ = -245) was left standing on the bench in direct light for one week. Separation of the product formed by preparative GC (column B) gave chrysanthenone (31%); $[\alpha]_D^{20}$ = -60°; λmax = 295 nm (ε = 206); ν max 3015, 1785, 1655, 1390, 1378, 878 cm^{-1}; m/e 107 (100%), 91 (73), 80 (49), 79 (42), 122 (38), 39 (28), 41 (27), 105 (26), 77 (19), 70 (14), $M^{\ddagger}$ m/e 150 11%). τ 8.02 (s, 3H), 7.99 (s, 3H), 8.29 (m, vinyl CH_3), 7.44 (m, 2H), 7.34 (m, 2H), 4.87 (br.s. vinyl proton).

p-Cymene-7-ol (35)

Cuminicaldehyde 3 g (0.02 mol) was added dropwise to 0.37 g (0.01 mol) of sodium borohydride in 2.5 ml of H_2O and 2.5 mls of 50% aqueous ethanol at 0°C. After stirring at room temperature for 2 hours, the mixture was saturated with salt, extracted with ether, dried (magnesium sulphate), analysed by GC and purified on a silica column to give 2.9 gm of (35) (97%); ν_{max} 3610, 1720, 1630, 1225, 1015, 815 cm^{-1} m/e 134 (100%), 105 (61), 79 (56), 150 ($M^{\ddagger}$, 51), 107

(43), 91 (43), 119 (38), 77 (35), 41 (19), 117 (18). τ 8.78 (d, iso-prcpyl, J = 7Hz), 7.15 (septet, C8 proton, J = 7Hz) 5.57 (s, 2H), 2.32 (s, 4H aromatic).

p-Cymene-8-ol (32)

p-Methyl acetophenone 3 g (0.02 mol) in 20 ml of ether (Na dry) was added during 1 hour to gently stirring 3M methyl magnesium bromide in ether (7.5 ml) at room temperature. Stirring was continued for a further $\frac{1}{2}$ hour after which the white solid magnesium alkoxide was destroyed by adding saturated ammonium chloride until the ether layer was just clear. The two layers separated, the aqueous layer washed with two 10 ml aliquots of ether, the combined ether extracts dried (magnesium sulphate) and purified by silica column chromatography to give 2.7 g of (32) (91%); ν_{max} (LF) 3800, 2970, 1510, 1360, 1260, 1020, 955, 865, 820 and 725 cm^{-1}; m/e 43 (100%) 135 (75), 91 (24), 132 (13), 107 (12), 150 (M^{+}, 10), 65 (9), 39 (7), 105 (6), 92 (5). τ 8.48 (s, 6H), 7.69 (s, AR-CH_3), 2.90 (AB qt, 4H, J = 8Hz).

p-Cymene-9-ol (37)

p-Cymene-8-ol 2.25 g (0.015 mol) was refluxed under nitrogen for 30 minutes at 20 mm Hg in the presence of a small crystal of iodine after which it was slowly distilled over at 70-75°C. An ether solution of the distillate was washed with two 5 ml aliquots of 10% sodium thiosulphate, dried (magnesium sulphate) and purified on a neutral alumina column to give 0.64 g of 4,α-dimethylstyrene (37%), λ_{max} = 248 nm (ε = 11,000); ν_{max} (LF) 1685, 1607, 1513, 1358, 1268,

815, 680 cm^{-1}; m/e 132 (M^{+}, 100%), 119 (84), 91 (41), 105 (41), 92 (31), 39 (24), 65 (21), 131 (16), 51 (16), 63 (14); τ 7.92 (s, vinyl proton) 7.74 (s, Ar-CH_3), 5.01 and 4.71 (br.s. exo-methylene), 3.01 and 2.73 (d, 4H J = 15 Hz).

A solution of 1.5 ml (1.7 g, 0.012 mol) of boron trifluoride etherate in 2 ml of THF (dry $LiAlH_4$) was added over a period of 1 hour at 25°C to a magnetically stirred solution of 0.009 mol of powdered $NaBH_4$ and 0.4 g (0.03 mol) of 4,α-dimethylstyrene. The whole apparatus was flushed with a steady stream of dried N_2. The excess hydride was decomposed with water (2 ml) and the trialkyl borane oxidised by addition 3N NaOH (3 ml) followed by dropwise addition of H_2O_2 at 30-32°C (3 ml). The reaction mixture was saturated with NaCl and the THF layer formed was separated off and washed with saturated NaCl solution[113]. The organic layer was dried (magnesium sulphate and purified on a silica column to yield 0.3 g of (37) (70%); ν_{max} (LF), 3380, 1725, 1680, 1605, 1550, 820 cm^{-1}; m/e 119 (100%), 91 (76), 134 (35), 65 (24), 39 (14), 43 (12), 63 (10), 120 (9), 89 (8), 51 (7) (M^{+}, 150, 2%); τ 8.81 (d, 3H J = 7Hz), 7.76 (s, Ar CH_3), 7.25 (septet, 1H J = 7 Hz), 6.53 (d, 2H, J = 6.9 Hz), 2.99 (br.s. 4H).

Myrtenol (31)

Selenium dioxide (4 g) was added during 15 minutes to a stirred solution of α-pinene (5 g, 0.035 mol $[\alpha]_D^{20}$ = + 48°) in ethanol, kept under reflux for 10 hours. The liquid was decanted and the residue washed with ether (2 x 25 ml). The washings were

combined with the mother liquor, the solvent removed in vacuo, and the product distilled (95-97°C/10 mm Hg) to yield a pale yellow oil (1.3 g) which corresponded to 95% myrtenal by GC analysis; m/e 79 (100%), 107 (54), 108 (31), 41 (29), 106 (27), 77 (26), 39 (26), 91 (24), 105 (16), 53 (16).

Reduction of myrtenal with sodium borohydride by the method described for p-cymene-7-ol, yielded myrtenol (1.2 g); $[\alpha]_D^{20}$ = + 53°; ν_{max} (LF), 3320, 3032, 1650, 1060 and 800 cm^{-1}; m/e 79 (100%), 91 (41), 41 (37), 108 (15), 39 (24), 77 (20), 93 (18), 95 (17), 67 (14), 55 (13); τ 9.08 (s, 3H), 8.83 (s, 3H), 8.75 (d, C7 endo-proton J = 8 Hz), 7.65 (m, 5H), 6.06 (br.s, 2H, hydroxymethyl), 4.51 (br.s, 1H vinyl).

trans-Pinocarveol (25)

This compound was obtained by lead tetraacetate oxidation of β-pinene by the method of Hartshorn and Wallis[117]. Lead tetraacetate (16 g) was added during 10 minutes to a stirred suspension of calcium carbonate (0.1 g) in dry benzene (10 ml) containing (+)-β-pinene (1 g; $[\alpha]_D^{20}$ = +20°) and the mixture stirred at 60°C for a further 20 minutes. The solid was removed by filtration, and the filtrate washed with aqueous sodium bicarbonate, water and dried (magnesium sulphate). Distillation under reduced pressure afforded benzene, an alkene mixture and a mono-acetate fraction (0.72 g) bp. 96-98°C/10 mm Hg. The mono-acetate fraction showed trans-pinocarvyl acetate (85%); $[\alpha]_D^{20}$ = -54°; ν_{max} (LF)

3070, 1775, 1640, 1370 and 1240 cm^{-1}; m/e 43 (100%), 91 (72), 92 (40), 119 (28), 41 (23), 93 (22), 109 (15), 77 (14), 105 (12); τ 9.29 (s, 3H), 8.69 (s, 3H), 7.94 (s, 3H, acetate), 6.95-8.44 (m, 6H) 5.95 and 4.93 (br.s. exo-methylene), 4.40 (d. C3 proton, J = 7.5 Hz).

Treatment of the crude mono-acetate fraction in methanol (5 ml) with aqueous potassium hydroxide (1 g in 0.75 ml H_2O) at 20°C for 12 hours, followed by isolation with ether followed by preparative GC on column B gave trans-pinocarveol (0.45 g), 40%); $[\alpha]_D^{20}$ = + 78°; ν_{max} 3470, 3070, 1640, 1372, 1368, and 890 cm^{-1}; m/e 41 (100%), 55 (95), 92 (93), 70 (70), 91 (67), 83 (63), 39 (53), 69 (44), 81 (40), 134 (33), (M^+, m/e = 152, 0.9%); τ 9.33 (s, 3H), 8.69 (s, 3H), 8.32-6.41 (m, 4H), 5.56 (d, C3 proton, J = 8Hz), 5.15 and 4.97 (br.s. exo-methylene).

The second alcohol isolated was myrtenol, with identical spectroscopic properties to the sample obtained by selenium oxidation of α-pinene.

cis-Pinocarveol (24)

Selenium dioxide oxidation of (+)-β-pinene with carbon tetrachloride as solvent by the method described for oxidation of α-pinene yielded pinocarvone (1.1 g, 20%); $[\alpha]_D^{20}$ = -65°; λ_{max} = 245 nm (ε = 11,700); ν_{max} (LF) 1710, 1630, 1388, 1330, 1150, 1115, 940 cm^{-1}; m/e 53 (100%), 81 (84), 108 (77), 150 (M^+, 47), 109 (37), 79 (36), 39 (36), 65 (35), 43 (21); τ 9.10 (s, 3H), 8.57 (s, 3H), 8.57 (s, 3H), 8.68 (d, C7 endo-proton J = 9.0 Hz), 7.72 (m, 1H), 7.44

(m, 2H), 7.24 (br.d, 2H, J = 5.5 Hz), 5.07 (d, 1H, exo-methylene J = 2Hz) 5.10 (d, 1H exo-methylene, J = 2Hz).

Bromine (1.2 g) in acetic acid (2 ml) was added dropwise during 30 minutes to a stirred solution of pinocarvone (1 g) in ether (20 ml) at 0°C. Isolation with ether gave a solid (2.25 g) which was dissolved in a mixture of acetic acid (20 ml) and water (2.5 ml). Zinc powder (4.5 g) was added during 20 minutes to the stirred solution kept at 10°C, followed by the addition of potassium hydroxide (15 g) in water (25 ml). Isolation with ether and fractionation by preparative GC on column B gave cis-pinocarveol (0.78 g, 68%; $[\alpha]_D^{20}$ = -61°; ν_{max} (LF), 3285, 3025, 1645, 890 cm^{-1}; m/e 83 (100%), 69 (92), 55 (92), 41 (62), 95 (41), 152 (M^+, 32), 97 (31), 81 (24), 39 (23), 110 (22); τ 9.27 (s, 3H), 8.75 (s, 3H), 8.78 (d, endo-C7 proton, J = 9.5 Hz) 7.43-8.34 (m, 5H), 5.43 (d, C3 proton, J = 7.5 Hz), 5.25 (d, exo-methylene proton, J = 2Hz) 4.95 (d, exo-methylene proton, J = 2Hz).

cis-Pin-3-en-2-ol (26)

Oxidation of (+)-α-pinene (1 g, $[\alpha]_D^{20}$ = 47°) with lead-tetraacetate by the method previously described yielded on distillation (99-100°C/10 mm Hg) containing predominantly cis-pin-3-en-2-yl acetate (0.8 g, 57%); $[\alpha]_D^{20}$ = 106°; ν_{max} (LF), 3020, 1780, 1440, 1365, 1245, 1030, 920, 680 cm^{-1}; m/e 119 (100), 91 (62), 43 (49), 134 (34), 92 (31), 41 (23), 93 (22), 109 (15), 77 (14), 105 (12); τ 9.10 (s, 3H), 8.69 (s, 3H), 8.58 (s, 3H), 8.00 (s, 3H acetate), 8.55 (m, 2 protons), 7.80 (m, 2H), 4.68 (br.s, 2 vinyl protons).

Hydrolysis of the acetate (0.75 g) with methanolic potassium hydroxide gave after preparative GC on column B cis-pin-3-en-2-ol (0.48 g, 80%); $[\alpha]_D^{20}$ = + 47°; ν_{max} (LF) 3380, 3015, 1650, 1095, 735 cm^{-1}; m/e 91 (100%), 41 (69), 109 (64), 119 (55), 81 (41), 92 (38), 94 (37), 77 (32), 79 (31). τ 9.13 (s, 3H), 8.66 (s, 3H), 8.28 (m, 3H), 8.84 (d, 4-endo C7 proton, J = 15 Hz), 7.82 (m, 3H), 6.72 (br.s, vinyl proton), 5.64 (br.s, vinyl proton).

iso-Piperitenone (48)

Following the method of Dauben et al[118], limonene (3.0 g, 0.02 mols) in methylene chloride (300 ml) at room temperature was added, in one portion, to a stirred slurry of chromium trioxide-pyridine complex (90 g) in methylene chloride (75 ml). After stirring for 25 hours the mixture was poured from the flask and the precipitate remaining washed with ether (3 x 100 ml). The washings and an additional 400 ml of ether were added to the methylene chloride solution. The solution was washed six times with 10% sodium hydroxide and the aqueous layer extracted with ether which was added to the original ether solution. This solution was washed with 10% HCl (3 x 50 ml), followed by 10% sodium hydroxide (50 ml) and finally with a saturated sodium chloride solution. The solution was dried (magnesium sulphate) and the solvent removed in vacuo to yield 2.8 g of a pale yellow oil. Analysis by GC showed the oil to contain limonene 15%, carvone 31% and iso-piperitenone 54%. Purification by preparative GC yielded 1.25 g of iso-piperitenone (34%) $[\alpha]_D^{20}$ = 64° λ_{max} = 233 nm (ε = 9,450); ν_{max} (LF) 1681, 1645, 899, and 883. cm^{-1};

m/e 82 (100%), 39 (21), 54 (16), 150 (M^+, 14), 135(12), 41 (11), 53 (8), 83 (6), 67 (5), 107 (5); τ 8.26 (s, 3H), 8.06 (s, 3H), 8.00-7.54 (m, 4H), 7.06 (t, C4 proton, J = 8Hz), 5.24 (s, 1H), 5.10 (br.s, 1H); 2,4-DNP, m.p. 155-156°C.

3,6,6-Trimethylcyclohepta-2,4-dieone (29)

Selenium dioxide oxidation of car-3-ene (0.75 g, 0.01 mol., $[\alpha]_D^{20}$ = 5.1°) in refluxing ethanol by the method previously described gave on work-up and purification by preparative GC, p-cymene-8-ol (15 mgs, 6%) and its ethyl ether (45 mgs, 21%); ν_{max} 1510, 1265, 1070, 820 cm^{-1}; m/e 163 (100%), 43 (67), 133 (54), 135 (50), 91 (11), 59 (8), 105 (8), 164 (8), 117 (7), 132 (7); τ 1.92 (t, CH_3, J = 7Hz), 8.57 (s, 6H), 7.71 (s, 3H), 6.91 (qt, 2H, J = 7Hz), 2.93 (AB qt, 4H, J = 8Hz).

Selenium dioxide oxidation on car-3-ene (5 g) in refluxing methylene chloride gave an oil which on distillation yielded a hydrocarbon fraction containing mainly car-3-ene (2.2 g, 28-30°C/0.5 mm Hg) and a second fraction (0.7 g, 55-62°C/0.5 mm Hg) which GC analysis showed contained p- cymene-8-ol (50%), p-mentha-1,5-diene-8-ol (15%) was also identified; λ_{max} = 263 nm (ε = 12,600); ν_{max} 3350, 1645, 800, 720 cm^{-1}; m/e 59 (100%), 94 (67), 79 (65), 91 (56), 43 (38), 93 (28), 77 (27), 119 (24), 92 (21), 41 (91); τ 8.2 (s, 6H), 8.3 (br.s, vinyl CH_3), 7.82 (m, 2H), 4.64 (m, C2 vinyl proton), 4.23 (br.s, 2H). Car-3-en-2-one (20%); $[\alpha]_D^{20}$ = + 212°; λ_{max} = 221 nm (ε = 7,820); ν_{max} 3028, 1660, 1450, 1420, 1240, 900, 840 cm^{-1}; m/e 150 (M^+, 100%), 107 (82), 108 (61), 134 (54), 67 (53), 91 (52), 41 (51), 39 (50), 79 (48), 80 (27); τ 8.90 (s, 3H), 8.78 (s, 3H), 8.28 (m, 3H), 8.43-8.70 (m, 2H), 7.48 (m, 2H), 3.74 (br.s, vinyl proton). Two minor

components (5 and 2%) were identified as eucarvone and an isomer 3,6,6-trimethylcyclohepta-2,4-dienone respectively. The former was identical to an authentic sample, the spectroscopic data of the latter was identical to literature values[121] $[\alpha]_D^{20} = 0$; λ_{max} = 294 nm (ε = 7,400); ν_{max} 3015, 1665, 1603, 1325, 750 cm^{-1}; m/e 107 (100%), 150 (M^+, 50) 91 (47), 135 (28), 79 (26), 39 (25), 108 (25), 41 (18), 77 (16), 80 (15); τ 8.90 (s, 6H), 8.00 (s, vinyl CH_3), 7.20 (s, 2H), 4.54 (d, C4 vinyl proton, J = 11.5 Hz), 3.98 (s, C2 vinyl proton), 3.92 (d, C5 vinyl proton, J = 11.5 Hz); 2,4-DNP m.p. 176.5-177°C.

Car-3-ene (5 g) was aerially oxidised (stirred 18 hours, 40°C, continuous bubbling of air), followed by reductive work-up with saturated sodium sulphite (50 ml) by stirring at 80°C for 2 hours. The product was extracted with ether (3 x 50 ml), dried (magnesium sulphate), the solvent removed in vacuo and the product distilled (80°C/1 mm Hg) to yield an oil (3.0 g) containing several products. Purification by preparative GC yielded 3,6,6-trimethylcyclohepta-2,4-dienone, (0.83 g, 14%) which was identical to the sample obtained by selenium dioxide oxidation. Other products identified were p-cymene (4%), car-3-ene (1.5%), 4,α-dimethylstyrene (13%), eucarvone (6%), car-3-en-2-one (2%), car-3-en-5-one (3%), p-cymene-8-ol (17%).

Car-3-en-2-one (10 mgs) in ether was stirred for 30 minutes with ethanolic potassium hydroxide (40%, 1 ml) at room temperature. The product (9 mgs) was identical to an authentic sample of eucarvone. Treatment of car-3-en-5-one (10 mg) in the same manner produced 3,6,6-trimethylcyclohepta-2,4-dienone.

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