SOME TERPENOID CONSTITUENTS

OF

AUSTRALIAN TIMBER

SPECIES

A report submitted in part fulfilment

of requirements for the degree of

Master of Science

by

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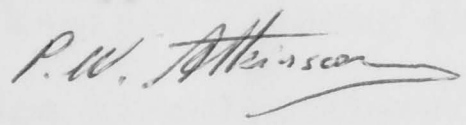
Except where it is acknowledged to have been performed by others, all the work described in this thesis was performed by me.



(P. W. Atkinson)

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ABSTRACT

Preliminary investigations were commenced on the function of chemical constituents, present in *Cedrela australis* and *Callitris columellaris*, both economically important Australian timbers, in influencing the host specificity of the insect pests, *Hypsipyla robusta* and *Diadoxus erythrurus* respectively. Although the number of test results precludes any definite conclusions at this stage, indications are that chemicals present in *Cedrela* function as an oviposition stimulant for *Hypsipyla*. A preliminary laboratory bioassay and breeding programme has also been established for *Hypsipyla*. No significant results were obtained from the *Diadoxus* investigation.

An examination of the constituents of *Callitris* resulted in the identification of various known mono-, sesqui- and di-terpenoids. Two new diterpenoids, Δ-13(16)-communic acid and 4-*epi* dehydroabietic acid, were identified; the latter has since been isolated and characterised by other workers. A new diterpenoid ester, an artifact of methyl *trans*-communate from high temperature gas chromatography, has been tentatively identified as methyl labda-8(20), *trans*-11(12), *cis*-13(14)-trien-19-oate.

A brief study of the chemistry of *Cedrela* resulted in the isolation of cedrelone from the wood oil.

SECTION I

BASIS FOR INVESTIGATION

It was initially proposed to undertake research on the chemical nature of attractants present in Australian timbers which permit insect pests to select their specific "host" timber for breeding. Exploratory work was to be commenced on problems involving economic timbers. The problems of "Cedar-Tip" moth and "Cypress Jewel" beetle, which hinder the commercial exploitation of Cedrela australis and Callitris columellaris (inland form) respectively, were selected for initial attack.

The basis for the investigation appeared sound. Towards the beginning of the 1950's the use of pesticides, especially those of the organo-chlorine type, for chemical control of insect pests became the target for considerable criticism. The harmful effects on vegetation and wildlife in treated areas had always been recognised as a potential hazard, and had usually received attention during preliminary field tests. However, the long-range aspects of the problem tended to be overlooked. The organo-chlorine content of soil, milk, meat, fish, poultry and even of the river and ocean system were examined and found positive. Resistance of some insects to insecticides was also becoming a problem. Any natural population consists

of a "genetic pool", and, as the environment changes, the genotype best fitted to survive will be favoured in the natural selection process. Thus, if within a population there exists a genotype which can resist destruction, it will be favoured. If it survives, it will breed at an accelerated rate since food and intra-specific competition are no longer limiting factors. Therefore, the application of insecticides must "select out" for survival the variety against which it is actually useless.

Newer control methods had to be devised(1). Over the centuries insects had managed to exist in hostile surroundings because they had developed extraordinary abilities, one of which was a highly specialised sense to detect and follow an odour. Insects were known to be able to successfully follow odours to sources of food, to host plants and animals, to the opposite sex, or to the right place to oviposit. It was the utilization of this highly efficient apparatus which received most emphasis as a possible new means of insect control. Rapid development in the field of natural and synthetic insect attractants followed. Powerful attractants were consequently found which greatly increased the efficiency of control or eradication of a variety of insect pests(2).

The most potent attractants, which may be effective at a distance of a quarter of a mile or more, are usually highly specific and have therefore proved valuable in the detection and estimation of insect populations. A good attractant can thus assure the early detection of an infestation before it can enlarge or spread. Control measures need only be applied to those areas where the insect is found and only as long as it continues to be present. In this manner, considerable economies may be effected and needless spreading of toxic materials avoided. The successful rapid eradication of the Mediterranean fruit fly (Ceratitis capitata) in 1957 from parts of south-eastern United States illustrates the power of attractants in this type of control. The synthetic attractants, "siglure"(3) and "medlure"(4), were used to lure the "medfly" to a previously defined area treated with insecticide.

A natural attractant has one possible advantage over a lure of a synthetic nature. On the basis that it has been a stable genetic feature of each species for a long time the natural attractant, on which a species relies for its survival, is unlikely to be "selected out" if it was used as a lure to destruction. The possibility that several such attractants exist in a population must,

however, always be considered. In the field of natural attractants the sex pheromones have aroused the most interest. A "pheromone", from the Greek "pherain" (to carry) and "horman" (to stimulate), is a substance secreted by an animal to influence the behaviour of other animals of the same species. Detected by the insect in extremely low concentrations, they are undoubtedly among the most potent physiologically active substances known today. Reports on the isolation and identification of these pheromones have been increasing over recent years and the work has been the subject of many reviews, the most extensive being that of Jacobson(5) in 1965. The potency of pheromones in the insect world is best illustrated by the sex attractant of the introduced pine sawfly (Diprion similis)(6). A virgin female, placed in a screened trap, attracted males within 30 seconds. After 5 hours 7,000 males had been attracted. Males arrived at the rate of approximately 1,000 per day until the female died on the fifth day. Even after death there was sufficient attractant left to lure males in dwindling numbers for another three days.

However, research into "secondary plant substances" (7) influencing insect-host plant specificity has not been so widespread. Early work in this field was the subject

of a symposium held in Amsterdam in 1951(8). It was generally accepted that although vision, phototaxis, geotaxis, and hygrotaxis undoubtedly all play a part in directing insects to the proper environment for oviposition and feeding, the ultimate forces operating in the final recognition of the host plants are largely chemical. The possibility that compounds effecting orientation from a distance were different from those initiating feeding was also discussed. The majority of work thus far has been restricted to those plant substances which induce feeding (2,9). However most of these reports describe little more than response of the insect to a partially purified active fraction. This has been largely due to the absence of a suitable method for isolation and identification of the pure active components. A rigorous approach, set out below, has recently been suggested and its success demonstrated (10).

1. Development of laboratory bioassay.

2. Production of large amounts of starting material.

3. A series of progressively refined isolation steps, each monitored by bioassay, until individual components are obtained in a very high state of purity. The presence of very small amounts of highly active impurities is an ever-present hazard.

4. Identification of the individual components. Usually these are obtained in mg. or μg. quantities so that identification rests heavily on spectrometric techniques.

5. Confirmation of postulated structures by comparison with rationally synthesised compounds.

6. Confirmation of biological activity of synthesised compounds in both laboratory and field. Ideally field bioassays should be performed at every major step in the chemistry.

This approach was successfully followed for the identification of the volatile substances responsible for the orientation of bark beetles to ponderosa pine. Initial observations had suggested an interrelation between successful initial attack of the host plant and an increased attraction as evidenced by mass attack. Further investigation indicated a secondary attractant, present in the frass produced during initial boring activity of the male beetle (Ips confusus), was responsible for increased orientation towards the host. Both male and female are effected and on arrival at the source of attractant they take part in boring, feeding, mating, and oviposition. A mixture of three terpene alcohols (Fig. I.) was identified as the secondary

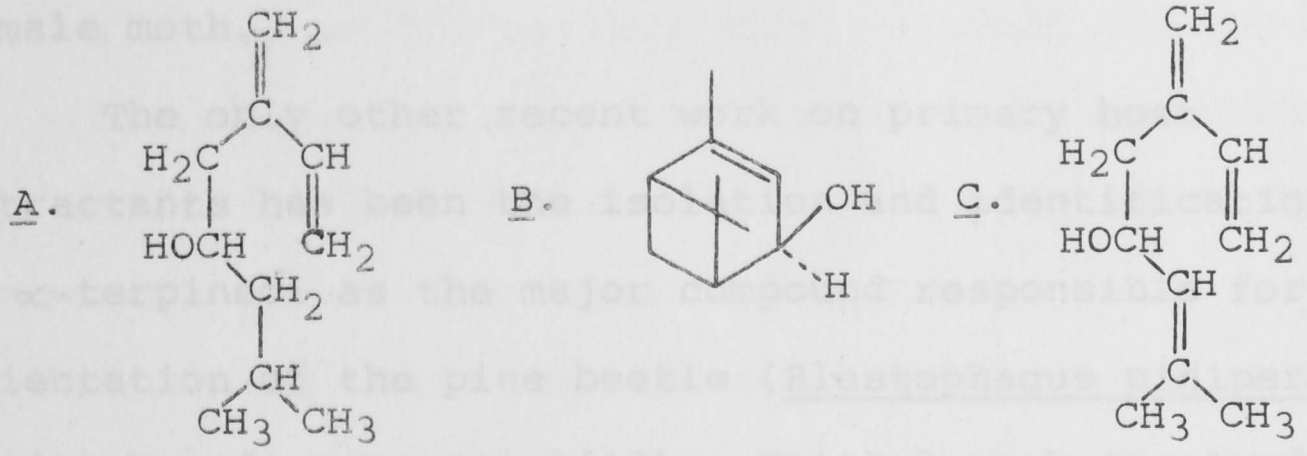


Fig.I. Compounds A, (-)-2-methyl-6-methylene-7-octen-4-ol; B, (+)-cis-verbenol; and C, (+)-2-methyl-6-methylene-2,7-octadien-4-ol.

attractant. In related work(11), a similar attractant, produced in the frass of the feeding female Western pine beetle (Dendrotonus brevicomis), was identified as exo-7-ethyl-5-methyl-6,8-dioxabicyclo(3,2,1)octane (see Fig.II.).

A further phenomena of insect-host plant specificity has recently been brought to light during investigations into the sex life of the polyphemus moth(12). An emanation from leaves of the red oak (Quercus rubra) has been found necessary for the mating of these moths (Antheraea polyphemus) under laboratory conditions. The reception of the oak volatile is prerequisite for the female's release of her sex pheromone, which in turn, is necessary for the sexual activation of the male. The oak emanation is therefore probably a primary host attractant for the

female moth.

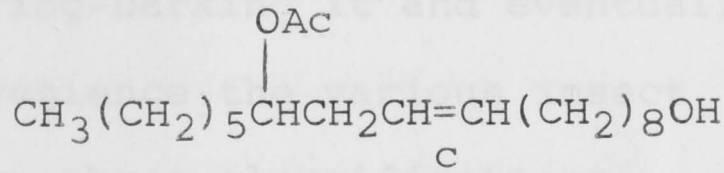
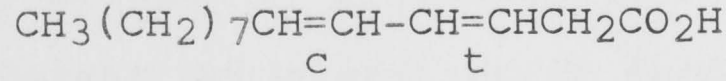
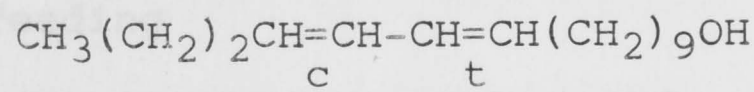
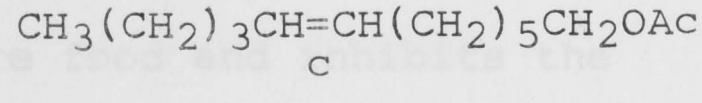
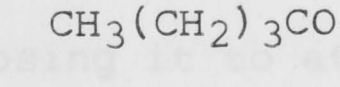
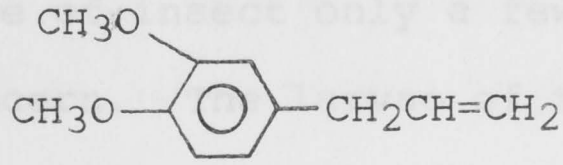
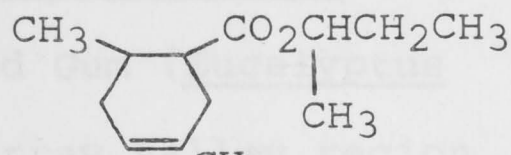
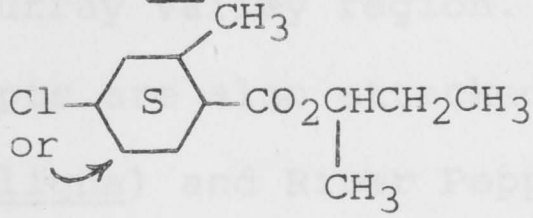
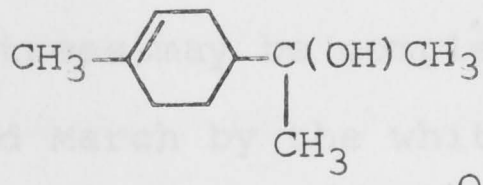
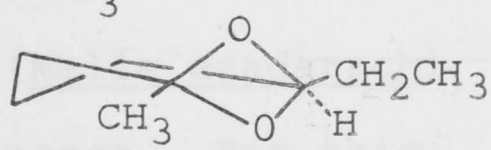
The only other recent work on primary host attractants has been the isolation and identification of ∝-terpineol as the major compound responsible for orientation of the pine beetle (Blastophagus piniperda) to its breeding material(13). Initial work involved the study of olfactory reactions toward pine phloem extracts, followed by some precise chemistry which finally led to the identification of the active component by gas chromatography. An authentic sample of ∝-terpineol was then shown to have similar attractant effects on the beetles.

Investigations into the problems of Cedar-tip moth and Cypress jewel beetle have largely involved the study of the "volatile" constituents, present in the host timbers, which could possibly act as primary host attractants to the insect pests. The question quickly arises: How volatile would these attractants need to be? Working from first principles it would seem likely that they would be at least reasonably volatile since natural selection would favour an insect which could detect its host from afar. At the same time it would need to be not so volatile that it would last only a short time. A general view has been that an attractant with 11 or 12

carbon atoms was the optimum size. However the discovery of effective insect attractants with up to 20 carbon atoms has shown that generalizations on molecular size may not be readily made. The relative evaporation among attractants has also been shown to vary greatly(14). In citing extremes, a 50 mg. sample of tert-butyl 6-methyl-3-trans-cyclohexene carboxylate, a synthetic attractant of the "medfly", evaporated under standard conditions in under 2 hours, but only 2 mg. of a similar quantity of "gyplure"(15) had evaporated after 3.5 months. It appears that the optimum volatility and size of the attractant can only be accurately determined by the insect itself. Some compounds, effective as insect attractants over reasonable distances, are shown in figure II.

Although only two cases were selected for initial investigation the occurrence of insect pest problems in Australian economic timbers is widespread(21). In most cases, however, the insect damage is not confined to one but many timber species thus making an investigation into a possible host attractant difficult. Trees are subject to injury by wind, fire, animals and man. When these agencies operate alone or together, sometimes assisted by defoliating and sap-sucking insects, the tree apparently becomes weakened and more susceptible to attack by other

FIG. II.

INSECT	TYPE OF ATTRACTANT(REF.)	ATTRACTANT
Gypsy moth (*Porthetria dispar*)	Synthetic sex(15.)	
Black carpet beetle (*Attagenus megatoma*)	Natural sex(16.)	
Silkworm moth (*Bombyx mori*)	Natural sex(17.)	
Cabbage looper (*Trichoplusia ni*)	Natural sex(18.)	
Sugar beet wireworm (*Limonius californicus*)	Natural sex(19.)	
Oriental fruit fly (*Dacus dorsalis*)	Synthetic food(20.)	
Medfly (*Ceratitis capitata*)	Synthetic sex(3.)	
Medfly (*Ceratitis capitata*)	Synthetic sex(4.)	
Pine beetle (*Blastophagus piniperda*)	Natural primary host(13.)	
Western pine beetle (*Dendrotonus brevicomis*)	Natural secondary host(11.)	
Bark beetle (*Ips confusus*)	Natural secondary host(10.)	Mixture of monoterpene alcohols (see page 7.)

insects which eat the bark and conductive region of the tree, partly or completely ring-barking it and eventually causing it to die. For convenience the various insect pests will be discussed under three classifications.

1. Foliage-eating
2. Sap-sucking
3. Conductive tissue-feeding

Defoliation by foliage-eating insects reduces the tree's ability to manufacture food and inhibits the movement of nutrient materials within the tree, thus effecting its vigour and predisposing it to attack by other insect pests. Of this type of insect only a few appear to cause any economic concern. The larvae of the "Gum-leaf Skeletonizer" moth (Roesilia lugens) have caused extensive damage to the River Red Gum (Eucalyptus camaldulensis) forests of the Murray Valley region. Several other species of eucalypts are also attacked, including Sydney Blue Gum (E.saligna) and River Peppermint (E.Lindleyana). White Cedar trees may be completely defoliated during February and March by the white cedar moth (Lymantria reducta). White Cedar (Melia azadarach) appears to be the only host for this insect. For this reason, and the ease with which larvae of the species can be trapped for breeding purposes, an examination of this

problem could prove fruitful. In Leeton (1967), a town noted for its tree-lined streets, this pest almost completely destroyed the white cedar population(22). The pest, of this type, causing most economic concern is the Cedar-tip moth which will be discussed in detail later.

A large number of different types of insects feed by sucking sap from the foliage and stems of trees causing discoloration, malformation and localised dead tissue. Only in cases where a large population present in a tree renders it more susceptible to attack by conductive tissue-feeding insects does any real concern arise. The psyllid (Glycaspis baileyi) occurring in the foliage of Sydney Blue Gum(23) was found to be associated with damage by the scolytid (Xyleborus truncatus).

Damage caused by insects to the bark and conductive tissue appears to be a widespread economic problem however. Living trees, predisposed by injury or fire scars, are the main target of attack. Although ring-barking of younger trees by the larvae of these insects sometimes causes death, the major problem arises when pupation channels, formed by larvae in the sapwood, render this part of the timber commercially unacceptable. Long-horned beetles (family Cerambycidae), occurring throughout the world, are well-known for this type of damage. In

Canberra, during the recent drought, many scribbly gums (E.rossii) ranging from young saplings to trees more than a century old were badly damaged or killed by attack of cerambycids of the genus Phoracantha. The fact that freshly felled and split Eucalyptus macrorhyncha, as well as fresh sawdust, are observed to attract swarms of longicorns(24) the problem is likely to yield to chemical examination. Longicorns however, although favouring Eucalyptus species, are not specific in their choice of host material which could complex the problem. The larvae of the Cypress jewel beetle also feeds on the conductive tissue of living trees and the type of attack is extremely similar to that of the longicorn. This also will be discussed in detail later.

Other timber borers of common occurrence, whose damage is most frequently encountered by householder and timber merchant alike, include Lyctus or powder post beetles(25), Anobium or furniture beetles(26), shot-hole borers (family Bostrychidae)(27), and pin-hole borers of families Scolytoidae(28) and Lymexylidae(29). There is a widespread incidence of each in felled timbers of both softwood and hardwood varieties. The stage of seasoning of the timber when attack occurs is fairly well defined for each species. All are of great concern to the timber

industry. With respect to the Lyctus and Anobium beetles the New South Wales Forestry Commission has stated it has facilities adequate for breeding and experiments designed to evaluate possible host attractants. An interesting point is that attack by Xylion spp. (family Bostrychidae) is initiated by the female beetle actually boring into the wood from outside(30). This appears as though a secondary host attractant may be formed in the frass of the female beetle, as in the cases of Ips confusus and Dendrotonus brevicomis.

A similar investigation into attractants responsible for insect pest-host specificity is presently being undertaken by Dr. I.R.C. Bick in Tasmania. An attempt is being made to isolate the scent involved in the host selection of the Sirex wasp (family Siricidae). This introduced pest has severely damaged the Pinus radiata plantations of Tasmania.

SECTION II

PROBLEMS FOR INVESTIGATION

Problems of insect pest-host specificity were selected for investigation primarily on the economic importance of the problem. The apparent ease or difficulty of bioassay techniques which would be involved was given only secondary consideration on the basis that field testing, if required, could be used for the preliminary assessment of the problem. Some evidence for the presence of a host attractant was, of course, essential. It was on these grounds that the following two problems were selected for investigation.

II.1. Red Cedar and the "Cedar-Tip" Moth

Red cedar (*Toona australis*, Syn. *Cedrela toona* var. *australis*) was once well distributed in the coastal rain forests of eastern Australia from Ulladulla in the south to Cooktown in the north. Considerable stands still exist in the Territory of Papua and New Guinea. This tree is very similar to the "Toon" of India (*Cedrela toona*) and is regarded by some botanists as synonymous. It is a member of the family Meliaceae, which contains several species of valuable timber trees such as rosewood and mahogany. Cedar timber itself is prized for cabinet work and has a rich red-brown colour. As well as being a fast growing

species it is durable, easy to work, and takes an excellent polish exhibiting its natural lustre.

Red cedar has been a favoured species since the Australian colony was first founded. Cedar-containing areas quickly came under the axe and today most stands, except in former inaccessible regions, such as the highlands of Papua and New Guinea, have been harvested. The trees cut represented the growth of centuries and it was not until late in the nineteenth century, when supplies of cedar were becoming limited, that concern was expressed about the virtual extinction of this valuable tree. A need to regenerate this species became evident.

Several attempts have been made since the beginning of the century to obtain regeneration both by artificial and natural means. Most have met with failure because of the depradation of the larvae of a moth known as the Red Cedar Tip Moth (or Twig Borer), Hypsipyla robusta (sub-family Phyticinae, family Pyralidae). The larvae attack the succulent leaders, eating out the pith and causing "die back"; a number of new leaders develop, and in turn these are attacked (see Fig.III.). The distortion of form leads to the production of very short logs or of "dog-leg trees", therefore effecting both log quality and yield.

Because of the high value placed on this timber in

FIG III Cedrela australis showing distortion of form caused by twig borer attack.

the furniture industry, any research into the problem would certainly appear to be of economic value. A questionnaire was forwarded to several Australian Forestry Departments enquiring into any present commercial activity in the cultivation of Toona australis. The following replies speak for themselves in assessing the economic grounds for any investigation into the problem of Hypsipyla robusta.

Papua and New Guinea: "Plantations of cedar are out due to twig borer attack. If this could be overcome, minor plantings would be made for cabinet timber requirements. Present stands are being harvested and replaced by Araucaria species."

Queensland: "Hypsipyla is such a very serious pest that it is impossible to consider using cedar in our forestry programmes. If red cedar were available in sufficient quantity, possible export markets could be found. If suitable log length could be obtained, small annual plantings of cedar would be made to provide future Australian and some overseas requirements."

New South Wales: "There is slight concern, little interest and slight activity. If one could guarantee that red cedar could be grown free of tip moth infestation then possibly there would be more interest."

Interest was first aroused in this insect in 1876 in India where Hypsipyla is regarded as a pest of the "Toon". Toona is also found in Burma, Ceylon, Java, Hawaii, South America and the West Indies. Hypsipyla is prominent in all these countries except Hawaii, where Toona has been introduced as an exotic to an area free of Hypsipyla. In Australia the first report of attack on red cedar was in Queensland in 1882 by L.A. Bernays. In 1914 investigations into the life history of the tip moth were initiated in India by C.F.C. Beeson who proved the existence of five generations attacking the tree at all stages of its growth(31). In Australia the first detailed investigations into the biology and life cycle of Hypsipyla were carried out by White in the early 1950's in North Queensland(32) and Bryant in 1956 at Wyong in New South Wales(33). The life cycle was shown to be similar to that in India with the first generation emerging in early spring and the fifth and over-wintering generation hibernating in late autumn. Although young shoots are generally attacked the larvae also feed on the flowers, fruits and succulent leaf midribs.

As yet there is no information why the moth attacks Toona or what radius of dispersal the moths have. However in all generations the female lays her eggs in the

new unexpanded leaves or the flowering shoots. This may suggest the presence of an oviposition attractant in the new foliage. The host selection of Hypsipyla appears not to be restricted to Toona. Although cedar is the main host species in Australia and the Territory, Swietenia, Khaya, Enantdophragma, and other genera of the family Meliaceae have been reported as subject to attack. If so, any attractant for Hypsipyla would appear to be common to a number of plants among the Meliaceae.

In an investigation into the problem it may be first necessary to establish a breeding programme for the moth. Previous attempts, however, by other workers have failed. The N.S.W. Forestry Commission were unable to induce oviposition even when portions of large trees were enclosed in cages in the field. Even when small trees were surrounded by cages (6'x6'x12') all attempts to procure oviposition resulted in failure. Beeson, in India, who had large numbers of insects and red cedars to work with, also failed to obtain oviposition on caged trees. It had been suggested that adults need to fly freely before oviposition. Mating has also never been observed and is believed to occur at night. Although the chances of breeding Hypsipyla in captivity appear slight, it seems possible that reasonable numbers of adults could

be obtained by collecting pupae in the field at appropriate times and holding them for emergence.

An interesting point is that, although Hypsipyla can cause severe damage to red cedar planted in the open, it has been observed that overhead and lateral shade considerably reduces the incidence of the pest. At about 50% light intensity, insufficient attack occurs to cause serious malformation of the tree and shading is not great enough to reduce vigorous growth significantly. Hoop, Kauri, Maple and other rain forest species have been used successfully as cover crops while weeds give excellent protection during the seedling stage. Although small plantations have become well established under cover the conditions are still regarded as economically unfavourable for any real increase in the planting programme of Toona.

Why are trees growing under canopy less attacked? Is it purely an avoidance of shaded areas by the moth or are the trees subtly different in some way the moth can detect? If shade offers some kind of mechanical barrier it would explain why oviposition was not observed on caged trees. Possibly the shading of the mesh was sufficient to discourage them from ovipositing. This phenomenon certainly complexes an already difficult problem.

An investigation of the problem of "Cedar-Tip" moth

would appear to be of economic value; the presence of an oviposition attractant in the fresh foliage seems possible. Breeding of the moth and the establishment of a suitable bioassay could, however, prove difficult and time-consuming.

II.2. White Cypress Pine and the "Cypress Jewel" Beetle

White Cypress Pine (Callitris columellaris; inland form)* is widely grown in New South Wales west of the dividing range, from Baradine in the north to the Victorian border and beyond in the south. Cypress is a valuable, indigenous timber used to meet a large percentage of Australian structural softwood requirements.

However, the Buprestids, Diadoxus erythrurus (White) and Diadoxus scalaris (L.&G.), the cypress pine jewel beetles, are recorded as pests of cypress under certain environmental conditions. Trees suffering from drought, shallow soils, or from competition with other trees for moisture and nutrient materials, are frequently attacked. Foliage-destroying insects, fire, and mechanical injury are also factors which are considered to predispose cypress to infestation by Diadoxus. The adult female lays her eggs in the damaged or weakened surface of the tree,

*(The nomenclature of Callitris spp. is discussed in Section III.1.)

and on hatching, the young larvae commence to feed in the phloem and wood-cambium. Whilst feeding, they etch the outer surface of the sapwood and compact their faeces and chewed wood in the channels behind them. In the case of younger trees, ringbarking and death may result. When fully fed, the larvae bore into the sapwood to pupate. The beetles then emerge through oval holes measuring approximately ¼ inch across (see Fig.IV.).

The presence of emergence holes in milled timber is of economic concern. Although some affected timber is considered suitable for normal constructional purposes, the emergence holes detract from its commercial value. There is thus a tendency to discard affected timber, or utilise it where appearance is of little concern.

Attack on Callitris spp. by Diadoxus was first recorded by von Lendenfeld in 1885 when he noted its occurrence in western New South Wales(34). The life cycle of Diadoxus has since been considered by various workers; the most recent report being that of Hadlington and Gardner(35) in 1959. The time taken to complete the life cycle is variable, and is dependent on the time of year when oviposition occurred. At least two generations are possible in one year and the adults are present from September to April. Larvae may be found beneath the bark

FIG IV Callitris columellaris (inland form) showing old larval activity and emergence holes of Diadoxus spp.

of attacked trees at any time during the year. Although Diadoxus has not been successfully bred under laboratory conditions, a laboratory bioassay may be devised using adults collected just prior to emergence from damaged trees. It may also be possible to trap emerged adults with light traps, although no previous experience with these is noted for Diadoxus. Normally adults can be kept in captivity for 1-2 weeks and this may be extended by using high humidity cages. It is not known what Diadoxus adults eat but, since most beetles are found near the inflorescences, it may be pollen.

The host selection of Diadoxus spp. however, is not restricted to C.columellaris(inland form). C.endlicheri and Cupressus macrocarpa var. lambertiana have also been recorded as hosts. Sound evidence is available for the presence of a host oviposition attractant in white cypress. If trees, growing in healthy regions, are exposed to a blow-torch, until the conductive tissues are exposed, jewel beetle attack is evident within the hour(36). The radius at which the beetles can detect damaged trees has not been determined. It appears as though an attractant is present in the sapwood or conductive tissues and any damage or weakening of the bark allows dispersal of the attractant which subsequently

effects adult Diadoxus in the region. This phenomenon suggests that most bioassay work could be successfully carried out in the field, thus eliminating the obvious difficulties of establishing a laboratory bioassay. Areas which have been fire-damaged, no more than twelve months previously, should provide a good population of Diadoxus adults under suitable weather conditions. Locations and accessibility of such areas are available from the N.S.W. Forestry Commission.

Recovery of Diadoxus infested trees after heavy rains is a common occurrence. Rains cause a fresh flow of resin which bridges many of the frass channels and kills any larvae which have not yet pupated. It appears that the larvae are not able to encroach on the cells actively producing resin and, at times, have been found isolated in polished chambers lined with opaque resin. Death is probably due to starvation. The resin certainly appears to act as a physical barrier rather than a chemical one.

The introduced species Cupressus macrocarpa var. lambertiana does not require injury before Diadoxus can initiate its attack. Comparison of its bark structure with that of both Callitris species showed that fibre layers were much less abundant and resin canal formation less frequent in Cupressus. The walls of the fibres of

the Callitris species were also thicker. These features could suggest that the dispersal of an attractant, present in the region of the conductive tissues, is possible through the bark of Cupressus without any prior injury as is necessary with Callitris. In addition to this, the introduced species does not appear to be able to resist the ingress of Diadoxus by the production of resin.

Although the problem of Diadoxus is not as extensive as that of the "Cedar-Tip" moth, an investigation still appears economically important. The evidence for the presence of an oviposition attractant and the possible use of field bioassay techniques would certainly be expected to simplify preliminary work.

SECTION III

THE CHEMISTRY OF WHITE CYPRESS PINE

The first problem investigated was that of the "Cypress Jewel" beetle. Initial work involved the preparation of crude extracts of the bark, oleoresin and sapwood of Callitris columellaris (inland form) for biological testing. A detailed chemical examination of various fractions of the extracts followed. This yielded pure compounds which could be later tested as possible host attractants for Diadoxus. Valuable experience was also gained in the techniques of isolation and identification of compounds from mg. samples of extract.

III.1. The Nomenclature of Callitris spp.

Before writing about a member of the genus Callitris Vent. it is first necessary to clarify discrepancies in the nomenclature of species of this genus.

In 1956, Garden, working at the New South Wales National Herbarium, completely revised the genus and claimed that many of the specific names in common use were not validly published in accordance with the International Code of Botanical Nomenclature(37). Garden distinguished between the three species C.intratropica R.T. Baker and H.G.Sm., C. columellaris F. Muell., and C.hugelii (Carr.) Franco and stated that the name C.glauca R.T. Baker and

H.G.Sm. is illegitimate and should be C.hugelii. However, in 1959, Blake, of the Queensland Botanical Herbarium, stated that the name C.hugelii had been misapplied and its correct status is probably that of a subspecies of C.columellaris(38). Blake also believed C.glauca and C.intratropica to be members of the one species for which the correct nomenclature was C.columellaris F. Muell. More recently the N.S.W. National Herbarium recommended that, until further investigations were completed, the three forms may be distinguished as follows(39):

The nomenclature C.glauca and C.hugelii will be replaced by C.columellaris (inland form). C.intratropica will not be used, but instead C.columellaris (intratropica form); what was C.columellaris will be referred to as C.columellaris (coastal form). This nomenclature will be used by the author.

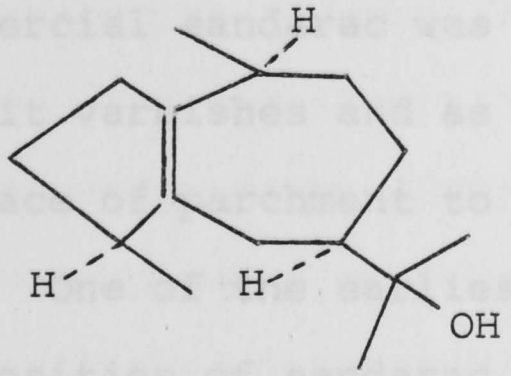
III.2. Literature survey

The first reported investigation of Callitris spp. was in 1910 when Baker and Smith compared the chemical constituents of the leaf oils of a wide range of species within the genus(40). All were shown to contain pinene, limonene, borneol, bornyl acetate, geraniol, geranyl acetate and possible terpinyl butyrate. Each species had its own characteristic oil with the above constituents

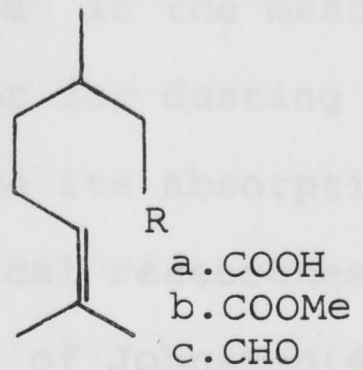
varying in amount in each well-defined species. Callitris columellaris (inland form), owing to its ready availability throughout Australia, was investigated in more detail. The leaf oil, predominantly pinene, was comparable with other Callitris species. Guaiol (I), and "callitrol", thought to be a phenol, were both isolated in high yield from the volatile wood oil.

White cypress pine was known for its high resistance to attack by subterranean termites and to decay by wood rotting fungi. These characteristics invited a continuation of the investigation into the chemical constituents of the wood oil. Trikojus and White(41) showed that "callitrol" was actually l-citronellic acid (IIa) and Neuhaus and Reuter(42) isolated a dehydro-geranic acid, 3,7-dimethylocta-2,trans-4,6-trienoic acid (III). The major contribution to this study in recent years was made by Rudman(43) who showed the heartwood contained, in addition to the above, α-eudesmol (IV), β-eudesmol (V), γ-eudesmol (VI), and cryptomeridiol (VII). He also isolated a further three compounds which were probably hydrocarbons; two of them appeared to be the parent hydrocarbons of guaiol and eudesmol.

An interest in the chemical composition of the oleoresin of the Callitris has also been evident. The



I



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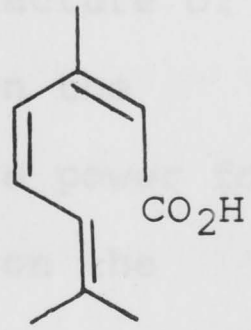

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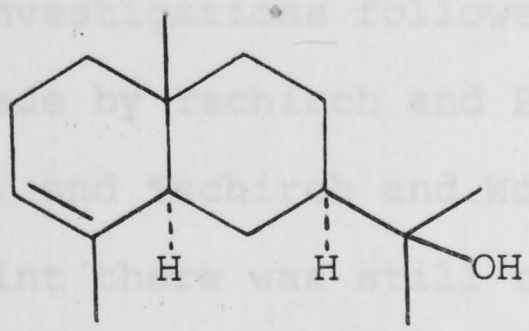

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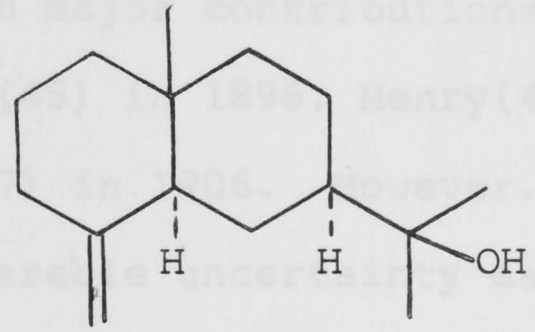

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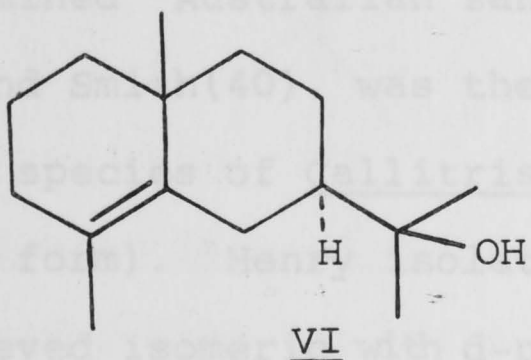

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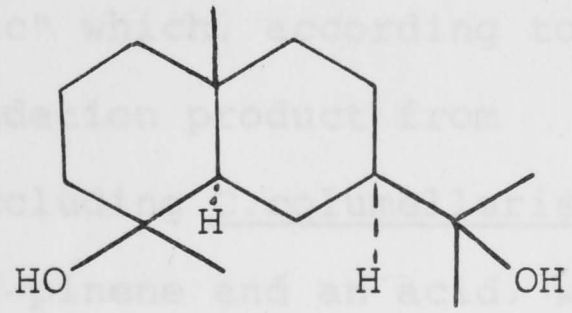


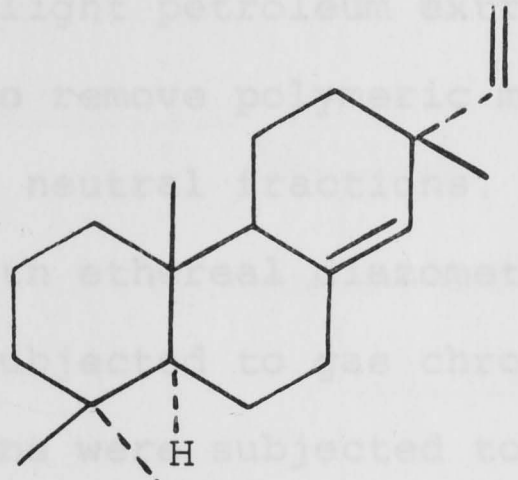
VII

oleoresin of the Callitris is contained in cells of the inner bark, and when this becomes injured in any way the oleoresin slowly exudes and forms "tears" on the exterior of the bark. Its composition and appearance closely resembles the original commercial sandarac of the North African tree, Tetraclinis quadrivalvis var. articulata.

Commercial sandarac was employed in the manufacture of spirit varnishes and as a powder for dusting on the surface of parchment to increase its absorptive power for ink. One of the earliest chemical researches on the composition of sandarac is that of Johnston(44), in 1839, who considered that it consisted of three resin acids. Other investigations followed with major contributions being made by Tschirch and Blazer(45) in 1896, Henry(46) in 1901, and Tschirch and Wolff(47) in 1906. However, at this point there was still considerable uncertainty as to the real composition of sandarac. Both Tschirch and Henry had examined "Australian sandarac" which, according to Baker and Smith(40), was the exudation product from various species of Callitris, including C.columellaris (inland form). Henry isolated d-pinene and an acid, which he believed isomeric with d-pimaric acid.

Further work was reported from time to time on the constituents of Tetraclinis oleoresin with the most recent publication being that of ApSimon and Edwards(48), from which earlier references may be traced. It was not until 1964, when Gough(49) reported a preliminary investigation into Australian sandarac from unspecified Callitris spp., that work on Callitris oleoresin was revived. He showed that the oleoresin contained poly-communic acid and

sandaracopimaric acid (VIIIa), as originally found by Henry. A neutral portion consisted mainly of alcohols and the corresponding aldehydes; compounds of the sandaracopimaric type probably predominated. This covers the published work on the chemistry of Callitris spp. up to the time when the present investigation was commenced in 1966.



	R
VIIIa.	H
b.	CH_3

III.3. Extractives of Callitris

In order that biological testing be simplified, the chemical constituents of the crude material were first divided into various fractions by both steam distillation and extraction with a wide range of common solvents. The crude material in this instance was the sawdust of both the fresh bark and sapwood of Callitris columellaris (inland form). The aim was to test each fraction for biological activity with respect to Diadoxus species; fractions indicating such activity would then be subjected to a series of progressively refined isolation steps, each

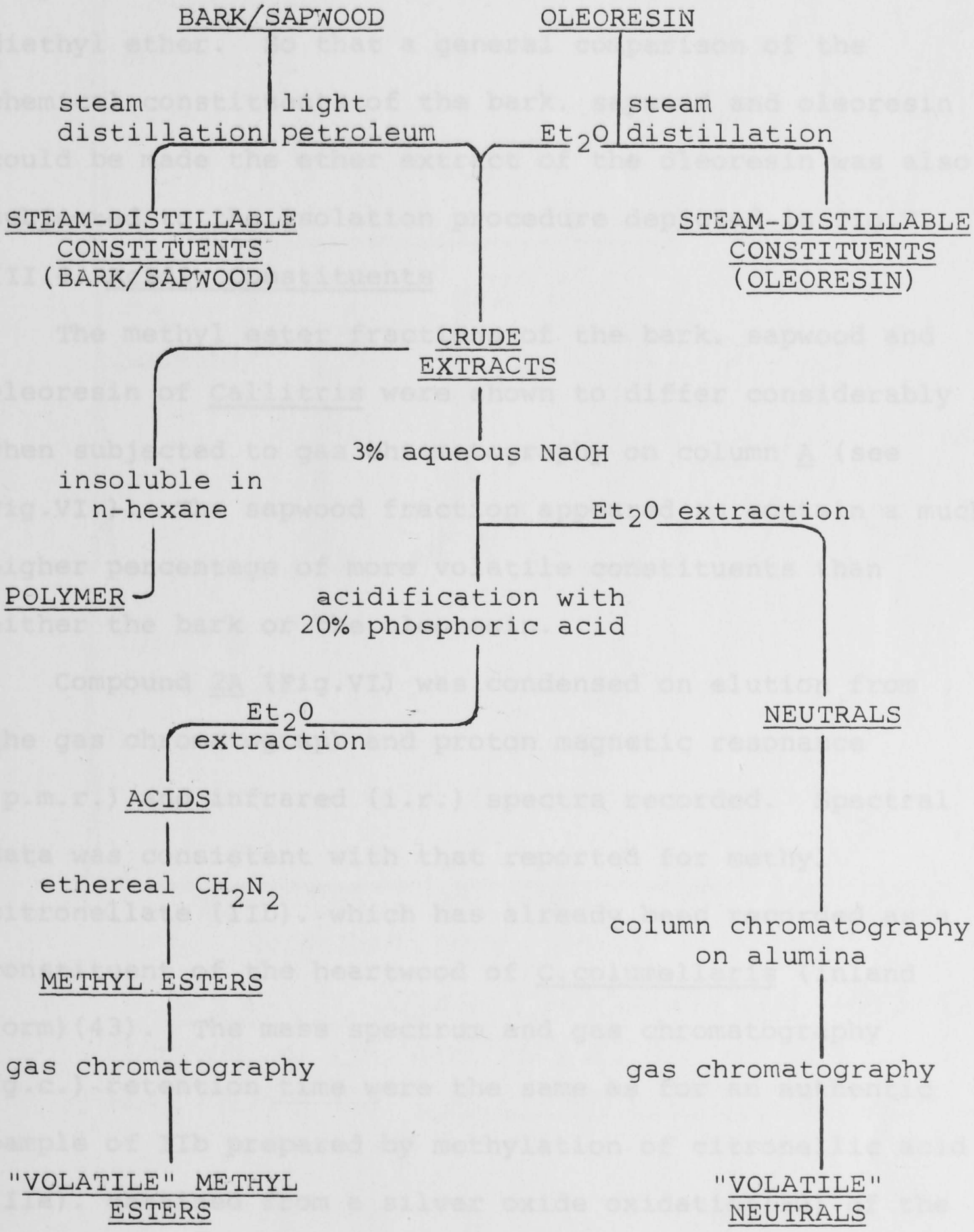
monitored by bioassay, until the individual active components could be obtained in pure form.

To establish the chemical and physical methods available to undertake such a series of isolation steps a detailed chemical investigation into the steam-distillable and light petroleum extracts of both the bark and sapwood was commenced. The isolation procedure used is depicted in Fig.V. The light petroleum extracts, after treatment with n-hexane to remove polymeric material*, were divided into acidic and neutral fractions. The acidic fractions were treated with ethereal diazomethane and the resulting methyl esters subjected to gas chromatography. Before the neutral fractions were subjected to gas chromatography they were further fractionated by column chromatrography on alumina. Any investigation of constituents was therefore restricted to steam-distillable compounds and neutral and methylated acidic compounds which could be successfully subjected to gas chromatography. Compounds other than these were regarded as probably being too involatile to be effective as insect attractants.

The oleoresin of Callitris was not extracted with all solvents since it was assumed that its chemical

*(Polymeric compounds are discussed in more detail in Section III.5.)

FIG.V.
ISOLATION PROCEDURE FOR CALLITRIS CONSTITUENTS



constituents were also present in the bark which is abundant in cells actively producing resin. An oleoresin sample was however both steam-distilled and extracted with diethyl ether. So that a general comparison of the chemical constituents of the bark. sapwood and oleoresin could be made the ether extract of the oleoresin was also subjected to the isolation procedure depicted in Fig.V.

III.4. Acidic Constituents

The methyl ester fractions of the bark, sapwood and oleoresin of Callitris were shown to differ considerably when subjected to gas chromatography on column A (see Fig.VI.). The sapwood fraction appeared to contain a much higher percentage of more volatile constituents than either the bark or the oleoresin.

Compound 2A (Fig.VI.) was condensed on elution from the gas chromatograph and proton magnetic resonance (p.m.r.) and infrared (i.r.) spectra recorded. Spectral data was consistent with that reported for methyl citronellate (IIb), which has already been recorded as a constituent of the heartwood of C.columellaris (inland form)(43). The mass spectrum and gas chromatography (g.c.) retention time were the same as for an authentic sample of IIb prepared by methylation of citronellic acid (IIa), obtained from a silver oxide oxidation(50) of the

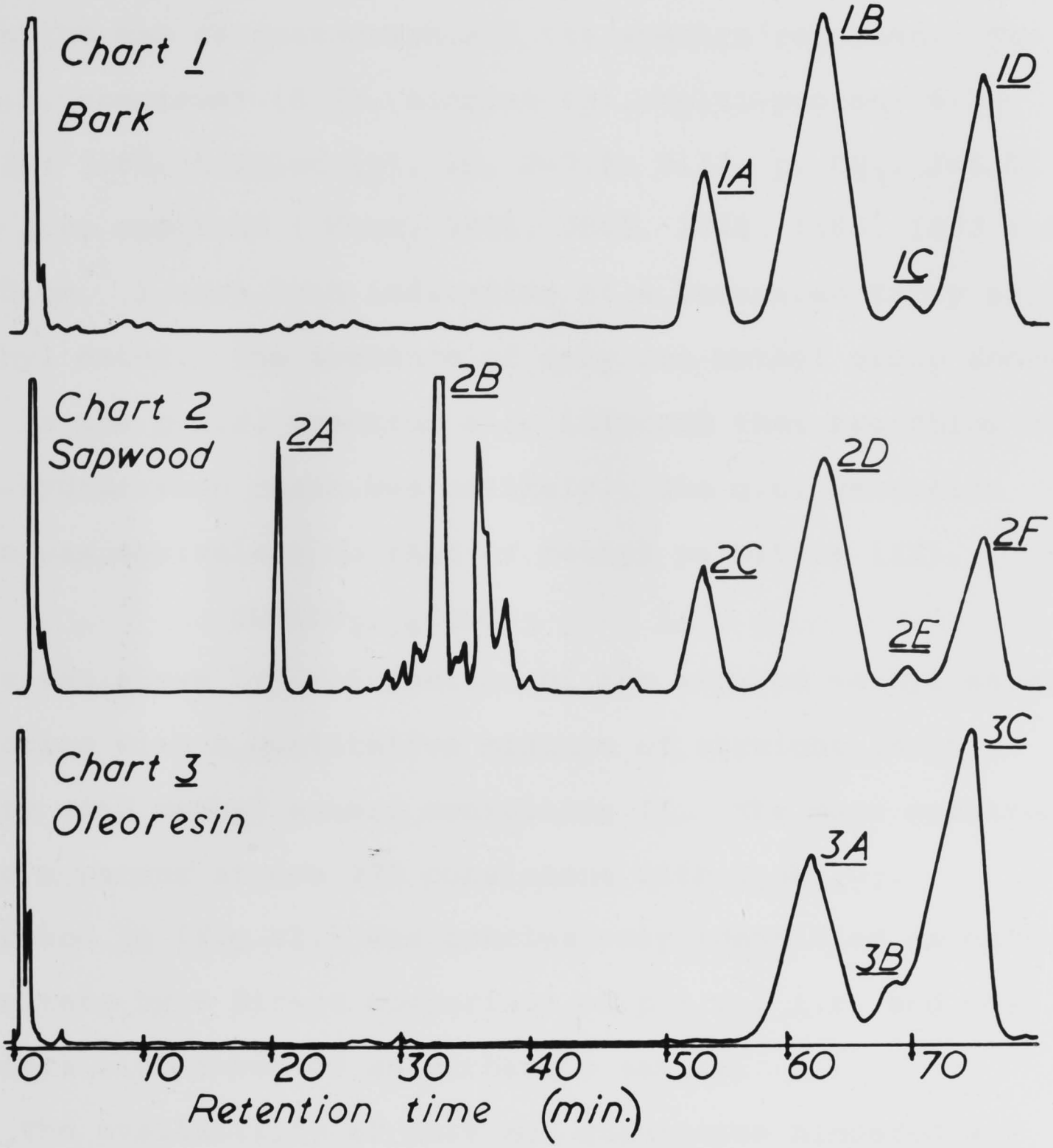


FIG. VI. GAS CHROMATOGRAPHS ON COLUMN A OF METHYLATED ACIDIC FRACTIONS OF CALLITRIS COLUMELLARIS.

corresponding aldehyde, citronellal (IIc).

Compound 2B (Fig.VI.) was also condensed on elution from the gas chromatograph and its spectra recorded. The p.m.r. spectrum* (8.73, singlet (s), multi-proton; 6.32, s, 3H; 7.66, triplet (t), 2H, J=7.5; 9.12, t, $C\underline{H}_3$, J=4.5) and i.r. spectrum (νmax. 2935, 2865, 1748, 1468, 1437 and 1170 $cm.^{-1}$) were both indicative of a saturated fatty acid methyl ester. The presence of only one methyl group above 8.8 in the p.m.r. spectrum also inferred that branching of the hydrocarbon chain was unlikely. The g.c. retention time was equivalent to that of methyl palmitate (IX).

$CH_3(CH_2)_{14}COOCH_3$ IX

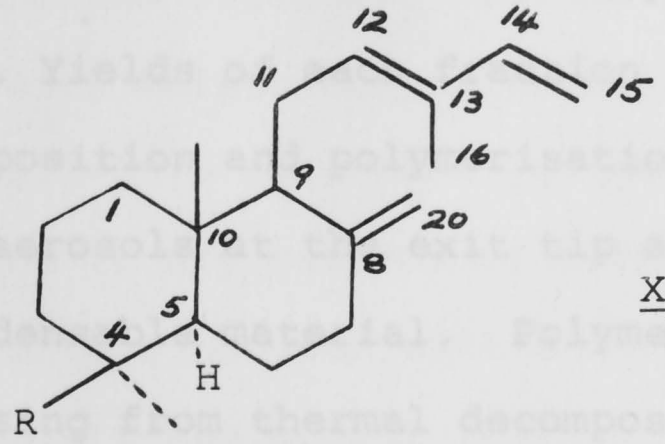
This was shown by co-injection of the sapwood methyl ester fraction with a qualitative mixture of straight-chain fatty acid methyl esters containing IX. The mass spectrum gave a parent at m/e 270 consistent with $C_{17}H_{34}O_2$.
Compound 2B (Fig.VI.) was conclusively identified as methyl palmitate by a direct comparison of p.m.r., i.r. and mass spectra with those of an authentic sample.

The availability of only mg. quantities hindered any further detailed investigation into the more volatile constituents of the sapwood methyl esters at this stage.

*(P.m.r. absorptions are quoted as τ values and splitting (J) as cycles per second.)

However, an i.r. spectrum (νmax. 2937, 2860, 1746, 1468, 1438 and 1172 $cm.^{-1}$) of a mixture of some of these compounds (g.c. retention times 33-42 min., Fig.VI.-Chart 2), also condensed on elution from the gas chromatograph, suggested that they were mainly fatty acid methyl esters.

A solution of bark methyl esters in methanol yielded a colourless crystalline compound on standing. Gas chromatography indicated a pure compound with retention time equivalent to peak 1B (Fig.VI.). Accurate mass measurement (mass spectrum) was consistent with a molecular formula of $C_{21}H_{32}O_2$. The ultraviolet (u.v.) (λmax. 232 mμ, logϵ 4.43) and i.r. (νmax. 1643, 1605 $cm.^{-1}$)spectra were both indicative of a conjugated diene structure. The p.m.r. spectrum showed three singlet methyls (8.26, 8.81, 9.44), one methoxyl (6.39) and six olefinic protons (3.40-5.53). A comparison of the mass spectrum (Fig.X. pg.51.) with those of some known diterpenoid esters of formula $C_{21}H_{32}O_2$(51) suggested methyl trans-communate (Xb) as the possible structure. Melting point (105-106^{o}), as well as u.v., i.r. and p.m.r. spectra were also in good agreement with values published for Xb(52). Reaction with methanolic potassium hydroxide solution yielded only starting material. This is in



R	
Xa.	COOH
b.	COOMe
c.	CH_3

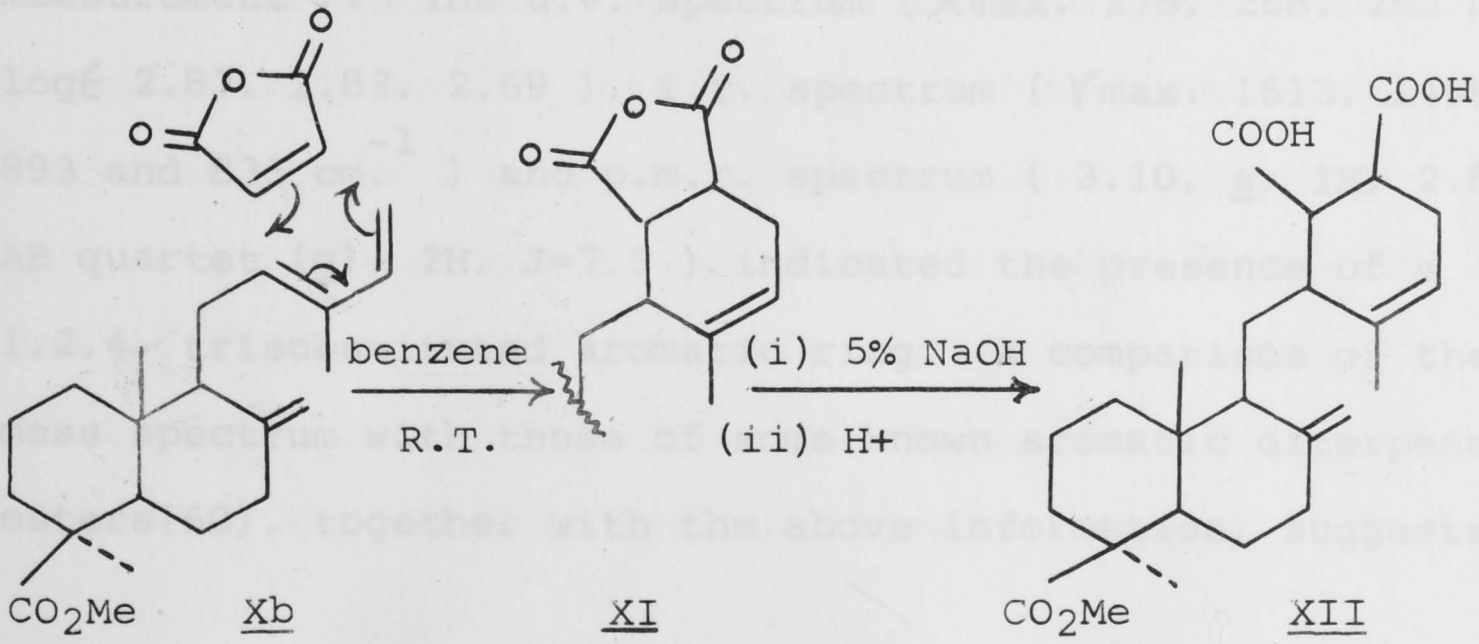
agreement with Xb which similarly fails to hydrolyse(53); a consequence of the axial orientation of the carbomethoxyl group at C_4(54). A mixed melting point determination and a direct comparison of the g.c. retention time and i.r. spectrum with those of an authentic sample confirmed the identity of the compound as methyl trans-communate.

The isolation of Xb initiated a further investigation into the diterpenoid constituents of the bark, sapwood and oleoresin acidic fractions. Work was commenced on the methyl esters of the bark diterpenoid acids depicted by gas chromatography (Fig.VI.-Chart 1). Attempts to separate the components by column and thin-layer chromatography on both alumina and silica gel were unsuccessful. Separation of diterpenoid esters on silver nitrate-impregnated silica gel columns and plates has, however, been recorded(55). Preparative gas chromatography on Carbowax 20M (column D) was eventually used to separate

the mixture into four fractions corresponding to peaks 1A, 1B, 1C and 1D. Yields of each fraction were low due to thermal decomposition and polymerisation on the column; formation of aerosols at the exit tip also lowered the amount of condensable material. Polymeric material and compounds arising from thermal decomposition were removed by column chromatography on silica gel.

Gas chromatography of fraction 1B on analytical column A yielded a single peak of retention time equivalent to that of methyl trans-communate (Xb). The p.m.r. spectrum showed all peaks consistent with Xb; however these appeared to constitute only a fraction of the total spectrum indicating that fraction 1B was actually a mixture. The u.v. spectrum (λmax. 232 mμ, logϵ 3.70) compared to that of Xb (λmax. 232 mμ, logϵ 4.43) was also indicative of a mixture. The percentage of Xb in the mixture, determined by p.m.r. integration values, was in the order of 20%. Assuming that Xb was the only compound in fraction 1B which contributes to the u.v. absorption at 232 mμ, the measured value of logϵ (3.70) corresponded to a percentage of 18.5% for Xb in the mixture. The similarity of the two percentages supported the above assumption and the presence, in the mixture, of compounds, other than Xb, which contained the conjugated diene

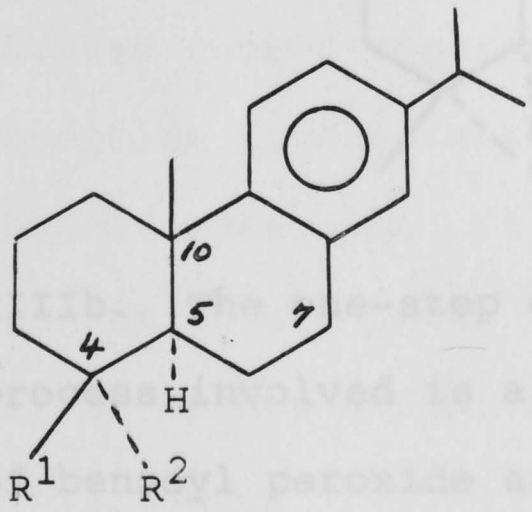
chromophore appeared unlikely. Xb has been reported to react readily with maleic anhydride to form the Diels-Alder adduct (XI)(53). Fraction 1B was thus treated with maleic anhydride in dry benzene at room temperature and the products further treated with a 5% sodium hydroxide solution to convert the adduct (XI) to the sodium salt of the corresponding di-acid (XII). The neutral fraction was removed by ether extraction and the basic solution acidified to yield a single crystalline di-acid. The p.m.r. spectrum showed three singlet methyls (9.47, 8.81, 8.22), one methoxyl (6.37), two one-proton singlets (5.46, 5.07), a one-proton multiplet (4.58) and a broad two-proton absorption (1.47). These values appeared consistent with those expected for di-acid XII which was the only expected acidic product from the above hydrolysis. Mass spectrum also gave a parent at m/e 432 consistent with $C_{25}H_{36}O_6$.



The neutral fraction from the above hydrolysis yielded also only a single compound; the p.m.r. spectrum showed three singlet methyls (9.17, 8.97, 8.81), one methoxyl (6.36) and four olefinic protons (3.95-5.30), corresponding to values published for methyl sandaracopimarate (VIIIb)(56). I.r. and mass spectra were also in agreement with those published for VIIIb(57,58). Treatment with potassium t-butoxide in dimethyl sulphoxide (59) yielded the corresponding acid identified as sandaracopimaric acid (VIIIa) by a mixed melting point determination and comparison of the i.r. spectrum with that of an authentic sample. VIIIa is already a known constituent of sandarac from the oleoresin of an unspecified species of Callitris(49) and its presence here is therefore not surprising.

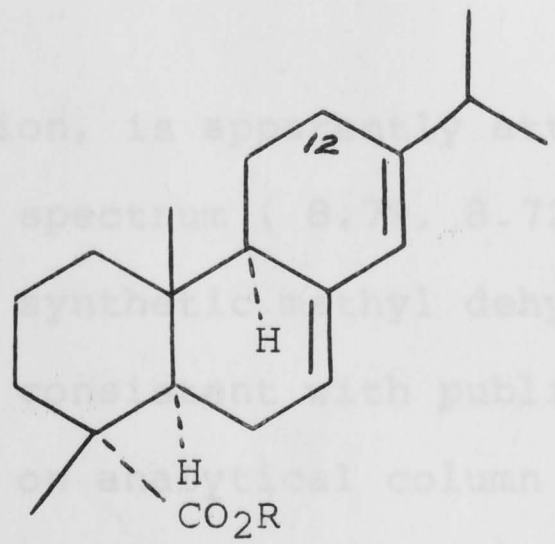
Fraction 1D (Fig.VI.) was collected as a crystalline solid of molecular formula $C_{21}H_{30}O_2$ (accurate mass measurement). The u.v. spectrum (λmax. 276, 268, 262 mμ; logϵ 2.87, 2.83, 2.69), i.r. spectrum (νmax. 1613, 1498, 893 and 833 $cm.^{-1}$) and p.m.r. spectrum (3.10, s, 1H; 2.85, AB quartet (q), 2H, J=7.5) indicated the presence of a 1,2,4- trisubstituted aromatic ring. A comparison of the mass spectrum with those of some known aromatic diterpenoid esters(60), together with the above information, suggested

methyl dehydroabietate (XIIIb) as the possible structure. However, a comparison of the p.m.r. spectrum with that published for XIIIb(61) showed them to be quite different.



	R^1	R^2
XIIIa.	Me	COOH
b.	Me	COOMe
c.	COOH	Me
d.	COOMe	Me
e.	Me	Me
f.	CH_2OH	Me
g.	CHO	Me

To check the published p.m.r. values, an authentic sample of methyl dehydroabietate (XIIIb) was prepared. Fieser and Campbell(62) had prepared 12-hydroxy abietic acid by oxidation of abietic acid (XIVa) with selenium dioxide; boiling with acetic acid had yielded dehydroabietic acid (XIIIa). Several attempts to repeat this work were unsuccessful. XIIIb had been prepared from methyl abietate (XIVb) by refluxing with N-bromo succinimide (NBS) and barium carbonate in carbon tetrachloride and treating the undetermined bromine-containing intermediate with sodium acetate in glacial acetic acid (63). A similar method was finally used to prepare XIIIb. Methyl abietate (XIVb), benzene and trace amounts of benzoyl peroxide, under vigorous reflux, were treated with NBS, with evolution of hydrogen bromide, to yield directly

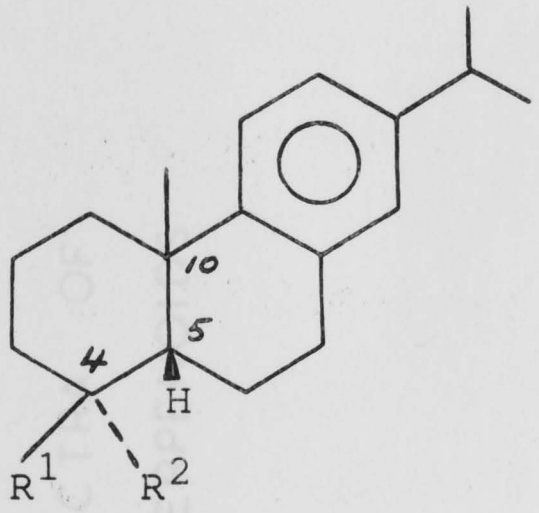


	R
XIVa.	H
b.	CH_3

XIIIb. The one-step dehydrogenation and rearrangement process involved is already known for NBS in the presence of benzoyl peroxide and is believed to involve a radical mechanism(64). Treatment of abietic acid (XIVa) with either bromine in carbon tetrachloride(65) or lithium in ethylenediamine(66) similarly yield directly dehydro-abietic acid (XIIIa). Work up of the reaction products of the above NBS-benzoyl peroxide reaction, however, indicated the presence of some unreacted starting material. This was easily removed by treating the reaction product, dissolved in glacial acetic acid, with small quantities of chromium trioxide in glacial acetic acid and water. Methyl abietate (XIVb) yields a mixture of acids and oxygenated neutral compounds which were separated out by column chromatography, leaving pure methyl dehydroabietate (XIIIb). Although XIIIb is known to undergo benzylic oxidation to yield the 7-oxo derivative(67), XIVb, being extremely susceptible to C-C double bond cleavage and

allylic oxidation, is apparently attacked preferentially.

The p.m.r. spectrum (8.79, 8.72, s,s, 2CH_3; 8.78, d, 6H, J=7.5) of synthetic methyl dehydroabietate (XIIIb) however proved consistent with published results; g.c. retention time on analytical column A also differed from that of ester 1D. It was therefore assumed that ester 1D was one of the three remaining possible stereoisomers of XIIIb; either XIIId, XVb or XVd.

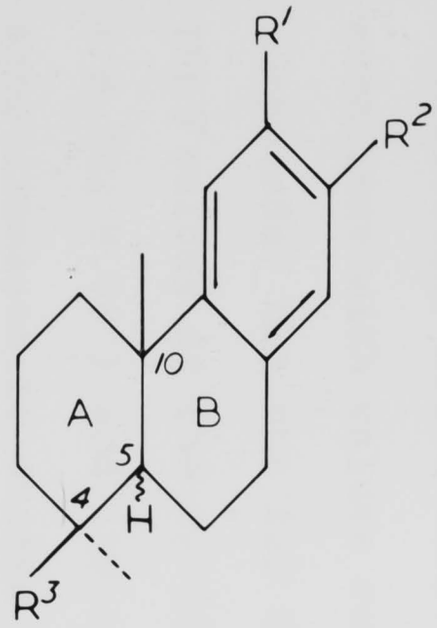


	R^1	R^2
XVa.	COOH	Me
b.	COOMe	Me
c.	Me	COOH
d.	Me	COOMe

Methyl dehydroabietate (XIIIb) and the unknown ester 1D, on hydrolysis with potassium t-butoxide in DMSO, both yielded their corresponding acids. Reduction of ester 1D with lithium aluminium hydride in ether gave the corresponding alcohol. The acid of ester 1D was shown, by direct comparison, to differ from an authentic sample of 5-iso dehydroabietic acid (XVc)(68). Two possible structures (XIIId and XVb), both with a 4β-configuration, remained.

A comparison was then made of the chemical shifts of

FIG. VII. P.M.R. SPECTRA OF SOME AROMATIC DITERPENOIDS



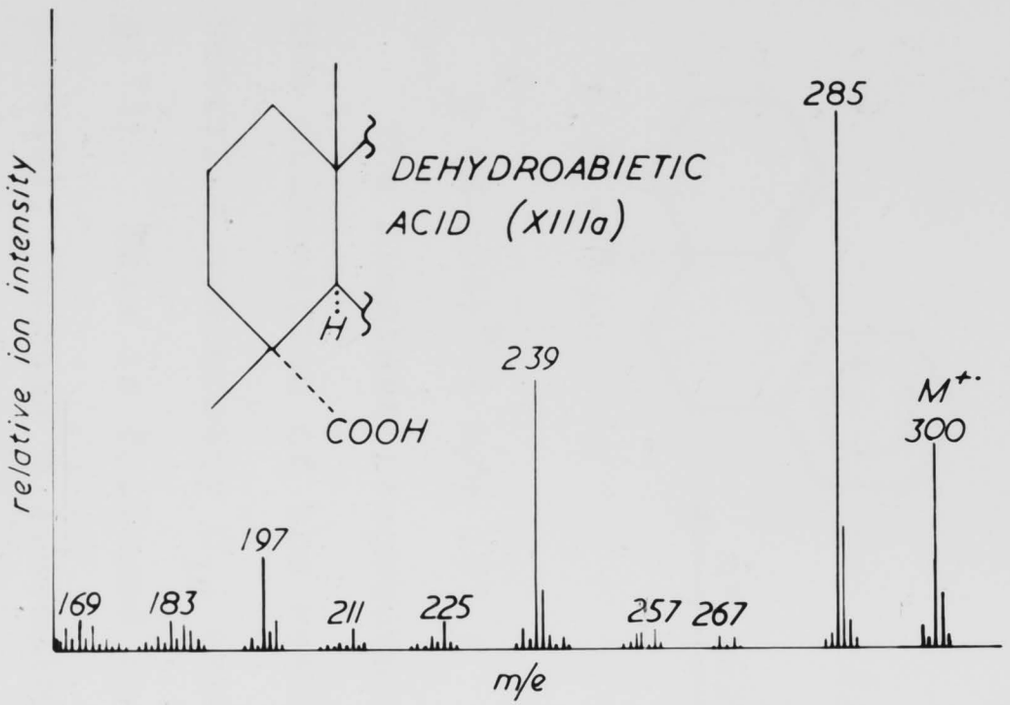
UNKNOWN ID	R1	R2	R3	C_4Me τ	C_{10}Me	τ-SHIFT acid-alcohol C_4Me, C_{10}Me	τ-SHIFT ester-alcohol C_4Me, C_{10}Me
Acid	H	$CH(Me)_2$	CO_2H	8.67	8.88		
Methyl ester	H	$CH(Me)_2$	CO_2Me	8.72	8.98	+.28, -.05	+.23, -.15
Alcohol	H	$CH(Me)_2$	CH_2OH	8.95	8.83		
A/B TRANS							
Deoxypodocarpic acid[74]	H	H	CO_2H	8.68	8.90		
Me deoxypodocarpate[69]	H	H	CO_2Me	8.73	8.97	+.27, -.08	+.22, -.15
Deoxypodocarpol[69]	H	H	CH_2OH	8.95	8.82		
Me O-Me podocarpate[69]	OMe	H	CO_2Me	8.73	8.96	—	+.24, -.14
O-Me podocarpol[69]	OMe	H	CH_2OH	8.97	8.82		
A/B CIS							
5β-Deoxypodocarpic acid[68]	H	H	CO H	8.91	8.78		
Me 5β-deoxypodocarpate[68]	H	H	CO_2Me	8.88	8.70	+.77, +.05	+.80, +.13
5β-Deoxypodocarpol[68]	H	H	CH_2OH	9.68	8.83		

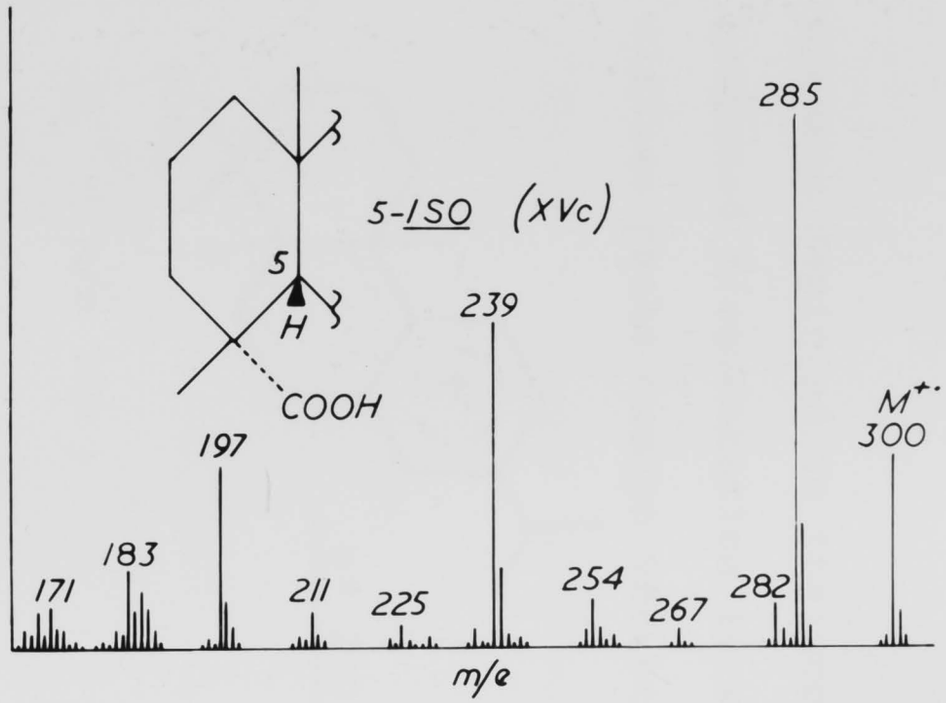
the C_4 and C_{10} methyl groups of ester 1D and its derivatives with some suitable A/B cis and trans, 4β-substituted, model compounds (Fig.VII.). Agreement was excellent with values reported for A/B trans model compounds, but not for those with A/B cis configurations The γ values of the C_4 and C_{10} methyl groups of ester 1D (8.72, 8.98) and its corresponding acid (8.67, 8.88) show diamagnetic shifts, due to the shielding effect associated with the carbonyl double bond(69), as compared with the values (8.95, 8.83) of the C_4 and C_{10} methyl groups of the corresponding alcohol. These diamagnetic shifts (Fig.VII.) are also consistent with those observed for the A/B trans model compounds, but not for those with A/B cis configurations. On these correlations, methyl 4β-dehydroabietate (XIIId) was assigned as the only possible structure for the unknown ester 1D.

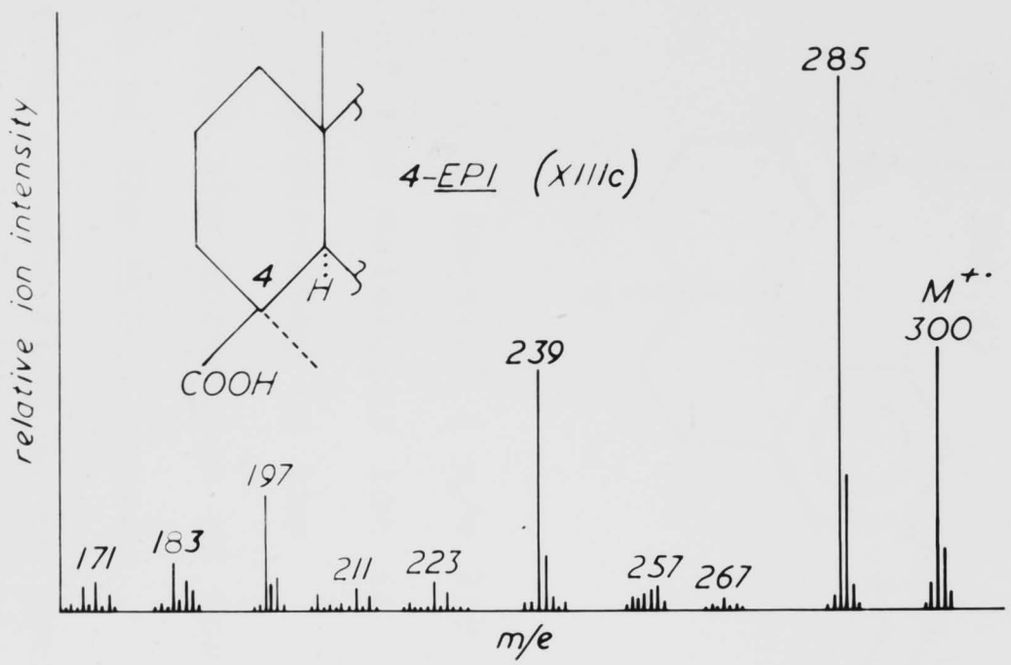
At this stage of the investigation two separate research groups recorded the isolation of a new diterpenoid ester from both "Australian sandarac" and the oleoresin of Callitris columellaris. The properties appeared identical with those of ester 1D isolated from the bark of Callitris. Both publications also arrived at methyl 4β -dehydroabietate (XIIId) (under the names methyl callitrisate and methyl 4-epi dehydroabietate) as the structure for the new

diterpenoid. Carman and Deeth(70) based their structure elucidation on a comparison of the corresponding hydrocarbon with dehydroabietane (XIIIe). The axial (β-) configuration of the carboxylate group at C_4 was indicated by the difficulty of hydrolysis of the ester, the pK_a value of the acid and the chemical shift of the $-CH_2OH$ protons in the p.m.r. spectrum of the alcohol. Gough(71) showed that dehydrogenation of the new ester and methyl dehydroabietate (XIIIb) afforded an identical series of products. Direct comparisons eliminated XIIIb and XVd as possible structures. Comparisons with the p.m.r. chemical shifts, i.r. spectra and O.R.D. data of suitable A/B cis and trans 4β-substituted compounds suggested an A/B trans configuration. Samples of XIIId, obtained from both research groups, have been shown to be identical in all respects to ester 1D. An authentic sample of 4,5-iso dehydroabietic acid (XVa)(72) has since been obtained and shown, by direct comparison, to be different to the acid of ester 1D; as expected.

The availability of small quantities of each of the four stereoisomers of dehydroabietic acid initiated the measurement and comparison of their mass spectra (Fig. VIII.). All stereoisomers yielded very similar spectra, each tending to follow the same major fragmentation







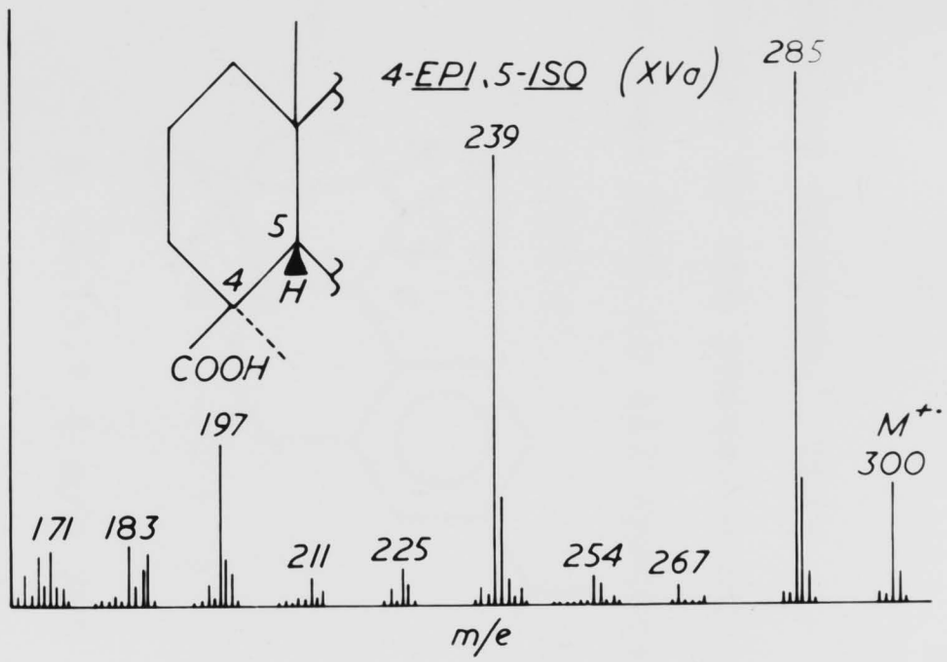
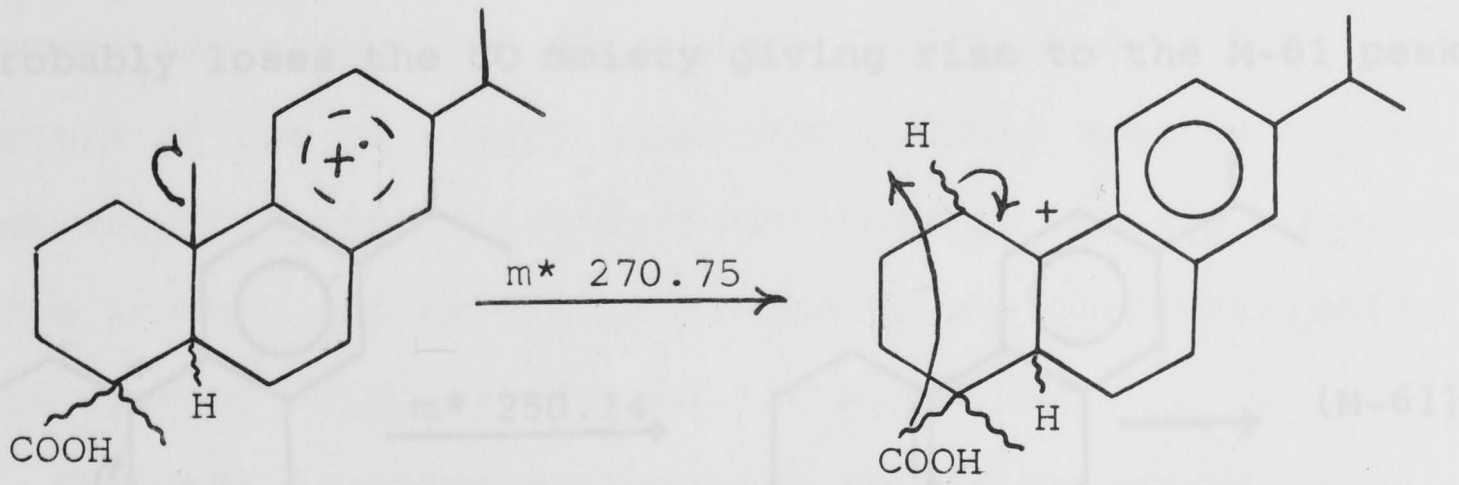


FIG. VIII. MASS SPECTRA OF THE STEREOISOMERS OF DEHYDROABIETIC ACID.

process depicted in the accompanying diagram. This proposed fragmentation is supported by the presence of diffuse peaks due to metastable ions (m*) in all spectra.



M·+ (m/e 300)

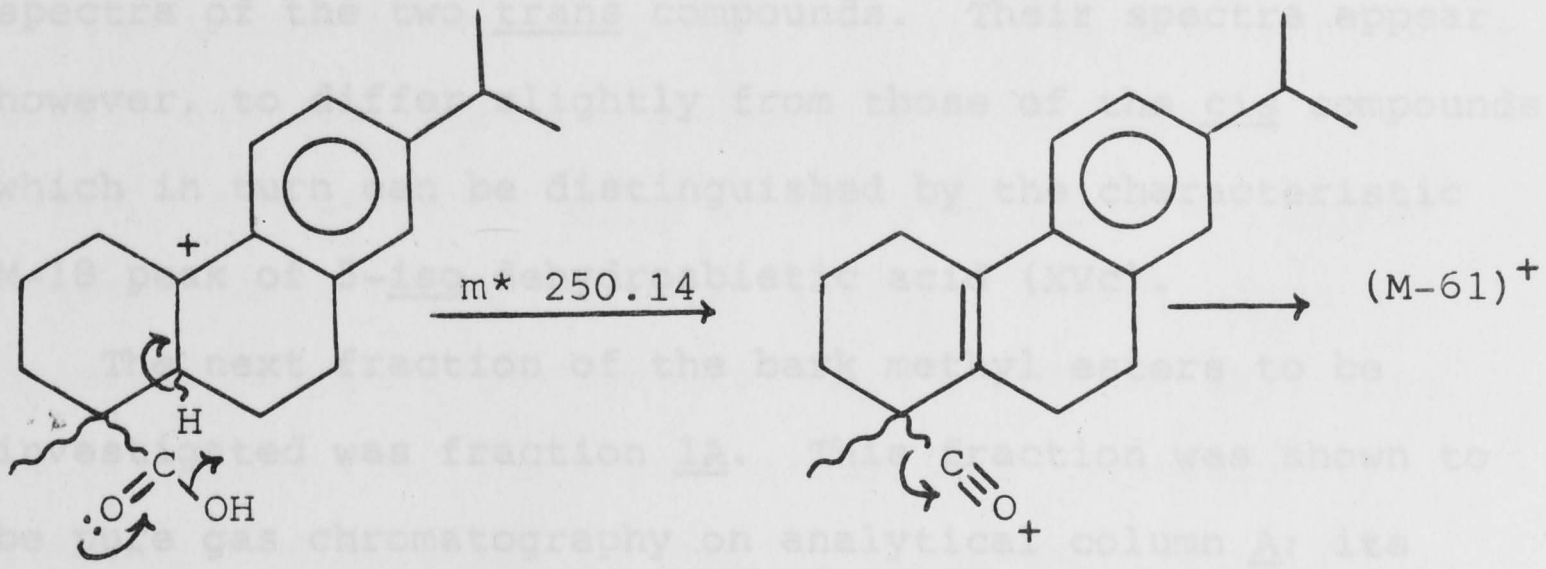
a (M-15)+ (m/e 285)

c (M-103)+ (m/e 197)

b (M-61)+ (m/e 239)

The simplest fragmentation of the molecular ion is the elimination of the methyl group attached to the benzylic quaternary C_{10} carbon with the formation of ion a(73). This is followed by loss of the HCOOH moiety, possibly through a 1,4-elimination across ring A, to form ion b which then eliminates the isopropyl group with back-

transfer of one of its hydrogens(73) giving rise to the M-103 peak (ion c). A metastable at m/e 250.14 suggests that ion a also eliminates H_2O to form ion d which then probably loses the CO moiety giving rise to the M-61 peak.



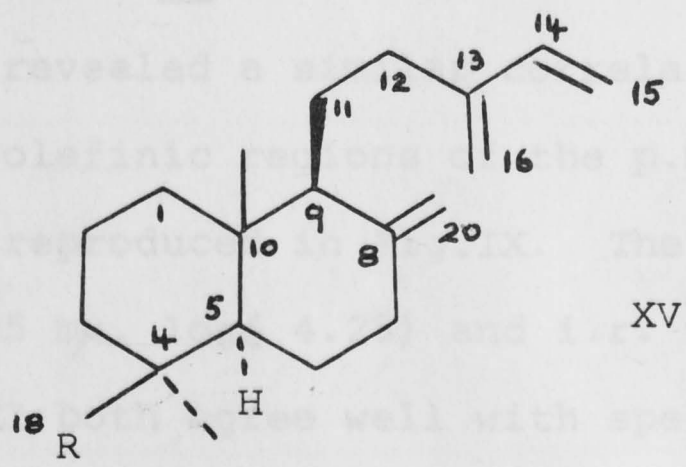
a d $(M-33)^+$ (m/e 267)

Of the four isomers, only 5-iso dehydroabietic acid (XVc) shows the characteristic M-18 peak (m/e 282) due to elimination of H_2O (m* 265.08) from the parent ion. A further loss of CO (m* 228.78) gives rise to the M-46 peak (m/e 254). The other cis isomer (XVa), although it shows no peak due to H_2O loss, also shows a relatively strong peak at m/e 254 compared to both trans isomers. The m/e 254 peak can also possibly arise from the direct elimination of the HCOOH moiety from the parent ion. Insufficient evidence, at this stage, is available to propose a mechanism which could satisfactorily explain the apparent preference of the cis isomers to eliminate the

HCOOH moiety, either step-wise or directly, from the parent ion.

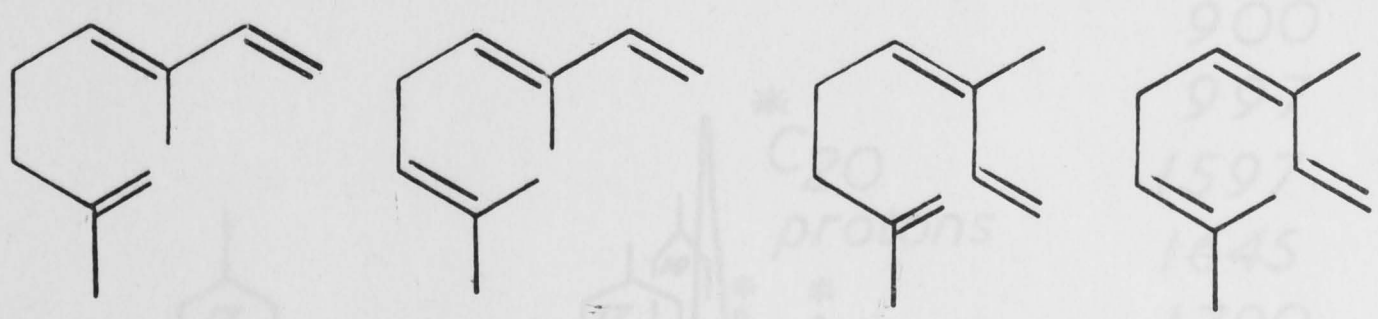
A comparison of the mass spectra of the four stereo-isomers has thus shown no apparent difference in the spectra of the two trans compounds. Their spectra appear however, to differ slightly from those of the cis compounds, which in turn can be distinguished by the characteristic M-18 peak of 5-iso dehydroabietic acid (XVc).

The next fraction of the bark methyl esters to be investigated was fraction 1A. This fraction was shown to be pure gas chromatography on analytical column A; its molecular weight, 316.2411 (accurate mass measurement), was consistent with $C_{21}H_{32}O_2$. The u.v. spectrum (λmax. 226 mμ, logϵ 4.28) and i.r. spectrum (γmax. 1642 and 1598 $cm.^{-1}$) were again consistent with a conjugated diene structure. Chemical and physical correlations with model compounds suggested methyl Δ-13(16)-communate (XVIa) as a probable structure for this new diterpenoid ester.



	R
XVIa.	COOMe
b.	CH_2OH
c.	Me

The p.m.r. spectrum (partly shown in Fig.IX.) showed two singlet methyls (8.88, 9.53), one methoxyl (6.42) and seven olefinic protons (3.40-5.38). In the case of methyl trans-communate (Xb) the configuration of the triene group had been elucidated by a comparison of p.m.r. and u.v. spectral properties with those of the acyclic monoterpenes trans-α, trans-β, cis-α and cis-β-ocimene (XVII to XX respectively(52). A very close agreement between the spectral data of Xb and XVII left little doubt that the triene group in Xb possessed a similar configuration to that of trans-α-ocimene (XVII).



XVII XVIII XIX XX

A similar comparison of the spectral data of the new diterpenoid ester 1A with that of a series of monoterpene trienes(75) revealed a similar correlation with α-myrcene (XXI). The olefinic regions of the p.m.r. and i.r. spectra of each are reproduced in Fig.IX. The u.v. spectrum (λmax. 225.5 mμ, logϵ 4.25) and i.r. spectrum in this region of XXI both agree well with spectra recorded for the new ester. The p.m.r. spectra are virtually the same

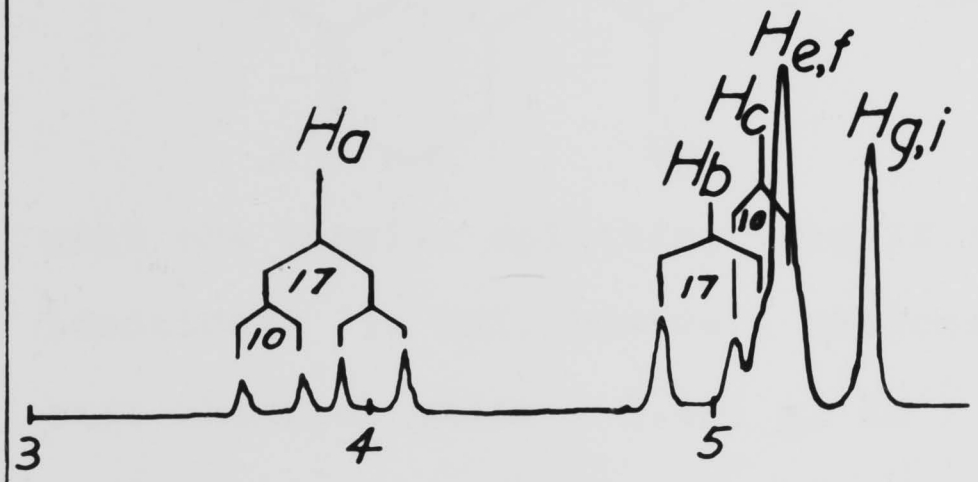
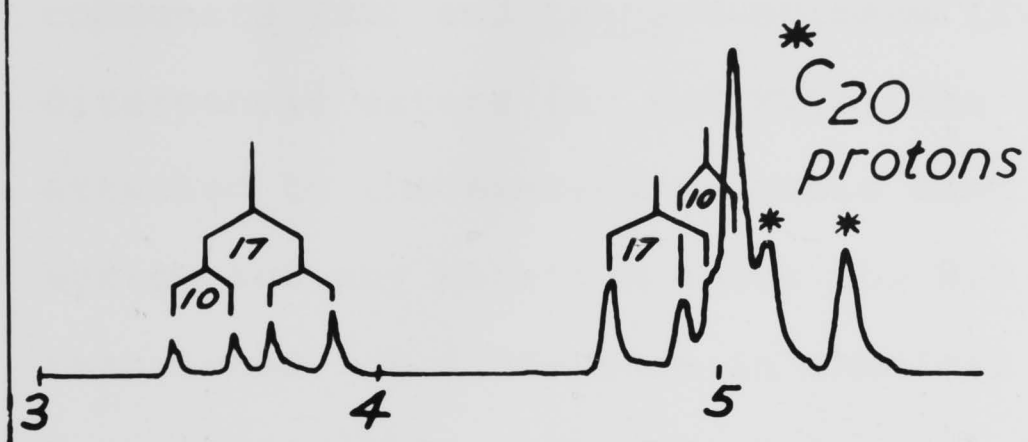
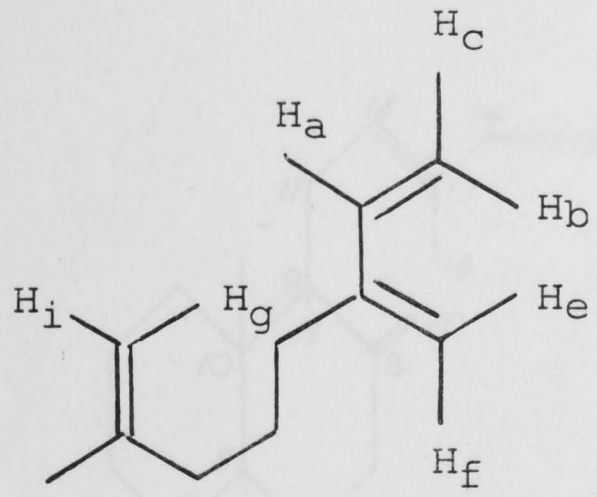
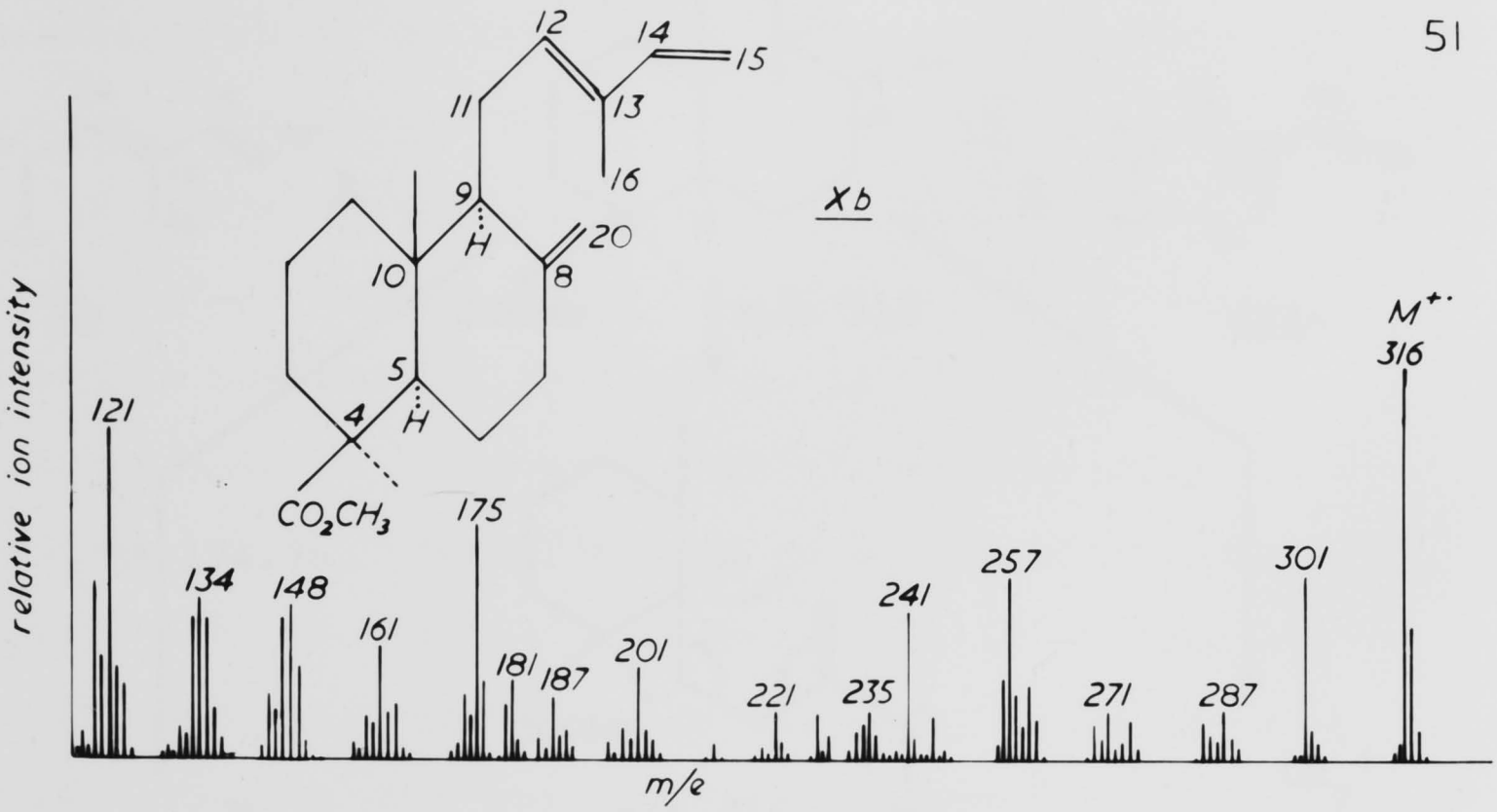
P.M.R. (τ)-$CDCl_3$	I.R. (cm^{-1})-CCl_4
	887-907 992 1596 1650 1791 1804 1980 3075
XXI	
	900 997 1597 1645 1790 1800 3075
XVIa	

FIG. IX. SPECTRAL CORRELATION BETWEEN α-MYRCENE (XXI) AND Me Δ-13(16)-COMMUNATE (XVIa).



XXI

with the vinylic splitting (Fig.IX.) in each case being identical. In XXI, however, protons H_g and H_i have the same chemical shift (5.48, s, 2H) while in ester 1A the chemical shifts of the two C_{20} protons differ (5.13 and 5.38, two one-proton singlets). This is consistent with a comparison of the p.m.r. spectra of methyl trans-communate (Xb) and trans-∝-ocimene (XVII). In both diterpenoid esters (Xb and XVIa) the C_{20} protons are attached to the exocyclic double bond of a trans-decalin system and any rotation about the 8,9 C-C single bond is restricted. A difference in chemical shifts of the two C_{20} protons thus arises because each is subjected to a different shielding effect due to the C-C double bonds of the conjugated diene system; no shielding effects would be expected in the acyclic monoterpenes due to the freedom of rotation about C-C single bonds. The above correlations suggest that ester 1A contains a triene system similar in structure to ∝-myrcene (XXI).



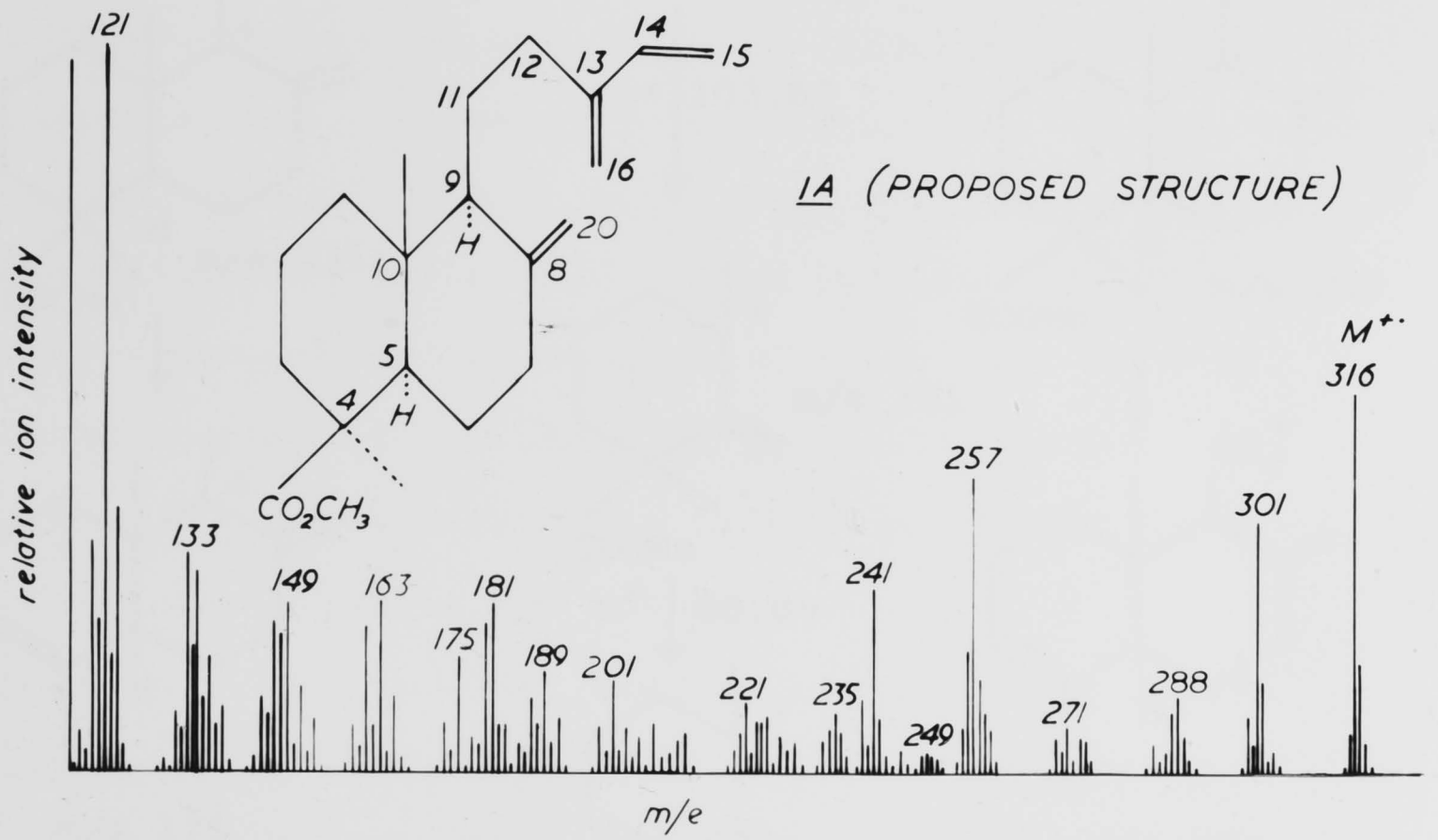


FIG. X. MASS SPECTRAL COMPARISON OF DITERPENE ESTER IA AND Me TRANS-COMMUNATE (Xb).

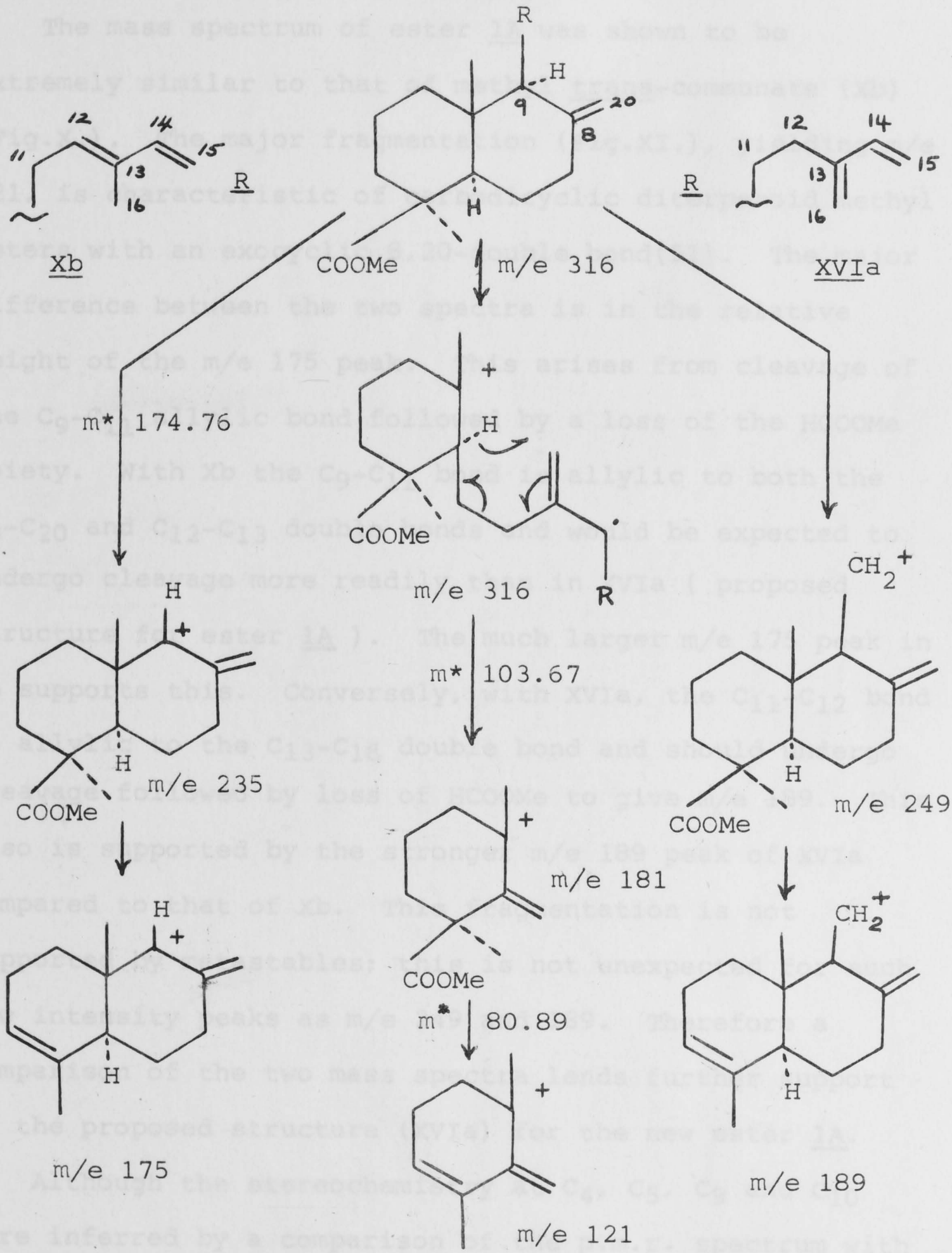
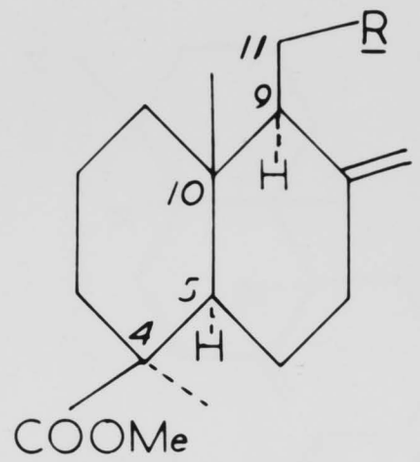


Fig.XI. Mass spectral fragmentations of methyl trans-communate (Xb) and methyl Δ-13(16)-communate (XVIa).

The mass spectrum of ester 1A was shown to be extremely similar to that of methyl trans-communate (Xb) (Fig.X.). The major fragmentation (Fig.XI.), yielding m/e 121, is characteristic of carbodicyclic diterpenoid methyl esters with an exocyclic 8,20-double bond(51). The major difference between the two spectra is in the relative height of the m/e 175 peak. This arises from cleavage of the C_9-C_{11} allylic bond followed by a loss of the HCOOMe moiety. With Xb the C_9-C_{11} bond is allylic to both the C_8-C_{20} and C_{12}-C_{13} double bonds and would be expected to undergo cleavage more readily than in XVIa (proposed structure for ester 1A). The much larger m/e 175 peak in Xb supports this. Conversely, with XVIa, the C_{11}-C_{12} bond is allylic to the C_{13}-C_{16} double bond and should undergo cleavage followed by loss of HCOOMe to give m/e 189. This also is supported by the stronger m/e 189 peak of XVIa compared to that of Xb. This fragmentation is not supported by metastables; this is not unexpected for such low intensity peaks as m/e 249 and 189. Therefore a comparison of the two mass spectra lends further support to the proposed structure (XVIa) for the new ester 1A.

Although the stereochemistry at C_4, C_5, C_9 and C_{10} were inferred by a comparison of the p.m.r. spectrum with those of suitable 10β, 9α-H, 5α, 4β-carbomethoxy model



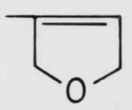
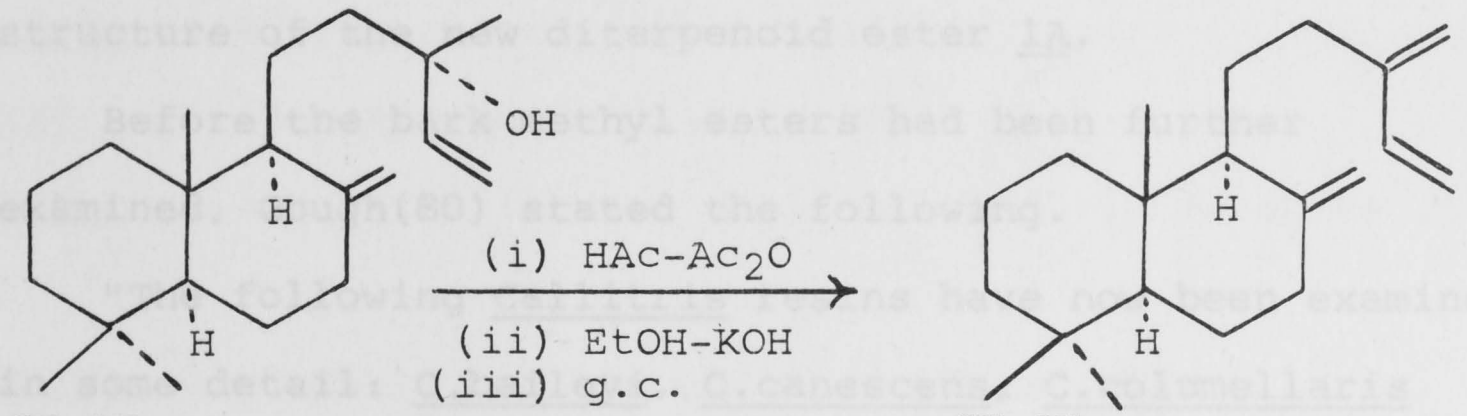
COMPOUND	R	C_4 Me	C_{10} Me	C_4 COOMe	Ref.
Me ester IA	$-CH_2CH(CH=CH_2)=CH_2$	8.88	9.53	6.42	
Me *trans*-communate	$-CH=CH(CH_3)CH=CH_2$	8.81	9.44	6.39	
Me *cupressate*	$-CH_2C(CH_3)(OH)CH=CH_2$	8.83	9.52	6.41	(76)
Me *isocupressate*	$-CH_2C(CH_3)=CHCH_2OH$	8.83	9.52	6.41	(76)
Me *sciadopate*	$-CH_2C(CH_2OH)=CHCH_2OH$	8.83	9.52	6.42	(77)
Me *lambertianate*	$-CH_2$ 	8.87	9.52	-	(77)

FIG.XII. P.M.R. SPECTRAL COMPARISON OF METHYL ESTER IA (PROPOSED STRUCTURE) AND MODEL COMPOUNDS OF 10β, 9α-H, 5α, 4β-CARBOMETHOXYL CONFIGURATION.

compounds (Fig.XII.), a chemical correlation with a known compound of the proposed configuration of XVIa was necessary to establish structure XVIa firmly as that of the new diterpenoid 1A. Reduction of 1A with lithium aluminium hydride in ether yielded the corresponding alcohol which should be identical with compound XVIb, reported as a reaction product from the acetic acid-acetic anhydride dehydration of torulosol (XXII)(53).



XXII XVIb

An authentic sample of XVIb was not available and only its u.v. spectrum (λmax. 227 mμ, logϵ 4.34) had been recorded. This necessitated repeating the dehydration reaction on torulosol. The major product (λmax. 226 mμ, logϵ 4.35) was isolated by gas chromatography and shown by direct comparison of g.c. retention times, as well as u.v., i.r. and mass spectra, to be identical to the alcohol derived from ester 1A. The p.m.r. spectrum of the alcohol also further substantiated the axial (4β-) configuration

of the carbomethoxyl group of ester 1A. The methylene protons of the hydroxymethylene group showed as an AB quartet (J=11) at 6.40τ, consistent with similar compounds with axial hydroxymethylene groups(78, 79). Those with an equatorial configuration usually reveal a quartet approximately .4τ upfield from those of axial configurations. Sufficient evidence was now available to conclude that methyl Δ-13(16)-communate (XVIa) was the structure of the new diterpenoid ester 1A.

Before the bark methyl esters had been further examined, Gough(80) stated the following.

"The following Callitris resins have now been examined in some detail: C.baileyi, C.canescens, C.columellaris (including C.hugelii and C.intratropica), C.endlicheri, C.macleayana, C.oblonga, C.preissii, C.rhomboidea and C.verrucosa.

The dominant compounds (60-80%) are trans- (mainly) and cis-communic acids (Xa and XXIIIa), usually more or less polymerised. Most samples examined contain 4-epi dehydroabietic acid (XIIIc) with a little 7-oxo-4-epi dehydroabietic acid (XXIVa), but the content is rather variable and it and its precursors are sometimes absent. Sandaracopimaric acid (VIIIa) is always present, together with variable but usually lesser quantities of isopimaric

acid (XXVa) and Δ-8-isopimaric acid (XXVIa). In addition there are several unidentified minor acids. The less oxidised precursors of the acids together with various mono- and sesqui-terpenoids make up the neutral fraction of the resins.

Taking the sandaracopimaric acid content as 100 parts, the nine C.columellaris samples analysed have the following compositions:

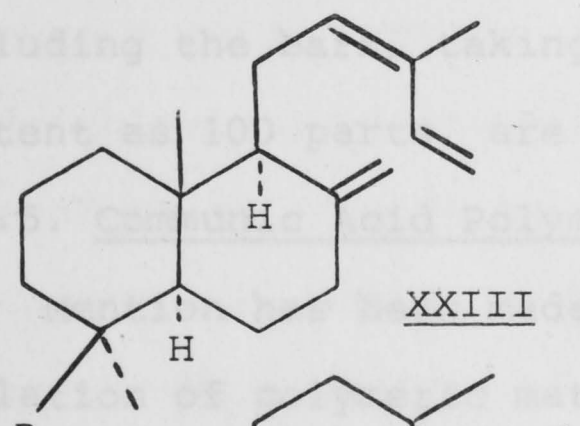
XXVIa 1-95 parts (only 3 above 10)

XXVa 5-48 parts (only 3 above 20)

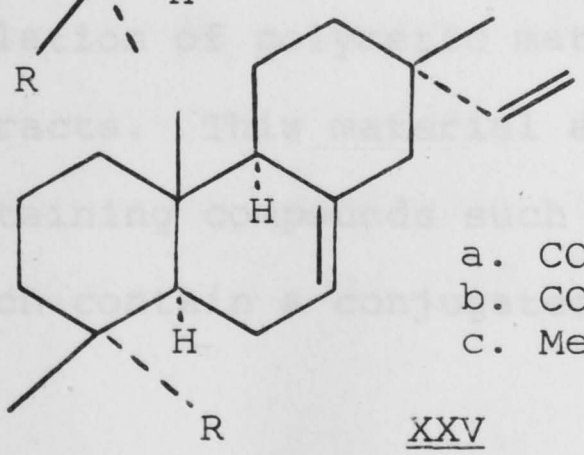
XIIIc 0-260 parts (absent in 1, below 50 in 4, above 150 in 1)

XXIVa 0-16 parts (of the order of 10% of XIIIc)

Broadly similar results are found for most other species. Occasional samples are pink-brown; these seem to be phenolic and lack diterpenes."

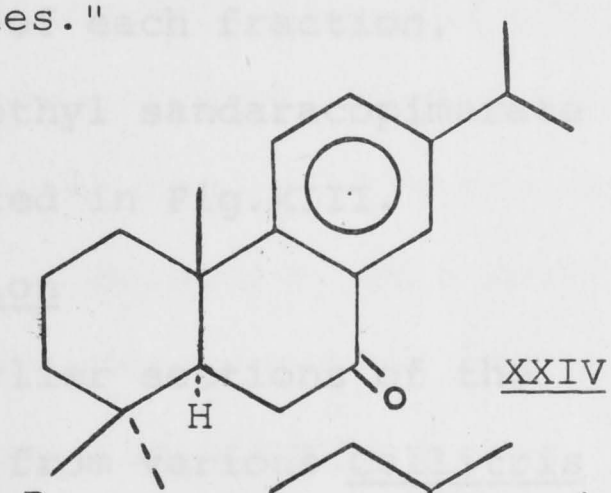


XXIII

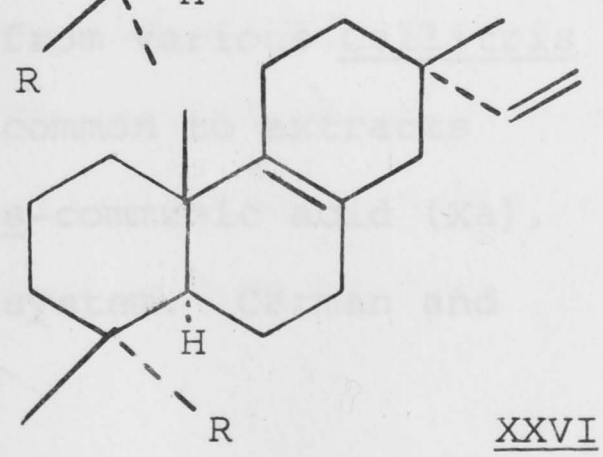


R
a. COOH
b. COOMe
c. Me

XXV



XXIV



XXVI

On receipt of Gough's communication, authentic samples of isopimaric acid (XXVa) and Δ-8-isopimaric acid (XXVIa) were obtained, methylated with ethereal diazomethane, and the methyl esters compared by g.c. retention times and spectral properties with the yet undetermined diterpenoid ester 1C of the bark extract. Correlations were consistent with methyl isopimarate (XXVb). Gas chromatography however showed no peak of equivalent retention time (R_d 0.71) to methyl Δ-8-isopimarate (XXVIb). All compounds indicated by gas chromatography (Fig.VI.-Chart.1.) had now been fully characterised.

The methylated acidic fractions from the sapwood and oleoresin (Fig.VI.-Charts 2 and 3 respectively) were then examined, by g.c. retention time and spectral property comparisons, for each of the characterised bark diterpenoid methyl esters. The compositions of each fraction, including the bark, taking the methyl sandaracopimarate content as 100 parts, are tabulated in Fig.XIII.

III.5. Communic Acid Polymerisation

Mention has been made in earlier sections of the isolation of polymeric materials from various Callitris extracts. This material appears common to extracts containing compounds such as trans-communic acid (Xa), which contain a conjugated diene system. Carman and

Compound	Composition		
	Bark	Sapwood	Oleoresin
Me Δ-13(16)-communate (XVIa)	37 (1A),	65 (2C),	-
Me trans-communate (Xb)	23 (1B),	75 (2D),	-
Me sandaracopimarate (VIIIb)	100 (1B),	100 (2D),	100 (3A)
Me isopimarate (XXVb)	10 (1C),	23 (2E),	34 (3B)
Me Δ-8-isopimarate (XXVIb)	-	-	-
Me 4-epi dehydroabietate (XIIId)	84 (1D),	104 (2F)	189 (3C)

Fig.XIII. Composition of Callitris diterpene acid methyl esters; methyl sandaracopimarate (VIIIb) taken as 100 parts. The corresponding g.c. peaks (Fig.VI.) are shown in brackets.

Cowley(81) have recently shown that treatment of the resin of Agathis robusta, known to contain both cis- and trans-communic acids (XXIIIa and Xa respectively), with dilute hydrochloric acid at reflux temperatures, yields two acids. One of these, "dundatholic acid" (potassium salt soluble in alcohol), was shown by gas chromatography of the methyl ester to be a mixture of the monomeric diterpene acid constituents of Agathis resin. The methyl ester of the other acid, "dundathic acid" (potassium salt insoluble in alcohol; m.p. 230-234°), was shown to have an i.r.

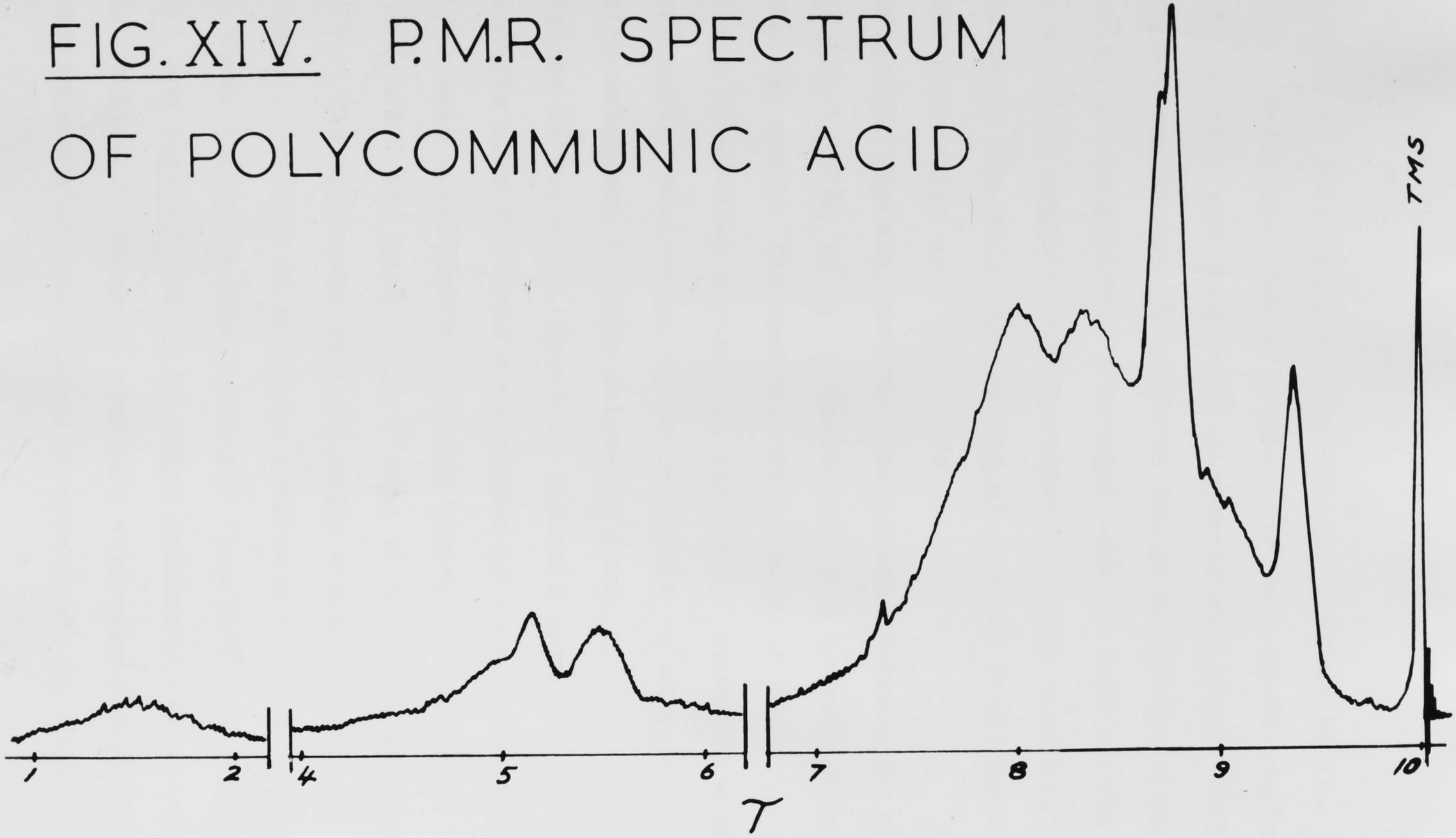
spectrum superimposable on that of synthetic methyl polycommunate obtained by polymerising trans-communic acid. Polycommunic acid is also a known constituent of the oleoresin of an unspecified Callitris species(49).

On the basis of the above information it was assumed that the n-hexane insoluble materials isolated from Callitris columellaris (inland form) were probably mixtures of monomeric and polymeric constituents. To show this, the n-hexane insoluble fractions were treated with aqueous potassium hydroxide and neutral constituents removed by ether extraction; acidification of the aqueous layer yielded the acidic fraction. Methylation and gas chromatography gave peaks consistent with the monomeric diterpenoid methyl esters already characterised in Section III.4. Further treatment of the acids with ethanolic potassium hydroxide(81) yielded potassium salts both soluble and insoluble in ethanol, thus indicating the presence of both monomeric and polymeric acids.

The alcohol-insoluble potassium salts from the oleoresin fraction were further investigated. Acidification and purification by precipitation with water from alcohol(81) gave a compound, presumably polycommunic acid, as a white powder, m.p. 177-192^{o}. Gas chromatography of the methyl ester showed no peaks. The u.v. (no λmax.

above 220 mμ) and i.r. (no absorption in the 1600 $cm.^{-1}$ region) spectra suggested the absence of a conjugated diene structure. This further suggested that polycommunic acid was probably being formed via a series of intermolecular Diels-Alder reactions, probably involving both trans- and Δ-13(16)-communic acids, already isolated as monomers from the bark and sapwood of Callitris. This intermolecular Diels-Alder mechanism is consistent with that proposed by Ohloff and coworkers(75) for the polymerisation of α-myrcene (XXI). The only significant absorptions in the olefinic region of the i.r. spectrum of polycommunic acid are at 3082, 1647 and 889 $cm.^{-1}$ which are characteristic of a gem-disubstituted alkene. Absorptions due to a monosubstituted alkene (vinyl) appear absent. The p.m.r. spectrum (Fig.XIV.) shows two methyl singlets (9.38, 8.79) which can be assigned to quaternary methyl groups corresponding to those at C_{10} and C_4 respectively in the communic acid monomers. No definite peak consistent with a methyl group attached to a double bond is observed. However a shoulder at 8.75 suggests the possibility of formation, during polymerisation, of another allylic quaternary methyl group. Broader peaks at 8.02 and 8.36 are probably due to saturated allylic and non-allylic C-H absorptions. Reasonably well-defined peaks at 5.43 and

FIG. XIV. P.M.R. SPECTRUM OF POLYCOMMUNIC ACID



5.13 appear to correspond to the two C_{20} protons in the monomers. This, with the i.r. spectral data above, seems to indicate that the C_8-C_{20} gem-disubstituted double bond of the monomers is not involved in the proposed Diels-Alder polymerisation mechanism. A broader peak at 4.80 in the p.m.r. spectrum suggests the presence of other olefinic protons while the broad absorption at 1.50 is consistent with the carboxylic acid structure.

Gough(49) has assigned an approximate composition of $(C_{20}H_{30}O_2)_n + H_{0.5}O_{1.5}$, where n is 2-5 or more, for polycommunic acid. The spectral data above is also indicative of several structural features. However, with the information available, it is difficult to assign any composite structure to this polymeric acid.

Values in Fig.XIII. (pg.58.) indicate that the ratio, of monomeric communic acid composition to total monomeric diterpene acid composition, is much higher in both the sapwood (.38) and bark (.24) than in the oleoresin (.00'). The oleoresin in turn shows a much higher percentage (54.4%) of n-hexane insoluble material present in the total light petroleum extract than both the bark (5.7%) and sapwood (0.5%). This indicates that polymerisation of monomeric communic acid-type compounds is greatest in the oleoresin; it also appears to be more

predominant in the bark than in the sapwood. This could suggest that the rate of polymerisation is a function of the amount of air contact and therefore oxygen initiation of some type of alkene polymerisation, not necessarily of the Diels-Alder type, involving the conjugated dienes of the monomers.

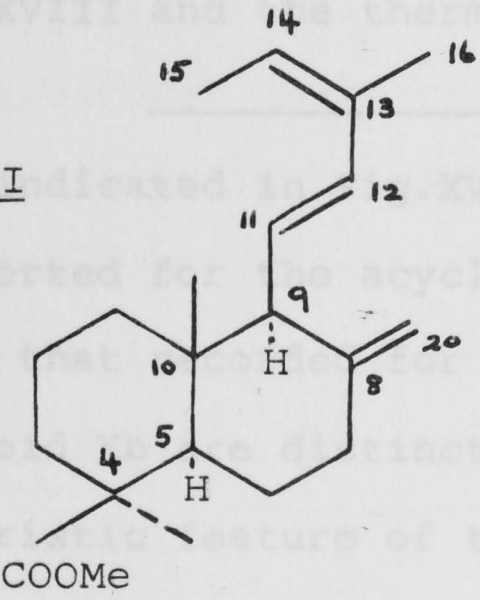
III.6. Isomerisation of Methyl trans-communate on Gas Chromatography

Methyl trans-communate (Xb), after isolation from Callitris and purification by recrystallisation, was subjected to gas chromatography on analytical column A. The chromatogram showed one major and several minor peaks. After condensing the eluted fraction and re-injecting onto column A two major peaks were now apparent. A further collection and re-injection again showed only one major peak with the peak of retention time consistent with that of Xb now absent. The column temperature was 245°, the block and injection port both at 300°. Reducing the column temperature to 230° and the block and injection port to 225° however virtually eliminated the above effect. Methyl trans-communate was therefore apparently undergoing a thermal change on chromatography. The similar retention times of Xb and the thermal product suggested that thermal isomerisation, probably of the triene system, was occurring

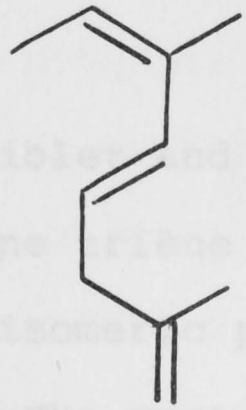
rather than thermal breakdown.

The thermal isomeric product could only be isolated pure in trace quantities due to the small quantities of pure methyl trans-communate available and the considerable drop in yields which resulted from several re-injections onto column A at such high temperatures. These temperatures also resulted in contamination of the isomer with column bleed (Carbowax 20M) which necessitated a further g.c. purification step on column B (SE 30) at lower temperatures. Enough sample however was finally collected for suitable analysis of the isomer.

XXVII

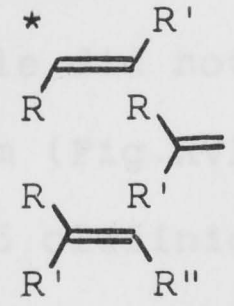


XXVIII



Spectral correlations with 2,6-dimethyl-trans-4(5), cis-6(7)-octatrien-(1,4,6) (XXVIII) and the two diterpenes, methyl trans-communate (Xb) and methyl Δ-13(16)-communate (XVIa), suggested XXVII as the probable structure for the isomer.

	Isomer	XXVIII
Ultraviolet	236.5 mμ	236 mμ
data (λmax.)	log ε 4.34	log ε 4.36
	1782 w	1785 w
Infrared data:	1648 m	1650 s
alkene bands	975 m	970 s
(film; $cm.^{-1}$)	892 s	895 s
	825 w	820 m



*alkene group responsible(82)

Fig.XV. Spectral correlation between monoterpene triene XXVIII and the thermal isomer of Xb.

As indicated in Fig.XV. the ultraviolet and infrared data reported for the acyclic monoterpene triene XXVIII(82, 75), and that recorded for the thermal isomeric product of diterpenoid Xb are distinctly similar. The most characteristic feature of the i.r. spectrum of the isomer was the presence of only one apparent absorption in the 1550-1700 $cm.^{-1}$ region; at least two bands were expected on the basis that u.v. data had clearly indicated the presence of a conjugated diene system. However, an examination of the i.r. spectra of known acyclic monoterpene dienes(82) revealed that this feature was also characteristic of the

monoterpene triene XXVIII; all other related compounds clearly showed the normal two bands. This spectral comparison therefore suggested that the thermal isomer of Xb probably possessed a triene group similar in configuration to that of 2,6-dimethyl-trans-4(5),cis-6(7)-octatrien-(1,4,6) (XXVIII).

Unfortunately the small sample available did not allow a full interpretation of the p.m.r. spectrum (Fig.XVI.) of the isomer. The integral, consistent with 5 olefinic protons between 3.40 and 5.60, a broad singlet at 8.23 (C_{13} Me), and a doublet at 8.36 (C_{14} Me), however, all appeared consistent with that expected for the proposed triene structure. More clearly defined singlets at 6.41, 8.81 and 9.40 could be readily assigned to methyl groups of a diterpenoid methyl ester configuration similar to those of both methyl trans-communate (Xb) and methyl Δ-13(16)-communate (XVIa). The correlation is shown below.

	τ values		
	C_4 COOMe	C_4 Me	C_{10} Me
Thermal isomer	6.41	8.81	9.40
Xb	6.39	8.81	9.44
XVIa	6.42	8.81	9.53

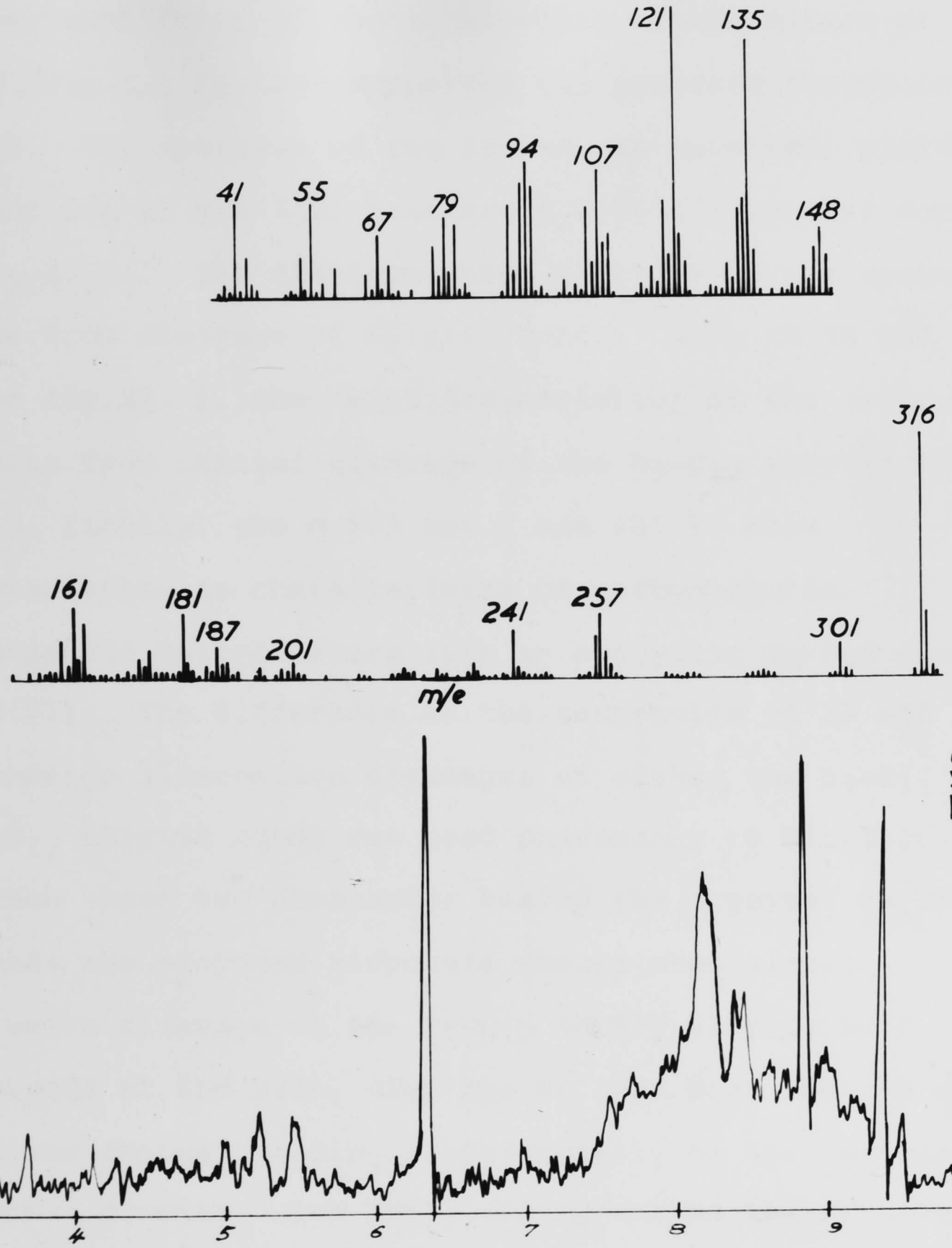
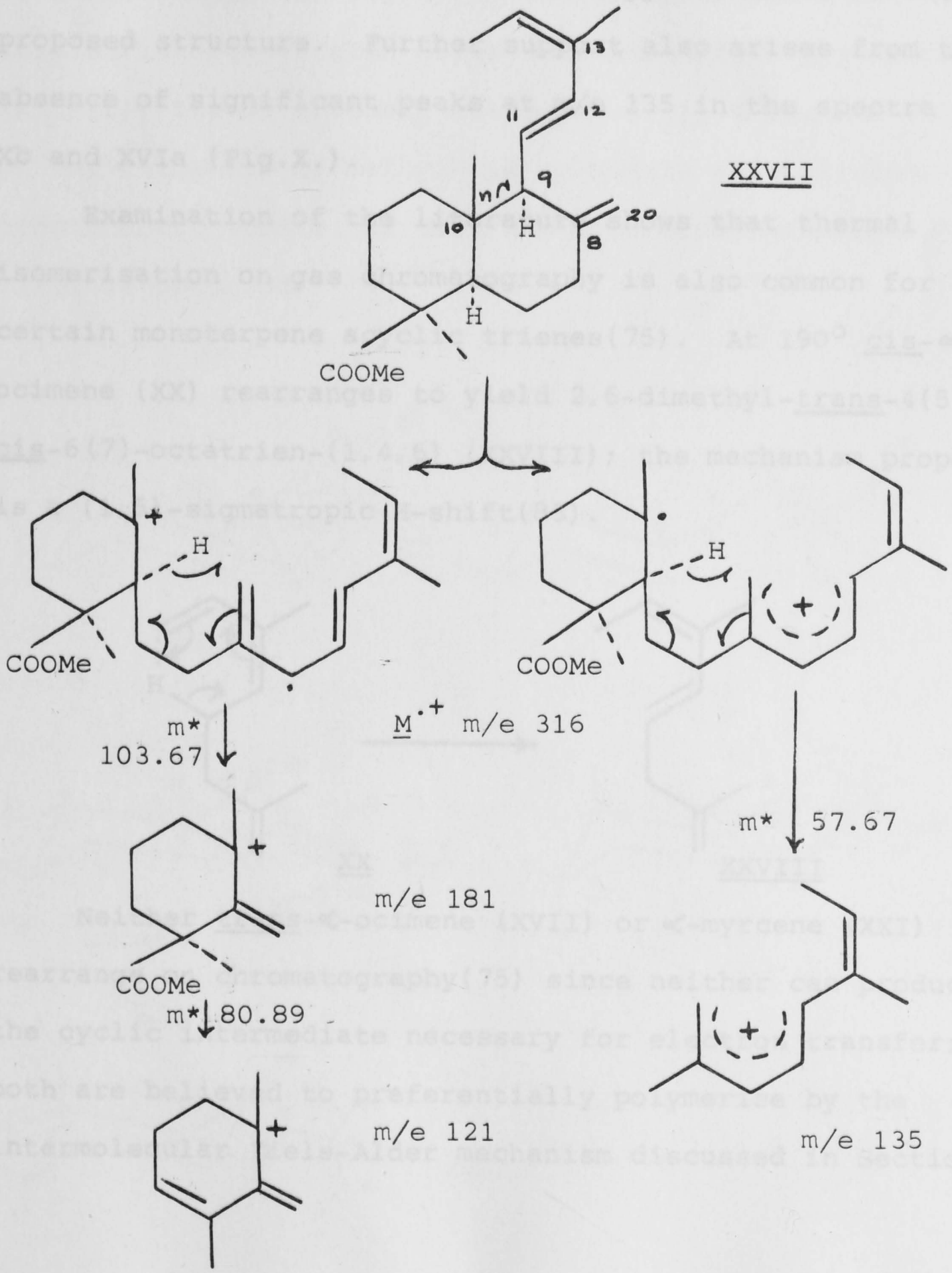


FIG. XVI. P.M.R. and MASS SPECTRA of the THERMAL ISOMER of METHYL TRANS-COMMUNATE (Xb).

A comparison of the mass spectrum (Fig.XVI.) of the isomer with those of the diterpenoid methyl esters Xb and XVIa (Fig.X.) further supported the proposed structure XXVII. The spectrum of the isomer, as expected, yielded a parent ion at m/e 316, consistent with a molecular formula of $C_{21}H_{32}O_2$. The distinguishing features of the spectrum arise from cleavage of allylic bonds. As with Xb and XVIa (see Fig.XI.), the major fragmentation of the isomer results from initial cleavage of the C_9-C_{10} allylic bond to yield, finally, the M-195 ion (m/e 121); this fragmentation is characteristic of carbodicyclic diterpenoid methyl esters with an exocyclic C_8-C_{20} double bond(51). The difference in the tendencies of Xb and XVIa to undergo alternative cleavages of either the C_9-C_{11} or C_{11}-C_{12} allylic bonds was used previously to distinguish between these two compounds; basing the argument on these grounds, the proposed structure should show almost exclusive cleavage of the C_9-C_{10} doubly allylic bond. With compounds Xb and XVIa, cleavage of this bond results in the positive charge residing preferentially on the tertiary C_{10} carbon. However, with XXVII the C_9 carbon is now situated alpha to two double bonds and a positive charge at C_9 would result in a most stable ion. Therefore in XXVII, cleavage of the C_9-C_{10} bond should result in the positive charge

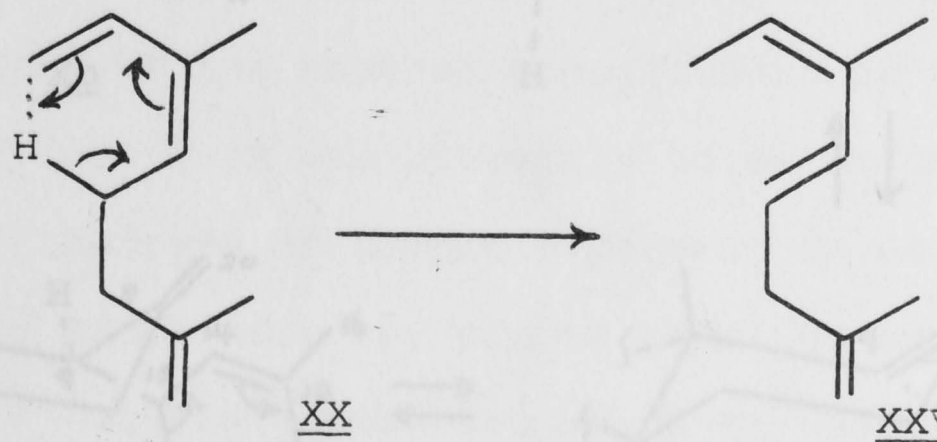
being shared between both C_9 and C_{10}; normal fragmentation via a cyclic mechanism(51) should then yield, as shown below, peaks at m/e 121 and m/e 135.



XXVII

The definite presence of both peaks m/e 121 and m/e 135, and the absence of significant peaks arising from cleavage of the C_9-C_{11} or C_{11}-C_{12} bonds, in the mass spectrum of the isomer, therefore supports XXVII as the proposed structure. Further support also arises from the absence of significant peaks at m/e 135 in the spectra of Xb and XVIa (Fig.X.).

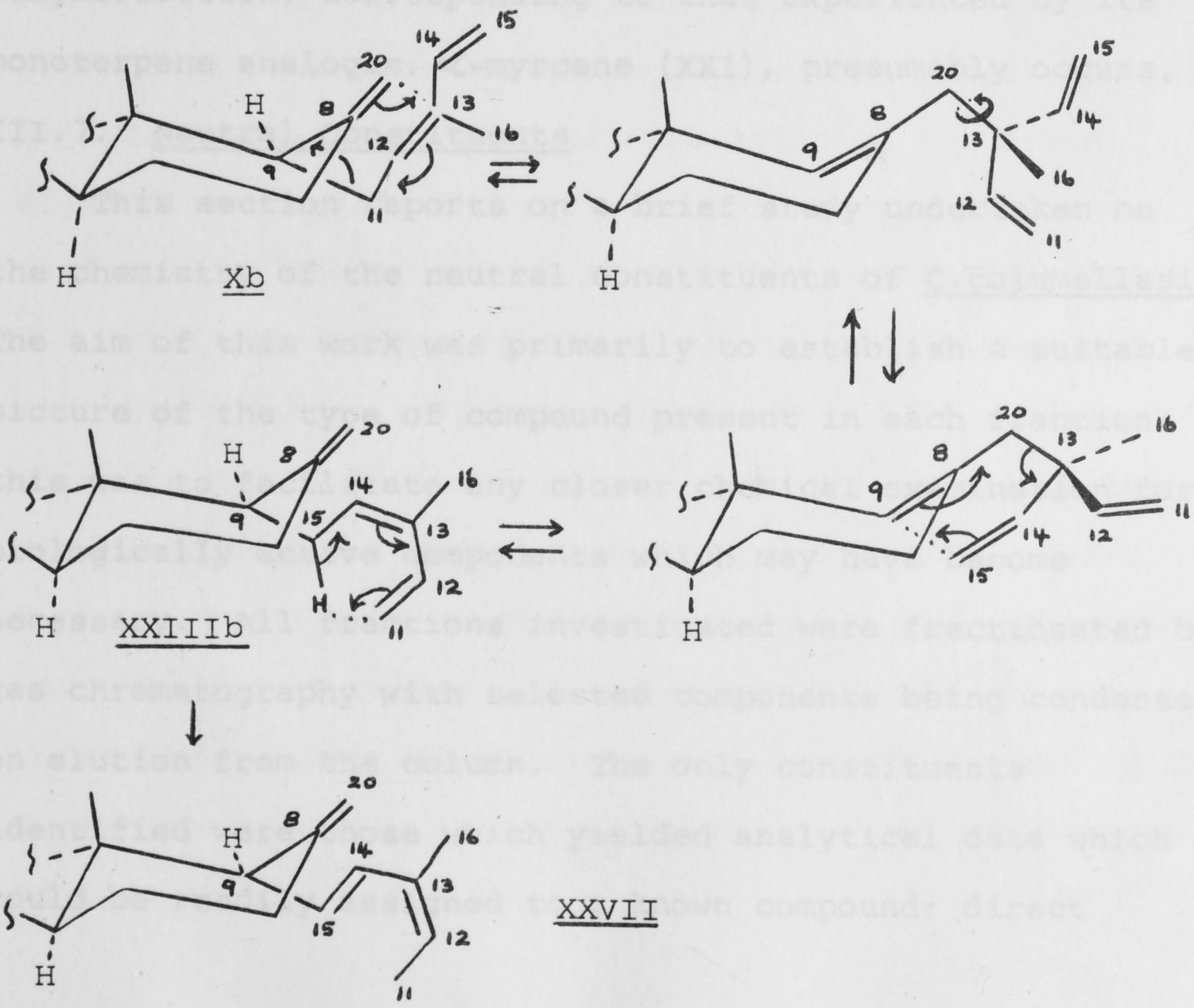
Examination of the literature shows that thermal isomerisation on gas chromatography is also common for certain monoterpene acyclic trienes(75). At 190° cis-∝-ocimene (XX) rearranges to yield 2,6-dimethyl-trans-4(5), cis-6(7)-octatrien-(1,4,6) (XXVIII); the mechanism proposed is a (1,5)-sigmatropic H-shift(83).



XX XXVIII

Neither trans-∝-ocimene (XVII) or ∝-myrcene (XXI) rearrange on chromatography(75) since neither can produce the cyclic intermediate necessary for electron transfer; both are believed to preferentially polymerise by the intermolecular Diels-Alder mechanism discussed in Section

II.5. The results just discussed, however, show that methyl trans-communate (Xb), the diterpenoid analogue of XVII, does rearrange on gas chromatography. A Cope mechanism, depicted below, is therefore proposed for an initial interconversion between Xb and methyl cis-communate (XXIIIb), the diterpenoid analogue of cis-α-ocimene (XX). On the formation of methyl cis-communate a (1,5)-sigma-tropic shift occurs, as with the monoterpene analogue, and XXVII is produced.



With trans-∝-ocimene (XVII), the monoterpene analogue of Xb, rotation about single bonds is more random and the possibility of the formation of a suitable Cope intermediate is unlikely. With Xb, however, the fixed stereochemistry about C_8 and C_9 makes a Cope quite probable. Methyl Δ-13(16)-communate (XVIa), which does not possess a 1,5-diene structure, would not be expected to undergo rearrangement on gas chromatography. A similar experiment to that which detected isomerisation of Xb on gas chromatography only showed a drop in peak height with XVIa. Polymerisation, corresponding to that experienced by its monoterpene analogue, ∝-myrcene (XXI), presumably occurs.

III.7. Neutral Constituents

This section reports on a brief study undertaken on the chemistry of the neutral constituents of C.columellaris. The aim of this work was primarily to establish a suitable picture of the type of compound present in each fraction; this was to facilitate any closer chemical examination for biologically active components which may have become necessary. All fractions investigated were fractionated by gas chromatography with selected components being condensed on elution from the column. The only constituents identified were those which yielded analytical data which could be readily assigned to a known compound; direct

comparisons with authentic samples, if available, then followed.

(i) Sapwood neutral fraction

Gas chromatography on column A of the total neutral fraction of Callitris sapwood (Fig.XVII.-Chart 4) showed one major component, corresponding to peak 4F; g.c. conditions however were such that compounds with retention times greater than those characteristic of diterpenoid methyl esters were not readily eluted from the column. The major component gave a mass spectrum (parent ion at m/e 222 and a strong M-18 peak) indicative of a sesquiterpene alcohol of molecular formula $C_{15}H_{26}O$. The p.m.r. spectrum (no peaks consistent with protons attached to carbon adjacent to oxygen) and i.r. spectrum (ν_{max} 3582 and 1125 $cm.^{-1}$) further suggested that the alcohol was tertiary. The p.m.r. spectrum also showed two equivalent methyl singlets (8.82), two methyl doublets (9.03, J=6.5; 9.00, J=6.5), and no olefinic protons. On this evidence the structure of the tertiary sesquiterpene alcohol, guaiol (I), a known constituent of the heartwood of C.columellaris(43) was assigned as probable. A direct comparison of the physical and spectral properties of sesquiterpenoid 4F and an authentic sample of guaiol showed the two compounds to be identical.

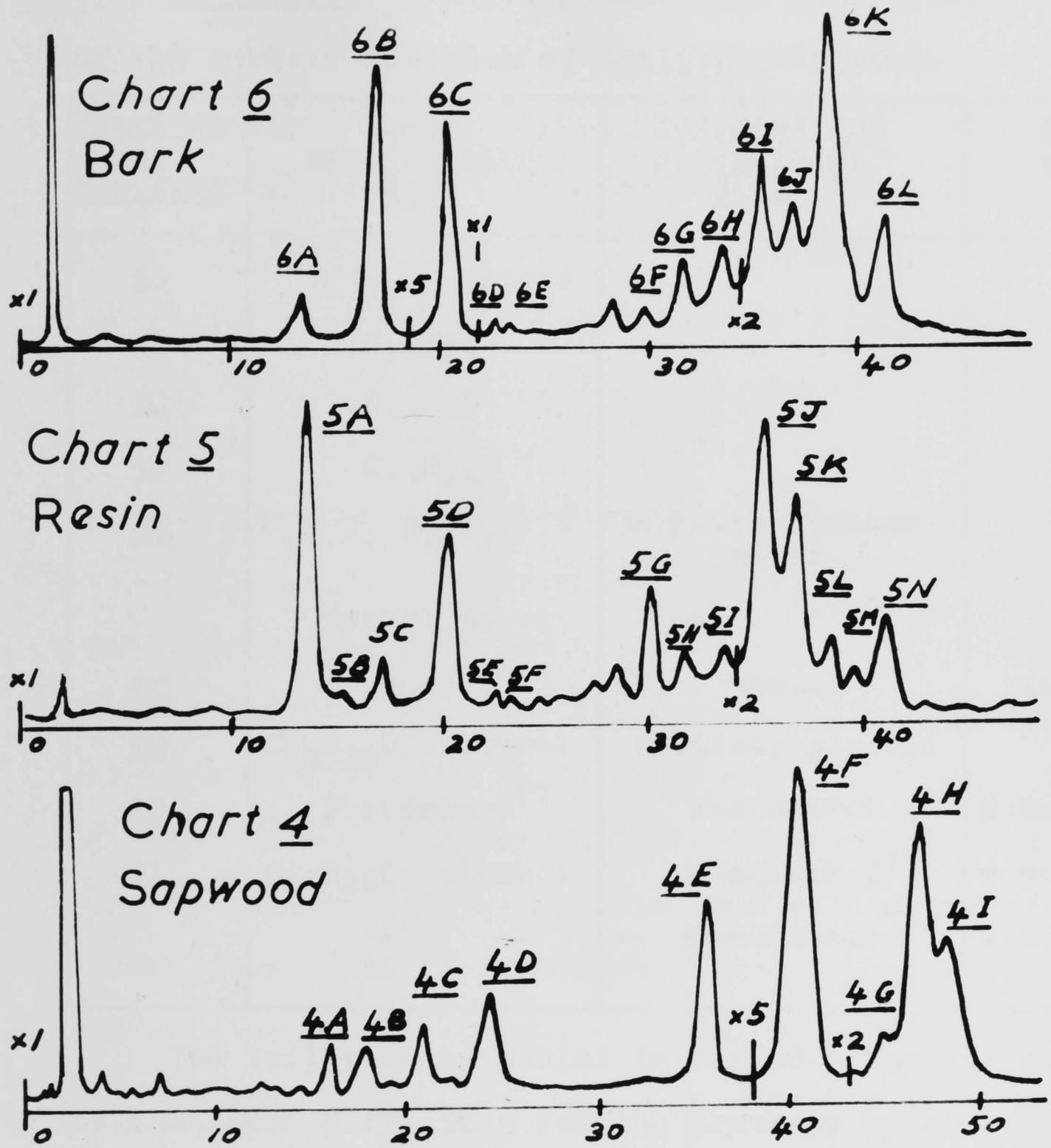


FIG. XVII. GAS CHROM'GRAMS OF NEUTRAL AND STEAM VOLATILE FRACTIONS OF CALLITRIS.

Fig.XVIII. Analytical data and identified compounds of the neutral fraction of Callitris sapwood.

Fraction No. Fig.XVII.	Mass spectrometry data	Infrared and ultraviolet data	G.c. data
4A	$C_{10}H_{16}O$, ketone		
4B	$C_{15}H_{24}$		
4C	$C_{15}H_{24}$		
4D	$C_{15}H_{24}$		
4E	$C_{11}H_{16}O_2$, methyl ester	α,β-unsaturated ester, $\lambda_{max.}$ 311.5 mμ	
4F	guaiol[I]	guaiol	guaiol
4G	$C_{15}H_{26}O$, alcohol	tertiary alcohol	
4H	γ-eudesmol[VI]	γ-eudesmol	γ-eudesmol
4I	$C_{15}H_{26}O$, alcohol	α-eudesmol[IV] + β-eudesmol[V]	α-eudesmol or β-eudesmol

The isolation of guaiol initiated a further examination of the sapwood fraction for the presence of other known heartwood constituents. The compounds identified, together with analytical data, are shown in Fig.XVIII.; most of these compounds appeared to correspond to the products isolated from the heartwood by Rudman(43). Cryptomeridiol (VII), however, was not detected, probably because of its relatively high retention time on Carbowax 20M(43).

The ultraviolet and infrared ($\nu_{max.}$ 1715, 1643, 1610, 960 $cm.^{-1}$) data for the compound corresponding to peak 4E appear consistent with those reported ($\lambda_{max.}$ 311 mμ; $\nu_{max.}$ $CHCl_3$: 1700, 1634, 1602, 950 $cm.^{-1}$)(84) for the methyl ester of 3,7-dimethylocta-2,trans-4,6-trienoic acid (III), a constituent of the volatile wood oil of C.columellaris (42); an authentic sample, however, was not available for a direct comparison.

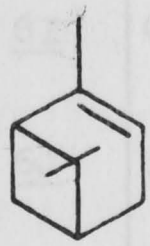
The presence of both eudesmol isomers, in the mixture corresponding to peak 4I, was indicated by infrared spectroscopy(85). All bands visible in an i.r. spectrum of an authentic mixture of α- and β-eudesmol were also evident in the sapwood sample; bands at 3020 and 1653 $cm.^{-1}$ appeared characteristic of the trisubstituted alkene of the α-isomer (IV), and bands at 3075, 1645 and 892 $cm.^{-1}$ characteristic of the gem-disubstituted alkene of the β-isomer(V). The g.c. retention times of the two isomers of the authentic mixture were shown to be identical, and the same as that of peak 4I.

(ii) Steam volatile fractions

The constituents of the steam volatile oils of the bark and oleoresin were shown to be extremely similar by gas chromatography on column A (Fig.XVII.-Charts 6 and 5 respectively). Only a variation in the relative

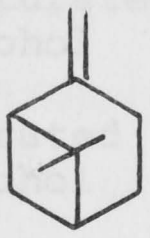
concentration of constituents was really apparent. The compounds identified, with relevant analytical data, are shown in Fig.XXIII.

The condensed material corresponding to peak 5D of the oleoresin gave a mass spectrum almost superimposable on that of 1,8-cineole (XXXIII); however a more intense peak than expected for pure XXXIII at m/e 68 indicated that peak 5D represented a mixture. Coinjection of 1,8-cineole and limonene (XXXII), which shows a base peak at m/e 68 in the mass spectrum(86), yielded only a single peak on column A; a good separation was obtained however on column C (LAC 446). Injection then of the mixture 5D on column C yielded pure limonene (~10%), eluted first, and pure 1,8-cineole (~90%). Limonene could not be detected by this means in the bark fraction; this was consistent with the

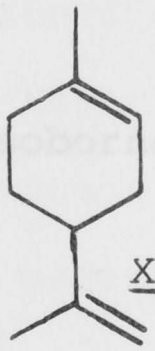


XXIX

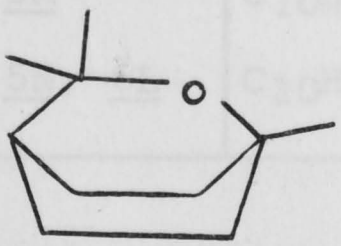
XXX



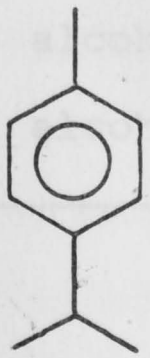
XXXI



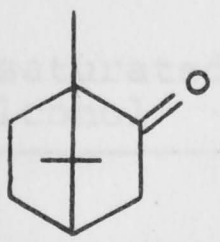
XXXII



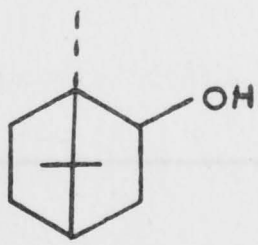
XXXIII



XXXIV



XXXV



XXXVI

Fig.XXIII. Analytical data and identified compounds of the steam volatile fractions of the bark[6] and oleoresin[5] of Callitris.

Fraction No. Fig.XVII.	Mass spectrometry data	Infrared and ultraviolet data	G.c. data
5A, 6A	∝-pinene XXIX	∝-pinene	∝-pinene
5B	camphene XXX		camphene
5C, 6B	β-pinene XXXI		β-pinene
5D	limonene XXXII		limonene
5D, 6C	1,8-cineole XXXIII		1,8-cineole
5E, 6D	p-cymene XXXIV		p-cymene
5F, 6E	cymene		
5G, 6F	$C_{10}H_{16}O$	unsaturated aldehyde	
5H, 6G	camphor XXXV		camphor
5I, 6H	$C_{10}H_{18}O$, alcohol		
5J, 6I	$C_{10}H_{16}O$, alcohol	unsaturated alcohol	
5K, 6J	isoborneol XXXVI	saturated alcohol	isoborneol
5L, 6K	$C_{10}H_{14}O$, ketone	∝,β-unsaturated ketone λmax. 252 mμ	
5M	$C_{10}H_{16}O$, alcohol		
5N, 6L	$C_{10}H_{14}O$, alcohol	unsaturated alcohol	

mass spectrum of 6C being identical to that of pure 1,8-cineole.

The monoterpene hydrocarbons identified in both fractions generally yielded single peaks on re-injection onto columns B (SE30) and C; any minor constituents which were apparent appeared to be in too low a yield for successful analysis. The oxygen-containing monoterpenes were mainly examined to determine functional groups present and to show the correlation between the constituents of the bark and the oleoresin; data reported for these constituents is therefore for that material condensed directly from column A.

It was later shown that the g.c. trace of the sapwood steam volatile oil differed considerably from those of the bark and oleoresin fractions; instead, it was virtually identical to that of the total neutral fraction shown in Fig.XVII. The only significant difference was a higher concentration of C_{10} compounds, with α-pinene, β-pinene, 1,8-cineole and p-cymene being identified as the major components in this group.

(iii) Bark neutral fraction

Gas chromatograms of both the bark and oleoresin total neutral fractions appeared too complex for any direct g.c. separation of constituents. The bark fraction was therefore

first chromatographed on an alumina column using n-hexane, n-hexane-ethanol (1:1 by volume) and ethanol as eluants. This was expected to yield a separation of hydrocarbon and oxygenated components(87). Fifteen fractions were collected and each subjected to gas chromatography on column A. Although overlapping of constituents between fractions was apparent, gas chromatography indicated a reasonable separation of hydrocarbon and oxygenated components as well as a further partial separation of the mono-, sesqui- and diterpene hydrocarbons. Of these fifteen fractions only two were investigated further.

Fraction 13

On gas chromatography of fraction 13 (1:1 n-hexane-ethanol as eluant) only a small amount of injected sample was eluted successfully from column A. This indicated that it consisted mainly of higher molecular weight oxygen-containing compounds, the corresponding alcohols and aldehydes(49) of the already characterised diterpenoid acids probably predominating. Injection of larger samples was required to observe the more volatile minor constituents (Fig.XIX.-Chart 7), whose retention times appeared consistent with a mixture of mono- and sesqui-terpenoids.

An interesting phenomenon arose when an attempt was

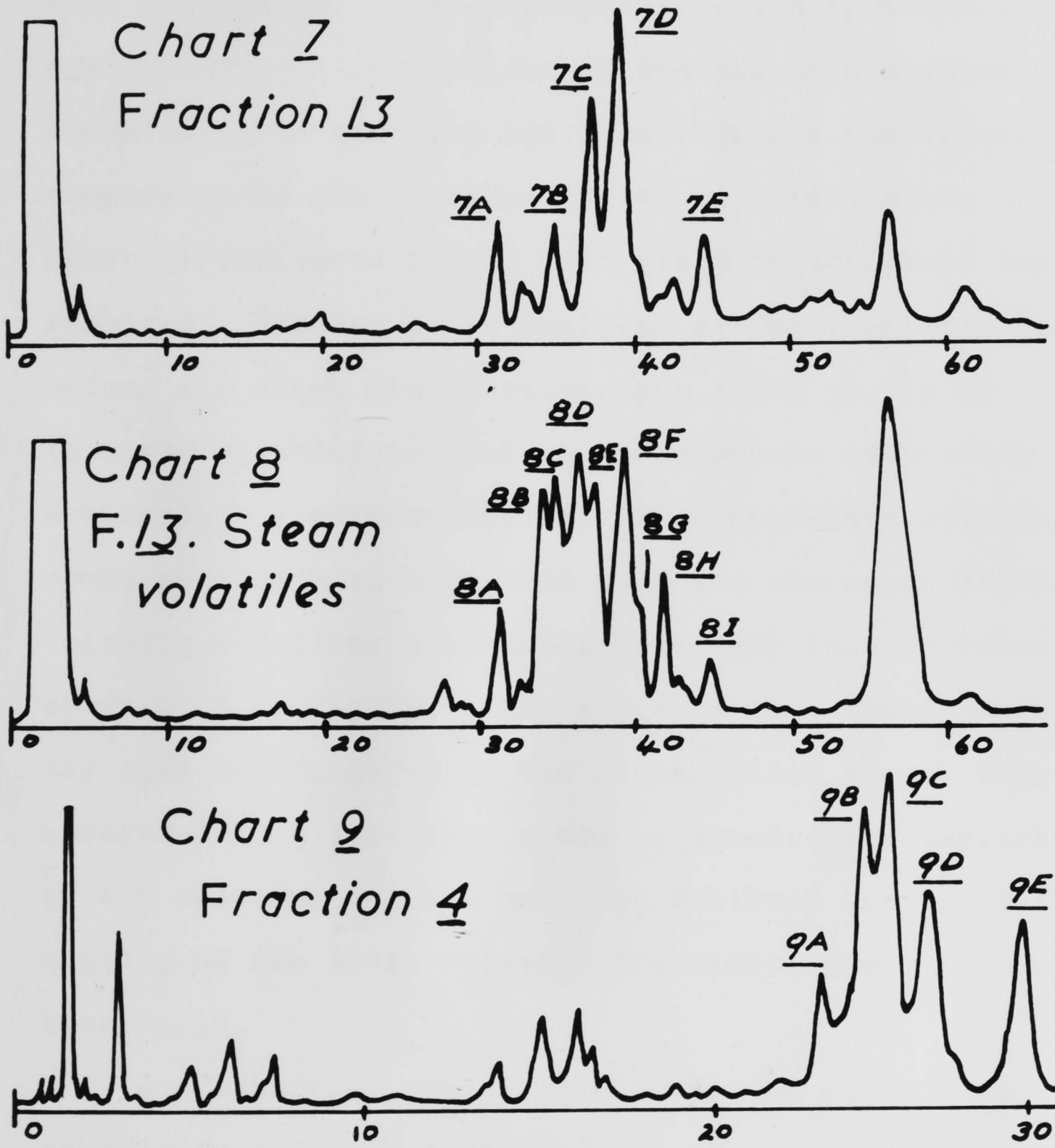


FIG. XIX. GAS CHROM'GRAMS OF NEUTRAL FRACTIONS OF CALLITRIS BARK.

made to separate these more volatile constituents by steam distillation. A comparison of the gas chromatograms of the steam volatile oil (Fig.XIX.-Chart 8) and the volatile components of the fractions prior to distillation (Fig.XIX.-Chart 7) indicated that a high yield of artifacts had resulted. Results of an analysis of the compounds present, before and after distillation, are shown in Fig.XX. The interesting point is that those compounds identified as artifacts all gave analytical data consistent with major components of both the crude bark and oleoresin steam volatile fractions previously examined; this correlation is shown also in Fig.XX. This suggests that these compounds may also be artifacts of the distillation step. This observation is important since it immediately implies that if any relevant results had been achieved during biological testing of the steam volatile fractions, they may not have been valid.

Mass spectral data recorded for the major components of fraction 13 also indicated the presence of two mono-terpenoid methyl esters, corresponding to peaks 7C and 7E. Neither compound had been previously detected in the steam volatile oil of the crude bark; this suggested a possible conversion to artifacts on steam distillation. An apparent drop, on steam distillation of fraction 13, in the relative

Fig.XX. Analytical data and identified compounds of bark fraction 13, before and after steam distillation; the corresponding crude bark and resin steam volatile components are also shown.

Fraction No. Figs.XIX. and XVII	Mass spectrometry data	Infrared and ultraviolet data	G.c. data
7A, 8A, 5H, 6G	camphor[XXXV]		camphor
8B*, 5J, 6I	$C_{10}H_{16}O$, alcohol	unsaturated alcohol	
7B, 8C	$C_{10}H_{16}O$, alcohol		
8D*, 5K, 6J	isoborneol[XXXVI]	saturated alcohol	isoborneol
7C, 8E	$C_{11}H_{16}O_2$, methyl ester	conjugated diene, λmax. 235 mμ	
7D, 8F, 5L, 6K	$C_{10}H_{14}O$, ketone	α,β-unsaturated ketone λmax. 252 mμ	
8G, 5M	$C_{10}H_{16}O$, alcohol		
8H*, 5N, 6L	$C_{10}H_{14}O$, alcohol	unsaturated alcohol	
7E, 8I	$C_{11}H_{16}O_2$, methyl ester		methyl perillate XXXVIIb

* Artifacts

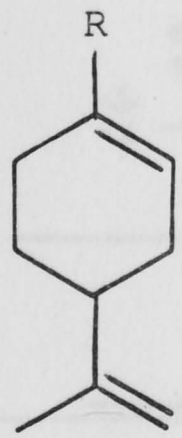
7: Fraction 13

8: Steam volatile oil of fraction 13

6: Steam volatile oil of crude bark

5: Steam volatile oil of crude oleoresin

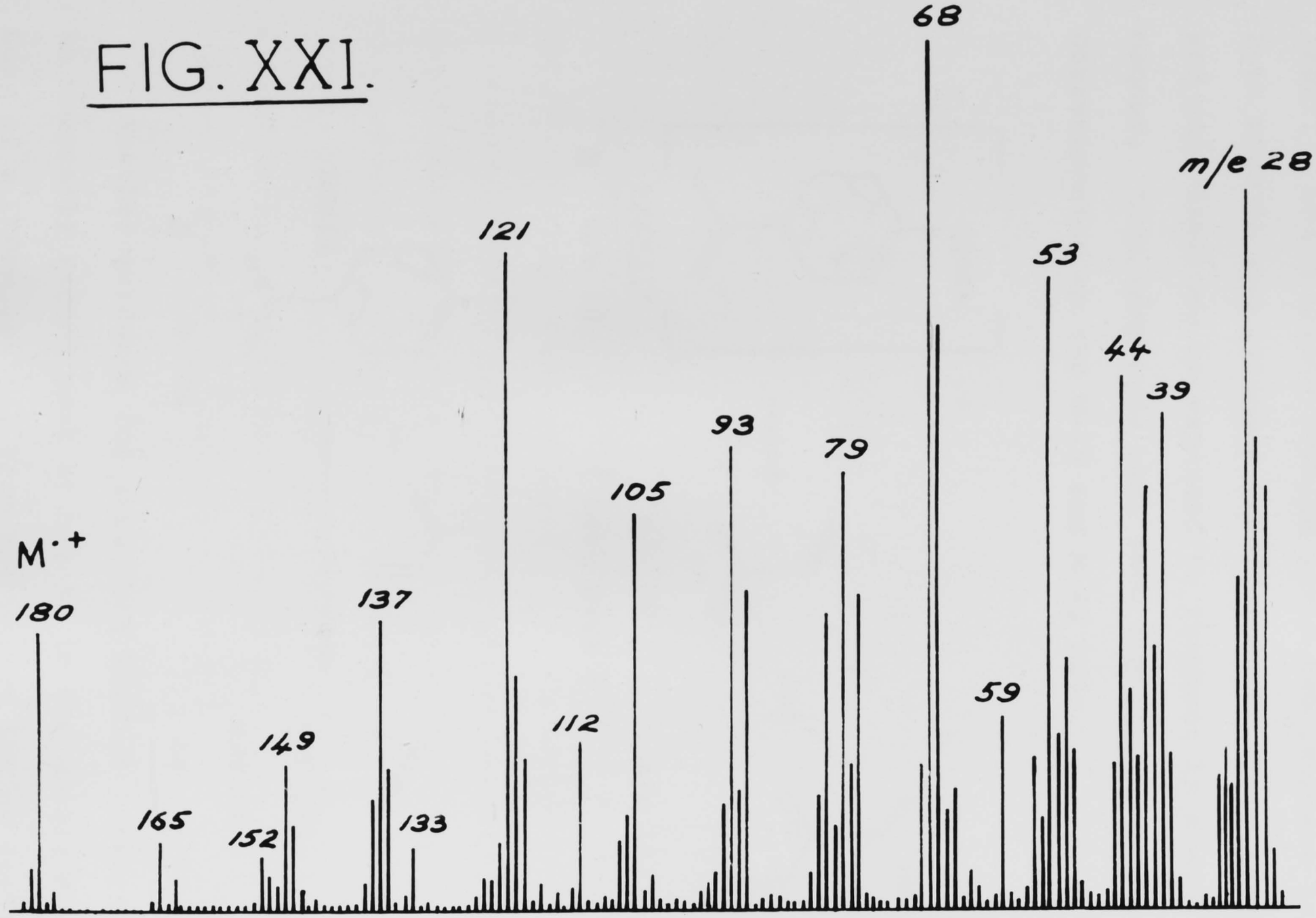
intensities of the g.c. peaks corresponding to these two compounds was further indicative of this possibility.



		R
XXXVII	a.	COOH
	b.	COOMe

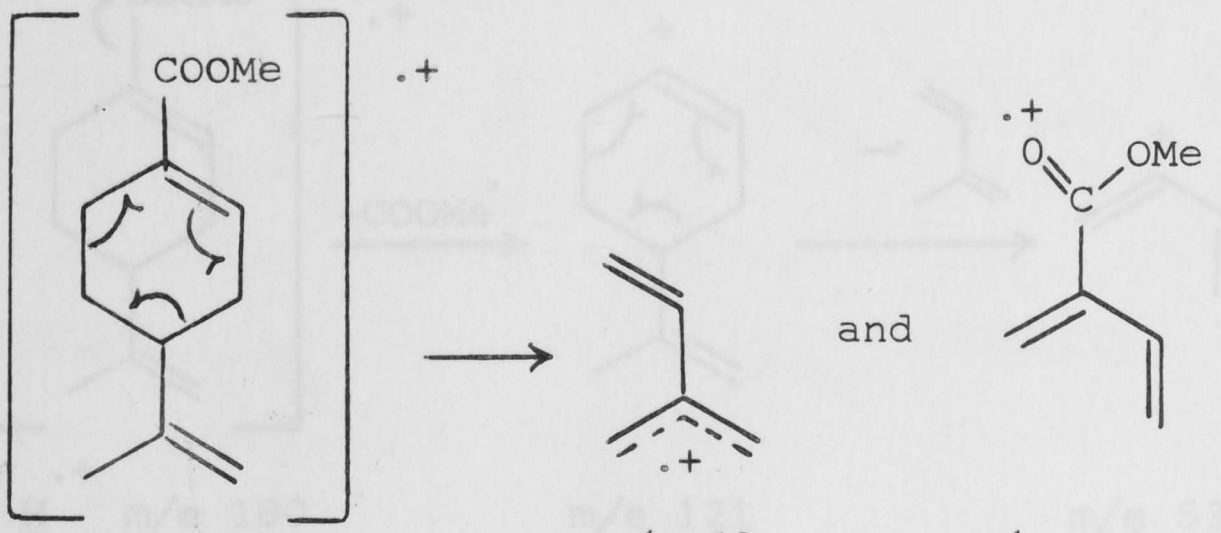
Of the two methyl esters only 7E was identified with certainty. With evidence based on the fragmentation pattern in the mass spectrum (Fig.XXI.), the structure of methyl perillate (XXXVIIb) was assigned as being probable for that of 7E. The mass spectrum showed a parent ion at m/e 180, consistent with a molecular formula of $C_{11}H_{16}O_2$; peaks corresponding to cleavage from the parent ion of moieties -OMe (m/e 149 and 31) and -COOMe (m/e 121 and 59) were further indicative of a methyl ester. Peaks at m/e 112 and 68 (base peak) however appeared most characteristic and it was the fragmentations which gave rise to these on which the assignment of the above structure was based. Cyclic fragmentation, involving a retro-Diels-Alder mechanism, of the doubly allylic C-C single bond of XXXVIIb, as shown, would yield both m/e 68 and m/e 112 peaks. This type of fragmentation is characteristic of the monoterpene hydrocarbon, limonene

FIG. XXI.



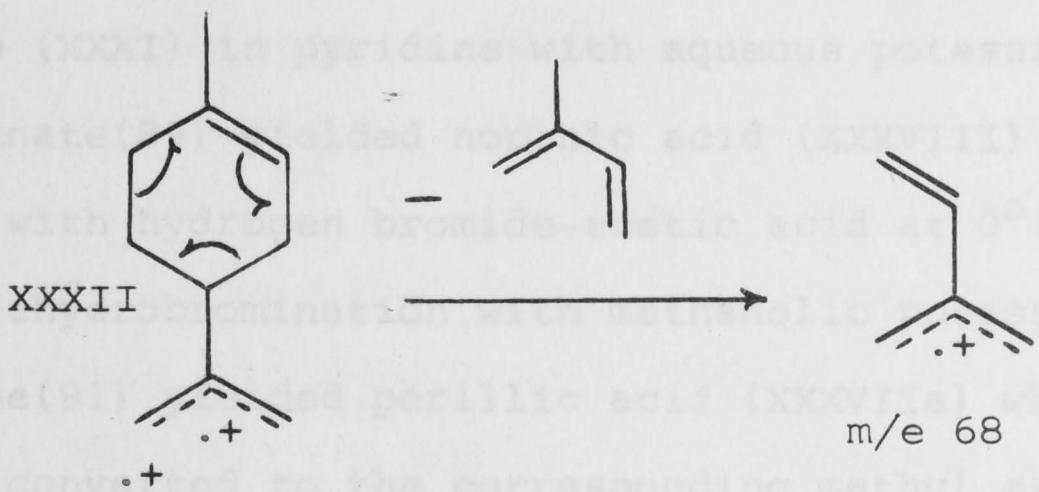
MASS SPECTRUM OF ESTER ZE.

(XXXII), which breaks down to yield two isoprene units(88); both XXXVIIb and limonene have identical diene structures and can therefore be expected to fragment in a similar manner. Both compounds also show significant peaks corresponding to the M-15 and M-43 ions.



XXXVIIb m/e 68 m/e 112

M .+ m/e 180 (base peak)

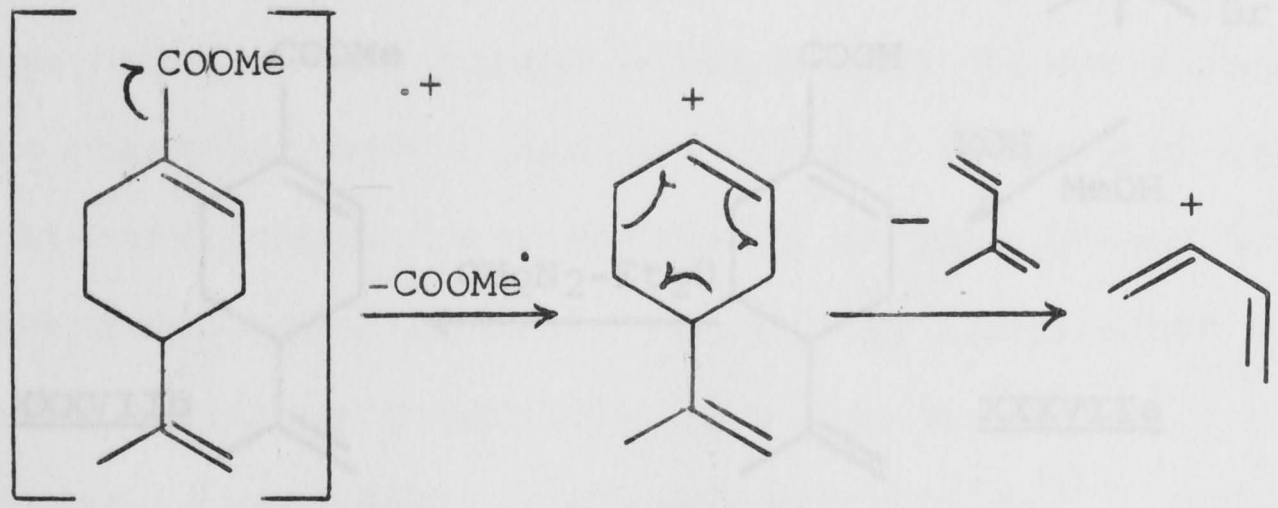


m/e 68

M .+ m/e 136 (base peak)

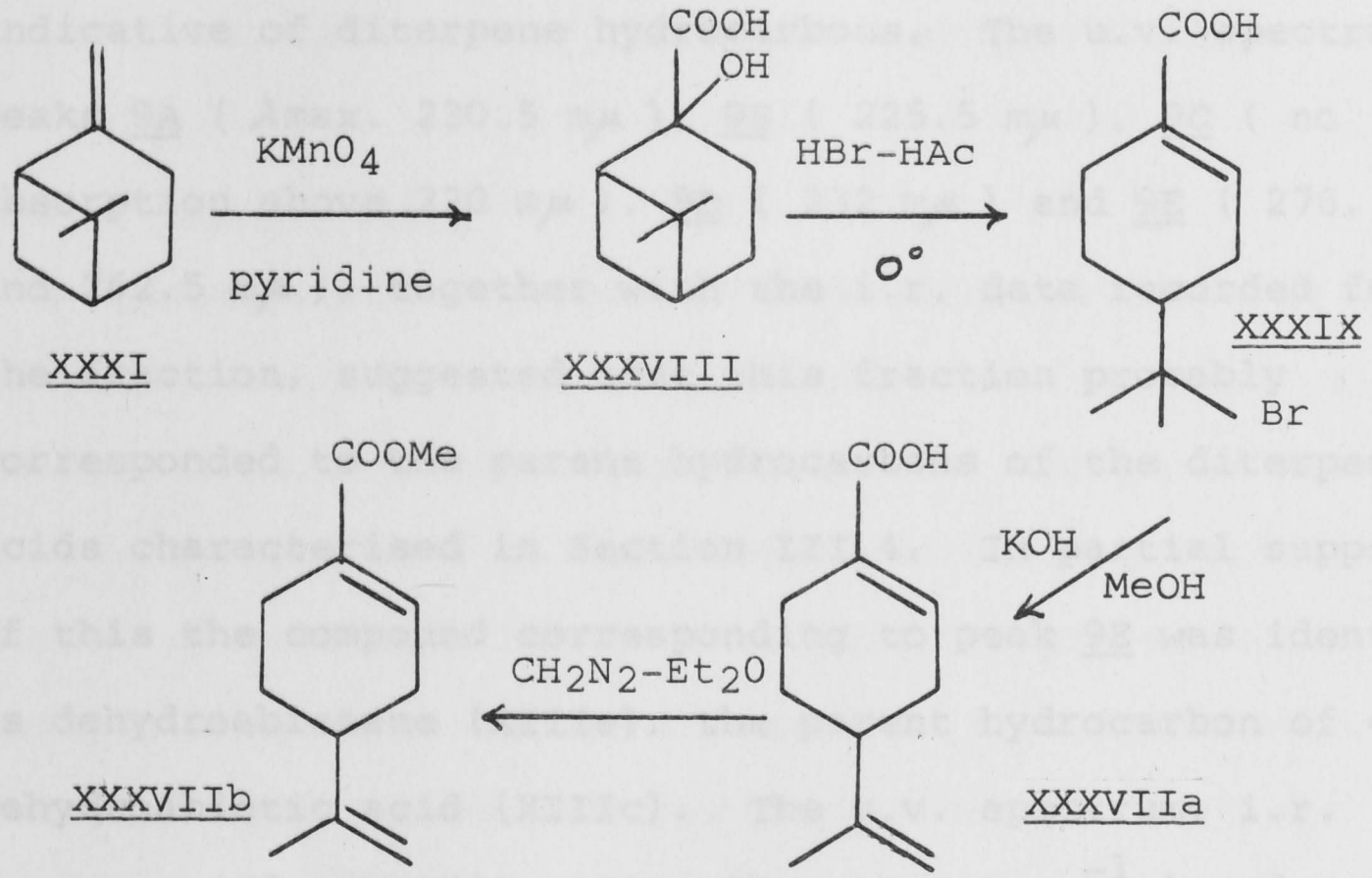
Further evidence for structure XXXVIIb is furnished by an unusually strong peak at m/e 53 in the spectrum of 7E. Loss of a COOMe radical from the parent ion of methyl

perillate would yield the M-59 (m/e 121) ion; this ion would then be expected to undergo a retro-Diels-Alder fragmentation of the doubly allylic C-C single bond to yield a strong ion at m/e 53.



M .+ m/e 180 m/e 121 m/e 53

As final proof of the assigned structure, an authentic sample of methyl perillate was prepared; oxidation of β-pinene (XXXI) in pyridine with aqueous potassium permanganate(89) yielded nopinic acid (XXXVIII) which, when treated with hydrogen bromide-acetic acid at 0^{o}(90), gave XXXIX; dehydrobromination with methanolic potassium hydroxide(91) yielded perillic acid (XXXVIIa) which was further converted to the corresponding methyl ester, methyl perillate (XXXVIIb), with ethereal diazomethane. Compound 7E was then identified as methyl perillate by a direct comparison of g.c. retention time and mass spectrum with those of the authentic sample.



Fraction 4

An i.r. spectrum of fraction 4 (n-hexane as eluant) was indicative of a mixture of hydrocarbons(92) with absorptions arising from C-H stretching (2950 and 2870 $cm.^{-1}$), $-CH_2-$ bending (1462 $cm.^{-1}$) and $C-CH_3$ bending (1462 and 1380 $cm.^{-1}$). Unsaturation was also evident with bands resulting from C-C double bond stretching (1640 $cm.^{-1}$), alkene C-H stretching (3080 $cm.^{-1}$) and alkene C-H bending (883, 903 and 985 $cm.^{-1}$). Conjugation of C-C double bonds (1607 and 1598 $cm.^{-1}$) and aromatic character (1502 and 818 $cm.^{-1}$) were also evident.

Retention times of the major peaks (9A to 9E), depicted by gas chromatography (Fig.XIX.-Chart 9), were

indicative of diterpene hydrocarbons. The u.v. spectra of peaks 9A (λmax. 230.5 mμ), 9B (225.5 mμ), 9C (no absorption above 220 mμ), 9D (232 mμ) and 9E (276, 268 and 262.5 mμ), together with the i.r. data recorded for the fraction, suggested that this fraction probably corresponded to the parent hydrocarbons of the diterpenoid acids characterised in Section III.4. In partial support of this the compound corresponding to peak 9E was identified as dehydroabietane (XIIIe), the parent hydrocarbon of 4-epi-dehydroabietic acid (XIIIc). The u.v. spectrum, i.r. spectrum (νmax. 1609, 1502, 886 and 818 $cm.^{-1}$) and p.m.r. spectrum (3.10, s, 1H; 2.87, AB q, 2H, J=5.5) were again indicative of a 1,2,4-trisubstituted aromatic compound. The mass spectrum showed a parent ion at m/e 270 consistent with a molecular formula of $C_{20}H_{30}$. This spectral evidence was sufficient to suggest XIIIe as the possible structure for hydrocarbon 9E; it was finally identified as dehydro-abietane by a direct comparison of the g.c. retention time and spectral data with those of an authentic sample, prepared according to the method of Carman and Deeth(70). Reduction of methyl 4-epi- dehydroabietate (XIIId) with lithium aluminium hydride yielded 4-epi-dehydroabietol (XIIIf) which was then oxidised to 4-epi-dehydroabietal (XIIIg) with chromium trioxide-pyridine; dehydroabietane

(XIIIe) was obtained by the Wolff-Kishner reduction of the aldehyde.

III.8. Experimental

Experimental or spectral data reported fully in previous sections is not repeated in this section. All methylations were with diazomethane(94), prepared from nitrosomethylurea à la Vogel(95). Unless otherwise stated, p.m.r. spectra were run in deuterochloroform with tetramethylsilane as an internal standard, i.r. spectra in carbon tetrachloride (1 mm. matched cavity cells) with a polystyrene reference, and u.v. spectra in 90% ethanol (1 cm. silica cells) with Holmium as reference. Other i.r. spectra were run as nujol mulls or films between NaCl plates.

P.m.r. spectra were normally run on a 60 Mc. Perkin-Elmer R.10 instrument; for smaller samples this instrument was used in connection with a Digital PDP-8/S computer. A Varian A.60 instrument in connection with a C.A.T. (C-1024) was also used for small samples; this instrument was made available by C.S.I.R.O. in Canberra. I.r. spectra were run on either a Unicam SP200 or SP200-G instrument; u.v. spectra were measured with a Unicam SP800 instrument. Mass spectra of mono- and sesquiterpenoids were run on an AEI MS10-C2 mass spectrometer with a heated inlet system. The less

volatile diterpenoids were run on AEI MS9 mass spectrometers, with direct inlet systems, made available by both Sydney University and the Research School of Chemistry, A.N.U.; these latter two instruments were also used for accurate mass determinations of samples.

Analytical g.c. was done on either an F.&M. 500 Temperature Programmed, or an Aerograph A90-P3, gas chromatograph; both instruments have thermal conductivity detectors. Samples collected from columns attached to these instruments were trapped in U-shaped melting point capillaries; cooling of traps was either by air, ice-water or liquid nitrogen, depending on the volatility of the eluted sample. Preparative g.c. was run on an Aerograph Autoprep 705 with a flame ionisation detector; standard preparative traps, cooled in ice-water, were used. G.c. columns used are listed below.

Column A: 12'x3/16"o.d. 20% Carbowax 20M on 60-100 sieved Embacel.

Column B: 5'x¼"o.d. 20% SE30 on 60-80 Chromosorb W.

Column C: 12'x3/16"o.d. 20% LAC-446 on 80-100 S.500.

Column D: 6'x3/8"o.d. 20% Carbowax 20M on 60-100 sieved Embacel.

Direct comparisons of g.c. retention times were made by co-injection of samples on relevant columns. The

relative amounts of samples represented by g.c. peaks were determined by weighing the paper contained within each peak.

If not otherwise stated, authentic samples were directly available within the Department in which the work was undertaken.

C,H analysis of compounds was carried out by the Australian Microanalytical Service at the University of Melbourne.

Extractives of Callitris

Three logs (~2'x1' diameter) and 200 g. of crude, brittle oleoresin of C. columellaris (inland form) were obtained from Beni S.F., Dubbo, in north-western N.S.W. All material was stored at -5° as soon as possible after collection until required. The logs were stripped of bark and the sapwood then removed by axe. A fine sawdust (3 mm. mesh) of both the bark and sapwood was prepared using a Wiley mill. Extractions of the sawdust were carried out on 50 g. (fresh weight) samples with 250 ml. solvent for one hour at reflux temperatures. Solvents used and the yields obtained are shown in the accompanying table. The crude oleoresin was completely soluble in warm diethyl ether. Steam distillations were carried out, on 500 g. samples of bark and sapwood and a 50 g. sample of oleoresin, at atmospheric pressure over a period of 3 hours.

Callitris Extractives

as a Percentage of Fresh Weight

	Bark	Sapwood	Oleoresin
Water	13.20	0.72	-
Ethanol	29.34	0.62	-
Methanol	30.00	0.74	-
Diethyl ether	8.58	0.10	100.00
Acetone	20.40	0.50	-
Chloroform	11.46	0.12	-
Benzene	6.74	0.08	-
60/80 petroleum	4.10	0.15	-
Steam volatile	0.04	<0.01	0.21

Larger quantities of bark (4.5 Kg.) and sapwood (5 Kg.) sawdust were extracted, in Kg. lots, with 2.5 l. quantities of 60/80 petroleum spirit. Oleoresin (40 g.) was dissolved in diethyl ether (200 ml.). Each of the three extracts was then treated with n-hexane, cooled to -5^{o}, and the polymeric material filtered off. Acidic constituents were removed from the non-polymeric extracts by extraction with 3% aqueous NaOH, acidification with 20% phosphoric acid and ether extraction. Ether extraction of the basic solutions yielded the neutral constituents. The relative yields of n-hexane insoluble materials, acids and

neutrals are shown below.

Callitris Extractives

as a Percentage of Total Extract

	Bark	Sapwood	Oleoresin
n-Hexane insolubles	5.7	0.5	54.4
Acids	74.6	36.0	35.2
Neutrals	19.7	63.5	10.4

Acidic Constituents

Analytical gas chromatography of the methyl esters of the acidic constituents (Fig.VI.) was carried out on column A (He flow rate, 250 ml./min.; column temperature programmed from 130° to 230° at 7.9°/min.; block and injection port at 225°). Rd is the ratio of the retention time of the relevant compound to that of methyl dehydro-abietate (XIIIb, 79.5 min.) on column A using the above conditions. The purity of all compounds condensed from column A was checked by re-injection on column A and column B (He flow rate, 50 ml./min.; column temperature dependent on the retention time of sample on column A).

Preparative g.c. was carried out on column D (H_2 and N_2 flow rates, 400 and 300 ml./min. respectively; column isothermal at 215°; injection and collection ports at 225°; detector at 300°). Forty 250 µL injections of the bark

methyl esters (30% A.R. acetone solution) were required to yield fraction 1A (0.047 g.), fraction 1B (0.245 g.), fraction 1C (0.029 g.), and fraction 1D (0.210 g.). Each fraction was purified by chromatography on silica gel (200 -300 mesh) using n-hexane as eluant; fractions 1A, 1B and 1D then yielded single peaks on g.c. on analytical columns A and B. Fraction 1C, contaminated with both fractions 1B and 1D, was further purified by g.c. on column A.

Samples of 4,5-iso dehydroabietic acid (XVa) and 5-iso dehydroabietic acid (XVc) were supplied by R.E. Ireland(72) and E. Wenkert(68) respectively.

Methyl citronellate (IIb)

Compound 2A (Fig.VI.), a single peak on g.c. columns A (Rd 0.26) and B, was condensed as a viscous oil; νmax(43) 3023w, 1743s, 1438s, 1379m, 1367w, 1290m, 1258w, 1199s, 1158s, 1112w, 1082m, 1018m $cm.^{-1}$; p.m.r. spectrum(43): 9.05 (d,3H,J=5); 8.40, 8.31, 6.32 (s,3xCH_3); 7.60-8.20 (m,4H); 4.86 (m,1H). The mass spectrum showed a parent ion at m/e 184 consistent with $C_{11}H_{20}O_2$. The compound was identified as methyl citronellate (IIb) by direct comparison with an authentic sample.

Oxidation of citronellal (IIc)

Citronellic acid (IIa) was prepared by mild oxidation of citronellal (IIc) with basic silver oxide(50). Silver

nitrate (1.5g.) in water (3 ml.) was added to sodium hydroxide (0.7 g.) in water (3 ml.). To this mixture was added, dropwise, citronellal (IIc, 0.66 g.) at 0°. The mixture was warmed to room temperature, filtered, and the filtrate extracted with ether to remove neutral constituents. The basic filtrate was then acidified and the acidic fraction (0.12 g.) extracted with ether. Gas chromatography of the methylated acids on column A yielded pure methyl citronellate (Rd 0.26) and several other unidentified methyl esters of higher molecular weight.

Methyl palmitate (IX)

Compound 2B (Fig.VI.), a single peak on columns A (Rd 0.45) and B, was condensed as a viscous oil which crystallised on standing. The compound was identified as methyl palmitate (IX) by direct comparison with an authentic sample.

Methyl trans-communate (Xb)

Colourless needles precipitated from a 30% methanolic solution of the methylated acidic fraction from the bark. Filtration and recrystallisation from methanol-ether yielded a compound pure by g.c. (Rd 0.77); $\nu_{max.}$ 3085w, 1790w, 1727s, 1643m, 1605m, 1154s, 987s, 892s $cm.^{-1}$; p.m.r. spectrum (52): 9.44, 8.81, 8.26, 6.39 (s, 4xCH_3); 5.53, 5.16 (s, 2x1H); 3.40-5.30 (ABXm, 3H: H_A 5.12, H_B 4.98, H_X 3.67; J_{AB}=1, J_{AX}=10,

J_{BX}=17); 4.60 (t,1H,J=6). Accurate mass: 316.2411 ($C_{21}H_{32}O_2$ requires 316.2402). The compound was identified as methyl trans-communate (Xb) by direct comparison with an authentic sample supplied by H.C. Deeth of the University of Queensland. Similar comparisons showed Xb also present in the sapwood, but not in the oleoresin.

Methyl sandaracopimarate (VIIIb)

Preparative g.c. of the methyl esters of the bark acids yielded fraction 1B (Fig.VI.). Fraction 1B (0.19 g.) in dry benzene (3 ml.), containing maleic anhydride (0.062 g.), was stirred at room temperature for 12 hours(53). Benzene was evaporated and the residue treated with 5% NaOH (2 ml.) at 65° for 10 hours. The neutral fraction was extracted with ether and the basic solution acidified with dilute hydrochloric acid. The acidic fraction was extracted with ether, washed with water to remove any remaining maleic acid, and the ether evaporated to yield the yellow crystalline di-acid (XII); m.p. 106-108°; νmax. (nujol) 2500 -3700br, 1690-1720s, 1643m, 1153s, 890m $cm.^{-1}$.

The neutral fraction (0.121 g.) yielded a clear oil pure by g.c. (Rd 0.77); νmax.(57) 3085w, 1728s, 1636m, 1240s, 994m, 908s, 854m $cm.^{-1}$; p.m.r. spectrum(56): 9.17, 8.97, 8.81, 6.36 (s,4xCH_3); 4.77 (s,1H); 3.95-5.30 (ABXm,3H: H_A 5.12,H_B 5.11,H_X 4.20; J_{AB}=2,J_{AX}=10,J_{BX}=18). Accurate

mass: 316.2411 ($C_{21}H_{32}O_2$ requires 316.2402). The compound was identified as methyl sandaracopimarate (VIIIb) by direct comparisons of g.c. retention time and i.r. spectrum with those of the methyl ester of an authentic sample of sandaracopimaric acid (VIIIa) supplied by L.J. Gough(49); VIIIb was similarly detected in both the sapwood and oleoresin.

Sandaracopimaric acid (VIIIa)

VIIIb (0.12 g.) in DMSO (6ml.) containing potassium t-butoxide (0.30 g.) was heated on a boiling water bath for 5 hours(59). The reaction mixture was then poured into ice-water (30 ml.), acidified, and the product extracted with ether. Evaporation of the ether yielded an oil (0.107 g.) which on recrystallisation from aqueous ethanol yielded colourless needles; m.p. 168-169°; νmax. 3085w, 2400-3500br, 1700s, 1638m, 1282s, 1001m, 917s, 862m $cm.^{-1}$ Found: C, 79.3; H, 9.8%. $C_{20}H_{30}O_2$ requires: C, 79.4; H, 10.0%. The compound was identified as sandaracopimaric acid (VIIIa) by direct comparison with an authentic sample(49).

Methyl 4-epi dehydroabietate (XIIId)

Fraction 1D (Fig.VI.), collected by preparative g.c. of the bark esters, was recrystallised from aqueous methanol to yield colourless needles, m.p. 78-79°, pure by g.c. (Rd 0.91); νmax. 1730s, 1613w, 1498w, 1240m, 1195m, 1160m,

1143m, 1036m, 994m, 893m, 833m $cm.^{-1}$; p.m.r. spectrum: 8.98, 8.72, 6.34 (s, 3xCH_3); 8.78 (d, 6H, J=6); 7.22 (m, 3H); 3.10 (s, 1H); 2.85 (ABq, 2H, J=7.5). Accurate mass: 314.2241 ($C_{21}H_{30}O_2$ requires 314.2246). The compound was identified as methyl 4-epi dehydroabietate (XIIId) by a mixed melting point determination and comparisons of g.c. retention time, i.r. and p.m.r. spectra with those of authentic samples supplied by H.C. Deeth(70) and L.J. Gough(71); XIIId was similarly detected in both the sapwood and oleoresin.

4-Epi dehydroabietic acid (XIIIc)

XIIId (0.075 g.) was treated with potassium t-butoxide (0.19 g.) in DMSO (4ml.) in the same manner as VIIIb. Work up yielded a colourless solid (0.069 g.) which on recrystallisation from aqueous ethanol gave 4-epi dehydroabietic acid (XIIIc); m.p. 167-168°; λmax. 276, 268, 262(sh) mμ; logϵ 2.87, 2.82, 2.66; νmax.(nujol) 2400-3700br, 1700s, 1608w, 1501w, 1275s, 887w, 830m $cm.^{-1}$; p.m.r. spectrum: 8.88 8.67 (s, 2xCH_3); 8.80 (d, 6H, J=7); 7.17 (m, 3H); 3.10 (s, 1H); 2.87 (ABq, 2H, J=7). Found: C, 80.1; H, 9.5%. $C_{20}H_{28}O_2$ requires: C, 80.0; H, 9.4%. The mass spectrum also showed a parent ion at m/e 300 consistent with $C_{20}H_{28}O_2$.

4-Epi dehydroabietol (XIIIf)

XIIId (0.1 g.) in dry ether (12.5 ml.) was refluxed with lithium aluminium hydride (0.05 g.) for 6 hours.

Excess hydride was destroyed with moist ether, and the solution poured into water. Ether extraction yielded 4-epi dehydroabietol (XIIIf) as a viscous oil, pure by g.c. (Rd 1.56); λmax. 276,268,261.5(sh) mμ; logε 2.75,2.72,2.60; νmax. 3700-3100br, 3630s, 1613w, 1500m, 1028s, 978m, 889m, 827m $cm.^{-1}$; p.m.r. spectrum: 8.95,8.83 (s,2xCH_3); 8.77 (d,6H, J=7); 7.10 (m,3H); 6.26 (ABq,2H,J=11); 3.08 (s,1H); 2.84 (ABq,2H,J=6). The mass spectrum showed a parent ion at m/e 286 ($C_{20}H_{30}O$ requires m/e 286).

Methyl isopimarate (XXIVb)

Fraction 1C (Fig.VI), from preparative g.c., was identified as methyl isopimarate (XXIVb) by direct comparison of g.c. retention time (Rd 0.84) and mass spectrum with those of an authentic sample supplied by L.J. Gough(80); XXIVb was similarly detected in both the sapwood and oleoresin.

Methyl Δ-13(16)-communate (XVIa)

Fraction 1A (Fig.VI.), from preparative g.c., was obtained pure as a clear oil (Rd 0.66); νmax.(film) 3085w, 1790w, 1725s, 1642m, 1598m, 1150s, 988m, 895s $cm.^{-1}$; p.m.r. spectrum: 9.53,8.88,6.42 (s,3xCH_3); 5.38,5.13 (s, 2x1H); 5.03 (s,2H); 3.40-5.10 (ABXm,3H: H_A 4.98,H_B 4.84,H_X 3.63; J_{AB}=1,J_{AX}=11,J_{BX}=17). Accurate mass: 316.2411 ($C_{21}H_{32}O_2$ requires 316.2402). Chemical and spectral

correlations showed the compound to be methyl Δ-13(16)-communate (XVIa); XVIa was also detected in the sapwood, but not in the oleoresin.

Δ-13(16)-Communol (XVIb)

XVIa (0.04 g.) was treated with lithium aluminium hydride (0.02 g.) in the usual way. Work up yielded Δ-13(16)-communol (XVIb) as an oil pure by g.c. (Rd 1.04); λmax. 226 mμ, logε 4.35; νmax.(film) 3400s, 3085w, 1780w, 1642m, 1597m, 1022s, 986m, 890s cm^{-1}; p.m.r. spectrum: 9.34, 9.02 (s, 2xCH_3); 6.40 (ABq, 2H, J=11); 5.42, 5.13 (s, 2x1H); 5.00 (s, 2H); 3.35-5.05 (ABXm, 3H: H_A 4.94, H_B 4.78, H_X 3.57; J_{AB}=1, J_{AX}=11, J_{BX}=17). The mass spectrum showed a parent ion at m/e 288 ($C_{20}H_{32}O$ requires m/e 288).

Dehydration of torulosol (XXII)

Torulosol (0.023 g.) was dehydrated with acetic acid (25 μl.)-acetic anhydride (25 μl.), according to the method of Arya et al(53). Work up yielded an oil (0.016 g.) which was subjected to g.c. on column B (He flow rate, 45 ml./min.; column temperature 218°). The chromatogram showed 5 peaks with the major component (37%), eluted first, having λmax. (226 mμ, logε 4.35) corresponding to that of Δ-13(16)-communol (XVIb), isolated as the major product of the reaction by Arya et al(53). All other components showed λmax. not corresponding to XVIb. The sample of torulosol

(XXII) was supplied by L.J. Gough.

Methyl dehydroabietate (XIIIb)

A 250 ml. three-necked flask was fitted with a stirrer, reflux condenser and funnel. Methyl abietate (XIVb, 18.5 g.) and benzoyl peroxide (0.1 g.) in dry benzene (20 ml.) was brought to vigorous reflux and a mixture of N-bromo-succinimide (8.9 g.) and benzoyl peroxide (0.1 g.) added portionwise as quickly as foaming permitted. As soon as the foaming subsided the solution was cooled in ice and the succinimide filtered off. Remaining succinimide was removed by washing with water. Chromatography on alumina with 1:1 benzene-light petroleum yielded a pale yellow oil (15.24 g.). P.m.r. spectrum showed the oil contained approximately 10% unreacted XIVb. A mixture of chromium trioxide (0.334 g.), water (0.25 ml.) and glacial acetic acid (0.75 ml.) was added to the oil (0.785 g.) in glacial acetic acid (4.25 ml.) over 1½ hours at 45-55° with vigorous stirring. The mixture was allowed to stand over-night at room temperature. Dilution with water and ether extraction yielded an oil (0.76 g.) which, after chromatography on alumina with n-hexane as eluant, yielded a colourless crystalline compound. Recrystallisation from aqueous ethanol-methanol gave pure methyl dehydroabietate (XIIIb), m.p. 62.5-63°; Rd 1.00; λmax. 276,268,262(sh) mμ;

logϵ 2.87,2.85,2.71; νmax. 1730s, 1612w, 1501w, 1250m, 1178m, 1129m, 1040m, 905w, 884w, 837w $cm.^{-1}$; p.m.r. spectrum (61): 8.79,8.72,6.34 (s,3xCH_3); 8.78 (d,6H,J=7.5); 7.13 (m, 3H); 3.11 (s,H); 2.87 (ABq,2H,J=7). Found: C, 79.8; H, 9.6%. $C_{21}H_{30}O_2$ requires: C, 80.2; H, 9.6%. The mass spectrum showed a parent ion at m/e 314 consistent with $C_{21}H_{30}O_2$.

Dehydroabietic acid (XIIIa)

XIIIb (0.5 g.) was treated with potassium t-butoxide (1.25 g.) in DMSO (25 ml.) in the usual way. Work up yielded dehydroabietic acid (XIIIa) as colourless needles from aqueous ethanol; m.p. 167-168°; λmax. 276,268,262(sh) mμ; logϵ 2.88,2.87,2.75; νmax. 3400-2400br, 1700s, 1613w, 1501w, 1284s, 908w, 886w, 839w $cm.^{-1}$; p.m.r. spectrum: 8.87, 8.65 (s,2xCH_3); 8.77 (d,6H,J=7); 7.13 (m,3H); 3.09 (s,H); 2.85 (ABq,2H,J=7). Found: C, 80.2; H, 9.5%. $C_{20}H_{28}O_2$ requires: C, 80.0; H, 9.4%. Accurate mass: 300.2098 ($C_{20}H_{28}O_2$ requires 300.2089).

Oxidation of methyl abietate (XIVb)

A mixture of chromium trioxide (3.34 g.), water (2.5 ml.) and glacial acetic acid (7.5 ml.) was added to methyl abietate (XIVb, 5.32 g.) in glacial acetic acid (25 ml.) over 1½ hours at 50° with vigorous stirring. The reaction mixture was then allowed to stand overnight at room

temperature. Dilution with water, and ether extraction, yielded an oil (4.9 g.), which consisted of acids (36.5%) and neutrals (63.5%). Thin layer chromatography of both the acidic and neutral fractions was indicative of fairly complex mixtures; identification of components was not attempted. G.c. of the neutral fraction showed that XIVb had been completely oxidised, however.

Communic Acid Polymerisation

Methyl esters were prepared by treatment of acidic fractions with ethereal diazomethane(94,95). G.c. was carried out on analytical column A under the same conditions used for g.c. of the bark methyl esters (pg.89.). Extraction of the acidic constituents from the n-hexane insoluble fractions was with 3% KOH; acids were regenerated with dilute hydrochloric acid. Acids were then separated into monomers and polymer by treatment with ethanolic KOH as described by Carman and Cowley(81). Crude polycommunic acid from the oleoresin was purified(81) to give a readily filterable colourless powder, m.p. 177-192°. The p.m.r. spectrum (Fig.XIV.) was run in a mixture of deutero-chloroform and D_6-DMSO. The i.r. spectrum (nujol) showed bands at 3500-2400br, 3082w, 1700s, 1647m, 1265m, 1178m, 1060m and 889s $cm.^{-1}$

Isomerisation of Methyl trans-communate

G.c. of methyl trans-communate (Xb) on column A (He flow rate, 200 ml./min; column isothermal at 245°; block and injection port at 300°) yielded its double bond isomer XXX. A sample of pure Xb (0.005 g. in 20 µl. acetone) was injected onto the column and the eluted material condensed; two re-injections yielded the crude isomer XXX. Four such runs resulted in the collection of 0.003-0.004 g. of this product. G.c. on column B (He flow rate, 50 ml./min; column isothermal at 215°) then yielded pure XXX (0.001-0.002 g.); Rd 0.70; νmax.(film) 1782w, 1725s, 1648m, 1225m, 1155s, 975m, 892s, 825w $cm.^{-1}$

Neutral Constituents

Total sapwood neutral fraction

G.c. of the total neutral sapwood constituents (Fig. XVII.) was carried out on column A (He flow rate, 60 ml./min; column temperature programmed from 130° to 225° at 4°/min; block and injection port at 225°). The purity of samples condensed from column A was checked by re-injection onto column B (SE30).

The compound corresponding to peak 4F (Fig.XVII.), crystalline on collection, was recrystallised from ethanol to yield colourless needles; m.p. 91-92°; νmax. 3582s, 1385m, 1372s, 1328m, 1125s $cm.^{-1}$ The compound was identified as guaiol (I) by a mixed m.p. determination and

a direct comparison of g.c. retention time, i.r. and mass spectra with those of an authentic sample.

Compounds corresponding to peaks 4H ($\nu_{max.}$ 3582s, 1385s, 1372m, 1328m, 1125m, 940w, 918w, 900m $cm.^{-1}$) and 4I ($\nu_{max.}$ 3582s, 3075m, 3020sh, 1653sh, 1645m, 1380s, 1372m, 937w, 920m, 892s $cm.^{-1}$), both viscous oils and yielding parent ions at m/e 222 in the mass spectra, were identified as γ-eudesmol (VI) and a mixture of α- and β-eudesmols (IV and V) respectively by direct comparisons with authentic samples.

Experimental data for other constituents of this fraction has already been recorded in Fig.XVIII.

Steam volatile constituents

G.c. of the steam volatile fractions (Fig.XVII.) was carried out on column A (He flow rate, 40 ml./min; column temperature programmed from 60° to 205° at 7.9°/min; block and injection port at 225°). The purity of samples condensed from column A was generally checked by re-injection onto columns B and C.

Total bark neutral fraction

The total bark neutral fraction (10.5 g.) was chromatographed on alumina (125 g; Merck, active I, neutral) with n-hexane [1], 1:1 n-hexane-ethanol [2], and ethanol [3] as eluants. Volumes and yields of each

fraction are tabulated below. In order to determine the degree of separation obtained, each fraction was subjected to g.c. on column A under the conditions used (pg.100.) for the total sapwood neutral fraction.

FRACTION NO	ELUANT	VOLUME COLLECTED (ml.)	YIELD (g.)
1	[1]	80	-
2	"	25	0.08
3	"	15	0.09
4*	"	15	0.12
5	"	10	0.08
6	"	10	0.31
7	"	10	0.48
8	"	25	0.56
9	"	25	0.71
10	"	40	0.69
11	[2]	60	0.52
12	"	50	0.04
13‡	"	50	3.80
14	[3]	100	2.49
15	"	300	-
		Total Yield	9.97

Bark-Fraction 4*

All compounds examined in fraction 4 were condensed,

on elution from column A (He flow rate, 250 ml./min; column temperature programmed from 100° to 225° at 7.9°/min; block and injection port at 225°), as viscous colourless oils. The compound (λmax. 276,268,262(sh) mμ; γmax.(film) 1608w, 1502m, 1462s, 1393m, 1380s, 1368m, 886s, 818s $cm.^{-1}$; p.m.r. spectrum: 9.07 (s,6H); 8.82 (s,CH_3); 8.76 (d,2xCH_3,J=6); 7.13 (m,3H); 3.10 (s,H); 2.87 (ABq,2H,J=5.5) corresponding to peak 9E (Fig.XIX.) was identified as dehydroabietane (XIIIe) by a direct comparison of g.c. retention time, i.r. and p.m.r. spectra with those of an authentic sample synthesised from methyl 4-epi dehydroabietate (XIIId, 0.2 g.), as described by Carman and Deeth (70). The intermediate compounds, 4-epi dehydroabietol (XIIIf) and 4-epi dehydroabietal (XIIIg), were not purified; their presence in each case was confirmed by comparison of the i.r. and p.m.r. spectra of the crude products with those published(70) for the pure compounds. Yields were consistent with those obtained by Carman and Deeth(70). G.c. of the hydrocarbon fraction, resulting from the Wolff-Kishner reduction of the aldehyde, yielded dehydroabietane (XIIIe) as the only major component.

Bark-Fraction 13‡

The monoterpenoids examined in this fraction were condensed as colourless volatile oils or solids, on elution

from column A (Fig.XIX.) under conditions used for the steam volatile fractions. Fraction 13 was steam distilled at atmospheric pressure for 45 min; the steam volatile fraction was then subjected to the same g.c. conditions (Fig.XIX.) as the non-steam distilled fraction above. The compound corresponding to peak 7E (Fig.XIX.) was identified as methyl perillate (XXXVIIb) by a direct comparison of g.c. retention time and mass spectrum with those of an authentic sample, prepared as follows:

β-pinene (100 g; ~95% pure by g.c.) was treated with potassium permanganate-pyridine as described by Winstein and Holness(80); extraction of the acidic products with 2N sodium bicarbonate solution (200 ml.) yielded the insoluble sodium salt (28.9 g.) of nopinic acid (XXXVIII). The sodium salt (10 g.) was then treated with 4N hydrochloric acid; ether extraction yielded a crystalline compound (0.96 g.), which when recrystallised from ethanol gave pure nopinic acid; m.p. 124-125° (literature(93), 126-128°); νmax. 3578w, 3480br, 3600-2400br, 1725s, 1360m, 1380m $cm.^{-1}$; p.m.r. spectrum: 9.16,8.73 (s,2xCH_3); 3.23 (broad s,H); the mass spectrum showed a parent ion at m/e 184 consistent with $C_{10}H_{16}O_3$. Treatment of XXXVIII with ethereal diazomethane yielded crystalline methyl nopinate, pure by g.c.; νmax. 3582m, 3480br, 1745s, 1385s, 1370s $cm.^{-1}$; p.m.r. spectrum:

6.21, 8.24, 9.24 (s, 3xCH_3); the mass spectrum showed a parent ion at m/e 198 consistent with $C_{11}H_{18}O_3$.

Nopinic acid (XXXVIII, 0.1 g.) was then treated with hydrogen bromide-acetic acid(90) to yield the α,β-unsaturated acid (XXXIX), which on dehydrobromination with methanolic KOH(91) gave perillic acid (XXXVIIa). Both acids were crystalline and gave i.r. and p.m.r. spectra consistent with those published(90,91) for the expected compounds. The mass spectra also showed parent ions at m/e 248,246 (consistent with $C_{10}H_{15}O_2Br$) and m/e 166 (consistent with $C_{10}H_{14}O_2$) respectively. G.c. of the methylated fraction, resulting from treatment of XXXVIIa with ethereal diazomethane, yielded methyl perillate (XXXVIIb) as the only major component.

SECTION IV

THE CHEMISTRY OF RED CEDAR

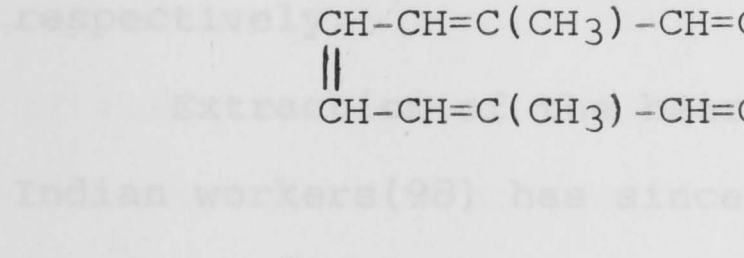
As with the Callitris investigation, initial work on the chemistry of Cedrela toona var. australis involved the preparation of crude extracts for biological testing. Yields of some of the extractives of both the timber and foliage proved, however, to be extremely low. This factor and the availability of only relatively small quantities of crude Cedrela material, due to the low population of this tree in New South Wales, hindered any detailed chemical investigation of minor constituents at this stage. A survey of the literature had already shown that published work on the major chemical constituents of Cedrela toona was quite extensive.

IV.1. Literature Survey

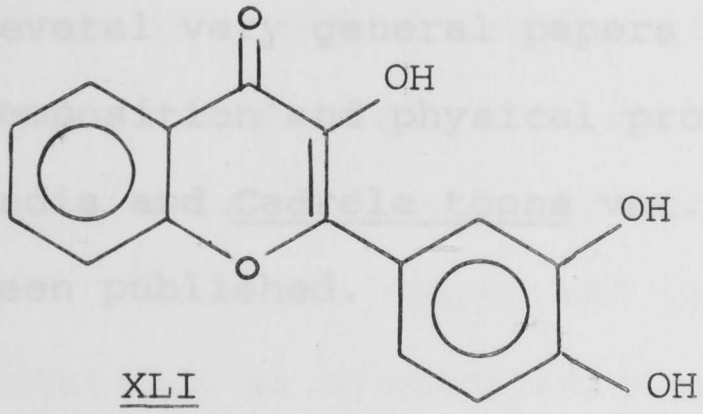
Perkin(96), in 1912, found the flowers of the "Toon" to be an interesting source of dyestuffs. A chemical investigation of the aqueous extract yielded both nytcanthin (XL) and quercetin (XLI).

The essential oil of the wood was first examined by Pillai and Rao(97), who, by steam distillation of the finely powdered wood, obtained it as a pleasant smelling liquid. It was found to consist mainly of a tricyclic sesquiterpene, l-copaene (XLII), l-cadinene (XLIII) and other bicyclic

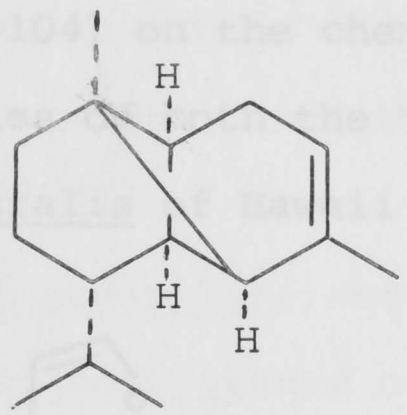
hydrocarbons, and the bicyclic alcohol, 1-cadinol (XLIV).



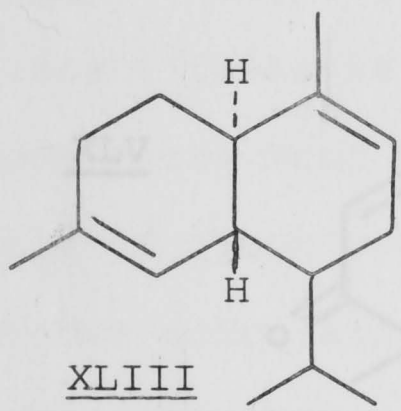
XL



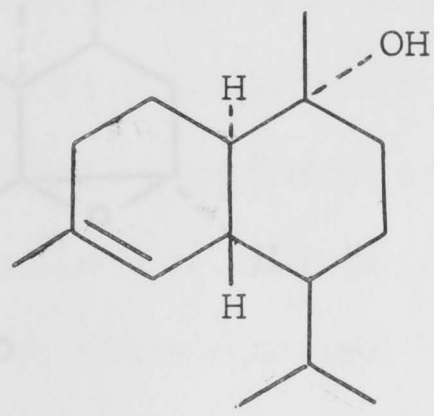
XLI



XLII



XLIII



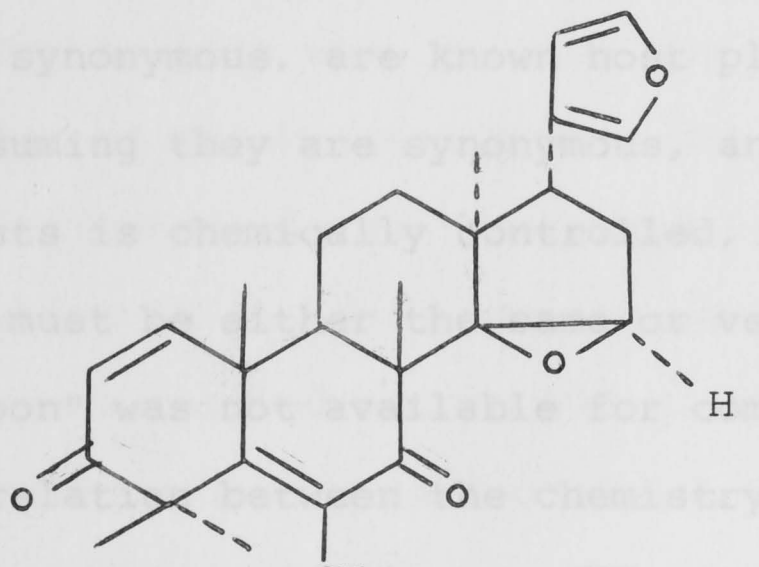
XLIV

Parihar and Dutt(100) isolated from the "Toon" wood an essential oil, a reddish-yellow colouring matter and a colourless, crystalline furan, cedrelone (XLV). The essential oil, on further examination by the same authors (99), was found to contain two liquid unsaturated tricyclic alcohols, with molecular formulae $C_{20}H_{34}O$ and $C_{22}H_{32}O$, and an unsaturated crystalline compound (m.p. 168^{o}) with molecular formula $C_{10}H_{16}O$. The alcohol and the crystalline

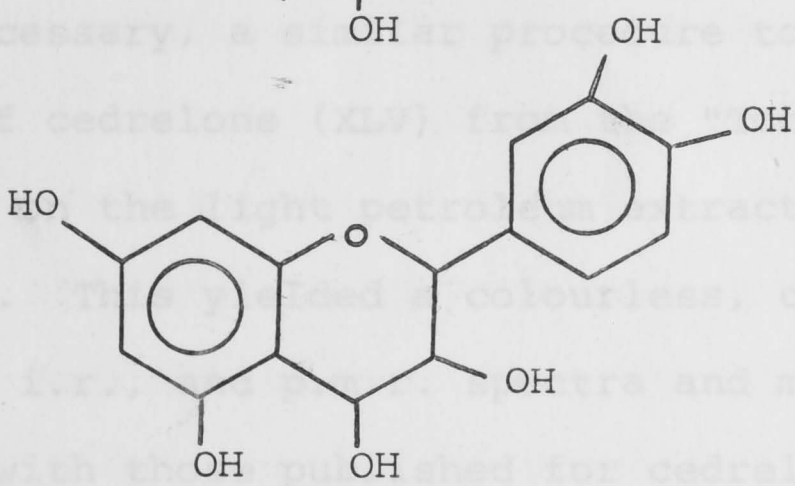
compound were named Toonol, Toonalol and Toonin respectively.

Extraction of the heartwood with acetone by other Indian workers(98) has since yielded a leucocyanidin (XLVI). Several very general papers (101-104) on the chemical composition and physical properties of both the "Toon" of India and Cedrela toona var. australis of Hawaii have also been published.

XLV



XLVI



IV.2. Extractives of Cedrela

The finely ground fresh wood and fresh foliage of

Cedrela toona var. australis were both steam distilled and extracted with a wide range of solvents. Gas chromatograms of the steam volatile fractions indicated a high concentration of compounds with retention times similar to those of standard sesquiterpene hydrocarbons and alcohols. This is consistent with earlier work published on Cedrela toona steam volatile constituents(97).

Both the "Toon" and Cedrela australis, regarded by some botanists as synonymous, are known host plants of Hypsipyla robusta. Assuming they are synonymous, and host specificity of insect pests is chemically controlled, then their chemical compositions must be either the same or very similar. As a sample of "Toon" was not available for comparison purposes, and some correlation between the chemistry of both species appeared necessary, a similar procedure to that used for the isolation of cedrelone (XLV) from the "Toon"(100) was carried out on the light petroleum extract of the wood of C.australis. This yielded a colourless, crystalline solid whose u.v., i.r., and p.m.r. spectra and melting point were consistent with those published for cedrelone(105,106). A parent ion at m/e 422 in the mass spectrum was consistent with a molecular formula of $C_{26}H_{30}O_5$. The chemistry of the two Cedrela species therefore appear similar; both are rich in sesquiterpene hydrocarbons and alcohols and contain the

crystalline furan, cedrelone (XLV).

IV.3. Experimental

The main log of a young Cedrela australis tree (2½" diameter at 4' above base) and fresh Cedrela foliage (73 lb.) were collected at Bulga and Wyong State Forests respectively in north-eastern N.S.W. All material was again stored at -5° as soon as possible until required. The wood and foliage were each finely ground (3 mm. mesh) using a Wiley mill. Extractions were carried out on 50 g. (fresh weight) samples of this material with 250 ml. solvent for 1 hour at reflux temperatures. Solvents used and the yields obtained are shown in the accompanying table. Steam distillations were carried out on 500 g. samples at atmospheric pressure over a period of 3 hours.

A larger quantity of ground wood (4 Kg.) was extracted, in 500 g. lots, with 2.5 l. quantities of 40/60 petroleum spirit. The resulting oil (7.49 g.) was then examined for the presence of cedrelone (XLV) according to the isolation procedure of Parihar and Dutt(100). Treatment with 2% aqueous KOH yielded the neutral fraction (5.30 g.) which was dissolved in benzene (75 ml.) and stored at 0°. A pale yellow compound (0.86 g.) crystallised out over a period of 12 days. Recrystallisation from chloroform-ethanol yielded pure cedrelone (XLV); m.p.(106) 206°; λmax. 279 mμ, logϵ

Cedrela Extractives

as a Percentage of Fresh Weight

	Wood	Foliage
Water	4.16	9.77
Ethanol	2.12	4.90
Methanol	2.53	5.26
Diethyl ether	0.15	1.53
Acetone	2.44	3.78
Chloroform	0.55	1.90
Benzene	0.21	1.62
60/80 Petroleum	0.16	0.79
Steam volatiles	< 0.01	< 0.01

3.97 shifting in base to 327 mμ, log ε 3.76; νmax.(nujol) (106) 3380m, 3120w, 1676s, 1614m, 1510w, 1250s, 1038s, 882m and 874m $cm.^{-1}$ The p.m.r. spectrum(105) indicated 5 tertiary methyl groups (9.23,8.88,8.70,8.50 and 8.42), a β-mono-substituted furan ring (3.79,2.81 and 2.61), an enone chromophore (two doublets at 3.86 and 3.03, J=10), an hydroxyl group (3.45) and an ether chromophore (6.18). Found: C, 73.2;H, 7.1%. $C_{26}H_{30}O_5$ requires: C, 73.9,H, 7.2%.

SECTION V

BIOLOGICAL TESTING

V.1. Field Testing of Callitris Extracts

During the term of the investigation two field trips were made into forest areas expected to yield the high population of Diadoxus adults required for biological testing of Callitris extracts. Both trips however coincided with unusual seasonal weather conditions which precluded any conclusions concerning the possibility of establishing a suitable field bioassay for Diadoxus; the problem now awaits a suitable opportunity for further field study. The limited field work which was undertaken has, however, laid a foundation for this future study and a discussion of the work and questions which arose from it is therefore of some importance.

It was the opinion of the N.S.W. Forestry Commission that field work be based at Baradine in north-western N.S.W. This was on the grounds that Baradine is closer to more forest areas, has a greater selection of forest type admixtures and densities, is away from the disadvantages of hilly country and is closer to more of the recently burnt areas which is important in assuring a high population of Diadoxus adults. A history of significant fires in white cypress pine forests in the area since 1963 was also

supplied by the Commission. Both field trips were conducted during the 1966-67 summer period (30.11.'66 to 10.12.'66 and 27.2.'67 to 4.3.'67) in the state forests listed below.

Forest	General Health of Trees	Diadoxus larval activity
Yarrigan S.F. 272	Drought-stricken	High
Baradine S.F. 672	Fire-damaged (31.10.'65)	High
Doona S.F. 512	Healthy	Absent
Breeza S.F. 110	Dead	Past activity only

Biological testing was conducted in Yarrigan and Baradine forests where larval activity and adult emergence holes were both evident; it was not possible, however, to determine how recently emergence had occurred. Tests were set up several hours before dusk, as suggested by Hadlington (36), and involved exposure of the conductive tissues of healthy trees with both axe and blow-torch, and spraying healthy trees and drought, fire and mechanically damaged trees with 1% n-hexane solutions (7-8 ml./tree) of the steam volatile fractions and total, acidic and neutral fractions of the light petroleum extracts of both the bark and sapwood. No Diadoxus adults were observed during lengthy observations of each test.

Diadoxus adults were however found in small numbers at

sawmills at Gunnedah, Baradine, Kenebri and Gwabegar; enquiries revealed that the first beetles were observed about mid-November and numbers were much lower than experienced in previous years. Further observations at the Gunnedah Sawmill indicated that Diadoxus sightings coincided with the arrival and milling of fresh loads of Callitris. This phenomenon further suggested that either Diadoxus were being brought in from the forest on the freshly-cut logs or they were being attracted to the mill, from Callitris growing in the near vicinity, by the fresh timber. The latter appeared most probable since an examination of Doona S.F., the source of timber being milled, revealed generally healthy trees with an apparent absence of Diadoxus activity. Attack by Diadoxus on felled timber has also been reported by Brennan(107) of the N.S.W. Forestry Commission.

Both field trips were accompanied by wet, windy conditions which resulted in well-below-average temperatures (maxima of 68-84^{o}F and minima of 60-66^{o}F) for the area. The fact that Diadoxus adults were not attracted to damaged host plants, as is normally expected, suggested that these conditions were probably unfavourable for biological testing regarding Diadoxus species. Hadlington, in an answer to a query on temperature and weather conditions most suitable for biological testing, confirmed this. Any conclusions

regarding the activity of Callitris extracts were therefore not possible. Negative results obtained in attempts to trap Diadoxus with light traps were also disregarded; it is probably noteworthy here that not a single adult beetle was observed in forest areas during either trip. Twenty four beetles however were caught at Gunnedah sawmill and brought back to Canberra where they again showed no attraction to Callitris extracts under laboratory conditions. Again no significance could be attached to this result since the history of each beetle before capture was not known and no distinction, other than by genitalia examination(36), could be made between male and female; also at no stage during a period of 10-12 days did they appear to enjoy captivity in a high humidity perspex cage (12"x12"x18").

Investigations have therefore indicated that for future field work weather conditions must be given full consideration and for a laboratory bioassay to be successfully established some method must be devised to obtain Diadoxus adults immediately after emergence; with respect to the latter an attempt to breed adults from larvae, observed in high numbers in the field, by means of an artificial diet (discussed in Section V.2.), could prove profitable. Another problem which must be overcome is that of the difficulty in distinguishing between male and

female; however, Moore, of the N.S.W. Forestry Commission, is presently undertaking an investigation on the biology and taxonomy of Diadoxus and will attempt to solve this problem.

V.2. Laboratory Bioassay of Cedrela Extracts

While awaiting suitable opportunity for further field study of the Diadoxus problem an examination of the problem of "Cedar Tip" moth was commenced. Preliminary evidence, although inconclusive at this stage, suggests that Hypsipyla is influenced by a compound or compounds, present in the fresh foliage of Cedrela, which act as an oviposition stimulant.

Investigations began on 31.5.'67 when a field trip was made to Sydney; this involved a brief tour of a small Cedrela plantation in Cumberland N.F. at West Pennant Hills and an examination of open growing specimens in local surroundings. The sole purpose of the trip was to observe, first-hand, the tip moth problem. A supply of Hypsipyla, mainly overwintering larvae, but a few pupae, were collected (20.7.'67) at Wyong S.F. The larvae had developed to the stage where further feeding was unnecessary and all pupated within the following few weeks. Stored at laboratory temperatures (about 20^{o}) the emergence of 7 adults was observed within the period 19.8.'67-21.8.'67; this allowed some early observations.

According to Beeson(31) the sexes of the adult moths are distinguishable by the terminal parts of the abdomen, females tufted and males not tufted. Attempts to determine the sex by these features however proved extremely difficult and it was only when oviposition was observed that the distinguishing features were finally established. Tufted abdominal tips were observed to some extent with both sexes. The main distinction is the small but definite aperture on the lower side of the terminal part of the female's abdomen; this is clearly visible to the naked eye. The oviposition observed is believed to be the first noted for Hypsipyla robusta under laboratory conditions; prior to this even adult moths on caged Cedrela trees in the field would not oviposit(33).

To ensure the availability of unfertilised females the remaining pupae were stored in individual containers until emergence. A series of tests was then conducted on oviposition, using virgin females, in the presence or absence of the light petroleum extract of the fresh foliage of Cedrela, using egg-count as the index. Tests were carried out in high humidity glass containers (6"x4" diameter) which were stored in positions of low light intensity; the extract (1 μl.) was absorbed on small pieces of filter paper and each test was examined at 24 hour

intervals. Copulation was at no stage observed, either in the presence or absence of extract. A summary of test results, tabulated in Fig.XXII.A. and B., are set out below.

(a) Females isolated from males and extract

(i) Majority died without oviposition.

(ii) Some oviposited, but only after several days.

(b) Females in presence of extract (no males)

(i) Majority oviposited on second or third day after emergence.

(ii) Others died without oviposition.

(c) Females in presence of males (no extract)

(i) Majority oviposited on third day after emergence.

(ii) Others died without oviposition.

(d) Females in presence of males and extract

(i) 50% oviposited within 24 hours of emergence.

(ii) All but one, which died without oviposition, oviposited within 48 hours.

Although the number of tests were too low for any definite conclusions to be statistically significant, indications are that oviposition occurs more readily in the presence of the Cedrela extract. Considerable variation in egg number (0-174) was observed, with no correlations apparent. According to Beeson(31) each female has the

Life Span of adult (days)	Eggs Oviposited within 24 hours,	48 hours,	72 hours.	Total eggs ovi'ted
3-4	0	0	0	0
2-3	0	0	*	0
5-6	0	0	0	0
2-3	0	0	*	0
8-9	0	0	0	6
5-6	0	0	22	22
4-5‡	0‡	0‡	3.6'‡	4.6'‡

TEST.I. Isolated female (no male, no extract)

3-4	0	0	20	20
2-3	0	0	*	0
2-3	0	0	*	0
5-6	0	1	1	30
3-4	0	12	56	68
2-3	0	0	22*	22
3-4‡	0‡	2.16'‡	16.5‡	23.3'‡

TEST.II. Female in presence of extract (no male)

* indicates death of female ‡ average value

Fig.XXII.A. Biological testing of unfertilised female Hypsipyla adults with Cedrela extract.

Life Span of Adult M	F	Eggs Oviposited within 24 hours,	48 hrs.,	72 hrs.	Total eggs ovi'ted
4-5	3-4	0	0	130	131Ø
4-5	6-7	0	0	2	58
3-4	2-3	0	0	*	0
4-5	7-8	0	0	0	9
5-6	5-6	0	0	14	53
4-5	3-4	0	0	0	0
3-4	3-4	0	0	14	14
3-4	9-10	0	0	0	0
4-5‡	5-6‡	0‡	0‡	20‡	33.1‡

TEST.III. Male (M) and female (F) (no extract)

1-2	3-4	6	24	24	24
4-5	5-6	60	153	173	174
7-8	7-8	0	3	5	20
2-3	2-3	9	13	*	13
3-4	2-3	0	2	*	2
1-2	7-8	0	1	2	2
3-4	4-5	0	9	9	9
2-3	2-3	0	0	*	0
3-4	5-6	1	4	4	4
3-4	3-4	14	19	36	36
3-4‡	4-5‡	9‡	22.8‡	26.8‡	28.4‡

TEST.IV. Male and female in presence of extract

* indicates death of female ‡ average value
Ø fertile eggs

Fig.XXII.B. Biological testing of male and female *Hypsipyla* adults with *Cedrela* extract.

capacity to lay 200-300 eggs. Fertile eggs were oviposited in one test only, in the absence of extract. All eggs, fertile or infertile, are colourless on oviposition; after 24 hours the development of the larvae within the fertile eggs gives them a characteristic red and white striped appearance. Extremely small orange coloured larvae emerged from the eggs 12 days after oviposition; unfortunately the inability to obtain either fresh Cedrela foliage or an artificial diet resulted in the death of all newly-hatched larvae.

Although preliminary results appeared promising at this stage, information supplied by both N.S.W. and Queensland Forestry Commissions indicated a shortage of Hypsipyla specimens and thus forced the investigation to lie idle for some period. A collection trip between 19.7.'68 and 20.7.'68 to both Bulga S.F. 285 and Dingo S.F. 779, in the Taree district, correlated this information and no larvae or pupae were observed in Cedrela stands. A need to confirm tentative test results however resulted in another collection trip to Strickland and Wyong State Forests between 29.10.'68 and 30.10.'68. Although no specimens were found under the bark of the heavily-barked larger trees, larvae were found to be active in both natural regeneration and fresh foliage of larger trees grown in the

open on grazing properties just outside the Wyong S.F. boundaries. The forty larvae collected were mainly very small and of a pale brown colouration; only 7 had turned blue, an indication that they would probably pupate without further feeding.

The smaller larvae were found to feed well on fresh Cedrela tips only while the freshness lasted; they were then transferred to an artificial diet (recipe on page 123) recommended by Dr. R.J. Bartell of the Division of Entomology, C.S.I.R.O. This move proved successful and the young larvae were raised through to the adult moth stage. Losses due to cannibalism however were high; this probably arose from the variation in sizes of the larvae and the effect of the ascorbic acid which functions as a feeding stimulant. Larvae were also found to eat through cardboard cups and were necessarily transferred to a glass container. It is suggested that future breeding be attempted with only small numbers of larvae per container of diet and that different sized larvae be separated; larvae showing signs of pupation should also be removed immediately since they too are very susceptible to cannibalism by still-active larvae.

Those adults which were successfully bred proved to be mainly males and only several test results, shown on page

Artificial Diet for Lepidoptera

Diet quanta:

A ingredients

Navy beans w/w.*	234 g.
Dried yeast	35 g.
Ascorbic acid	3.5 g.
Nipagin	2.2 g.
Formaldehyde; 10%	8.8 ml.
Sorbic acid	1.1 g.
Water	350 ml.

B ingredients

Agar agar	14 g.
Water (boiling)	350 ml.

*soak beans overnight; dry weight ca x ½ wet weight

Blend A ingredients thoroughly in blender. Boil water of B ingredients and dissolve agar; cool to ca 60°. Mix ingredients A and B thoroughly and pour into cups immediately. Allow to cool and gel. Surface of the diet should be cross scored before plating out larvae.

124, were obtained. Although generally consistent with previous results, this number of test results must be regarded as too small to confirm any proposal on the function of the Cedrela extract as an oviposition stimulant.

Test Type Fig.XXII.	Life Span M.	F.	Eggs oviposited within 24 hours,	48 hrs.,	72 hrs.	Total eggs
I	6-7		0	0	0	0
II	9-10		0	0	2	33
II	5-6		0	4	8	13
II	7-8		6	19	21	21
III	9-10	9-10	0	4	8	114
III	*	5-6	0	1	7	*
	7-8‡	7-8‡				

* Female escaped captivity ‡ Average value

Field work at Wyong also served to further demonstrate how effective shaded areas were as a deterrent of Hypsipyla attack. A closer investigation during larvae collection appeared to indicate that fresh foliage, in open grown natural regeneration and larger trees, which was almost continually shaded, received little attention from Hypsipyla. Where larvae had successfully initiated attack in these areas their efforts had been usually halted by a flow of fresh sap; sunny areas appeared to show this effect to a much lesser degree. A possible explanation is that a higher degree of moisture, present in shade-effected foliage,

allows sap to flow more readily; in sun-effected foliage the moisture content of the sap is considerably reduced, thus allowing larval activity to progress beyond the initial attack. Moisture-free sap was observed in the field to not hinder larval movement in the tips in any way. A more detailed examination of this observation could possibly explain the phenomenon of the successful growth of Cedrela under cover.

Although the problem of the "Cedar Tip" moth again lies idle, the development of a possible laboratory bioassay and breeding programme should facilitate future work. Also, interest in this project has been expressed by the Queensland Forestry Commission, who have some activity in this field planned for the near future, and the Department of Forests of Papua and New Guinea, who are believed to be in the process of investigating the life history of Hypsipyla in that region. This hopefully will mean a greater supply of larvae or pupae for breeding, with a consequent increase in testing facilities and field collaboration.

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