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NAME OF AUTHOR *NOM DE L'AUTEUR* Michael G Pence

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NAME OF SUPERVISOR *NOM DU DIRECTEUR DE THÈSE* D. C. A Ayer

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Edmonton.

NL-91 (4/74)

THE UNIVERSITY OF ALBERTA

METABOLITES OF *CYATHUS BULLERI*

by

MICHAEL GEOFFREY PAICE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

FALL 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled METABOLITES OF CYATHUS BULLERI submitted by MICHAEL GEOFFREY PAICE in partial fulfilment of the requirements for the degree of Master of Science.

W. A. Ayer
Supervisor

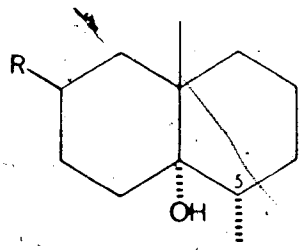
John Hoos

Date 19 75

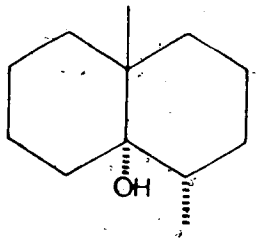
ABSTRACT

The bird's nest fungus Cyathus bulleri Brodie has been grown on a chemically undefined liquid medium in static culture. Fungal metabolites extracted from the medium were separated by chromatographic methods. Three of these metabolites were isolated in crystalline form; namely cybullol ($C_{12}H_{22}O_2$), compound F ($C_{15}H_{28}O_3$) and compound B ($C_{13}H_{12}O_4$).

The structure of cybullol (79) was partially determined by spectroscopic analysis of the parent compound and several of its derivatives. The stereochemistry at C-5 was established by chemical conversion of cybullol into geosmin (76), a naturally occurring compound first isolated from Actinomycetes.



79 R= ····OH
80 R= =O

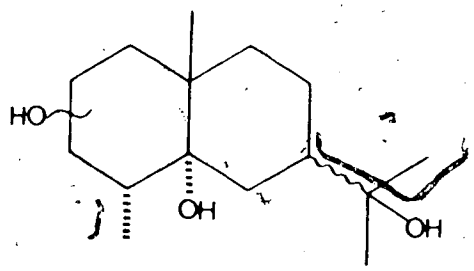


76

Both cybullol and geosmin are optically active; the absolute structures are as represented in 79 and 76. These were deduced from the positive Cotton effect observed in the

circular dichroism spectrum of keto-alcohol 80, formed from cybullol by mild oxidation.

Biogenetically, cybullol appears to be a degraded sesquiterpene. The co-occurrence of compound F, tentatively assigned partial structure 109, supports this hypothesis.



109

Spectroscopic data from compound F and its acetate derivative are in agreement with this partial structure. The relative stereochemistry was assigned by analogy with cybullol.

The identity of compound B, isolated in very small amounts, has not been established.

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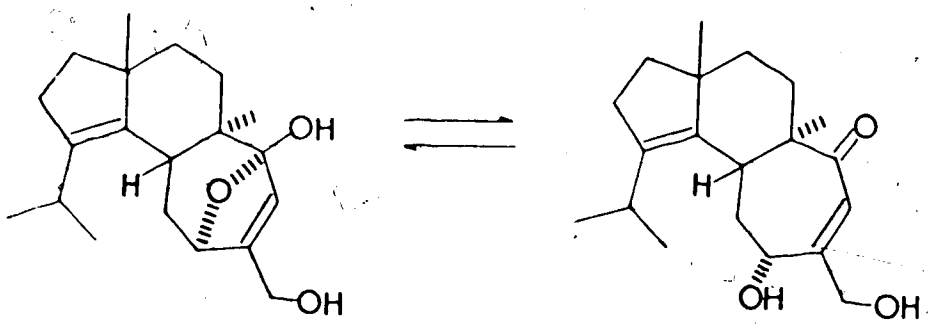
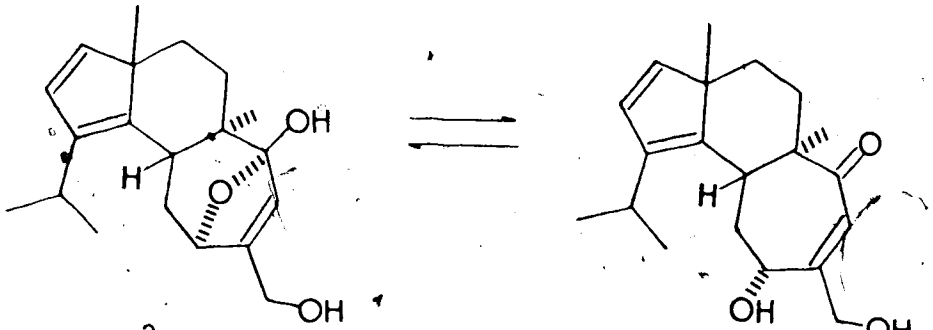
I INTRODUCTION

Since Fleming's celebrated recognition of the antibacterial activity of Penicillium notatum in 1929[1] the screening of fungal metabolites has uncovered a large and varied selection of antibiotics[2]. In these laboratories the birds nest fungus Cyathus helenae Brodie (Gasteromycetes) has been cultured, following reports by Olchowecki[3] and Johri[4,5] that its metabolites possessed antibiotic activity. The name 'cyathin' was given to the complex mixture responsible for this activity. Separation of the cyathin mixture and structure elucidation of the resulting components has identified a number of diterpenes, possessing a novel carbon skeleton. These cyathins are named according to their molecular formulae. A letter designation gives the number of hydrogens: 30 = A series, 28 = B series, etc. The number of oxygens is shown by a subscript. Isomers of previously isolated compounds are denoted by an allo- or neoallo- prefix.

Taube[6,7] has isolated and identified cyathin A_3 (1a, 1b) and allocyathin B_3 (2a, 2b). In the solid state cyathin A_3 is in the hemiketal form 1a while allocyathin B_3 exists in the hydroxyketone form 2b. The structures were determined by physical and chemical methods: X-ray analysis



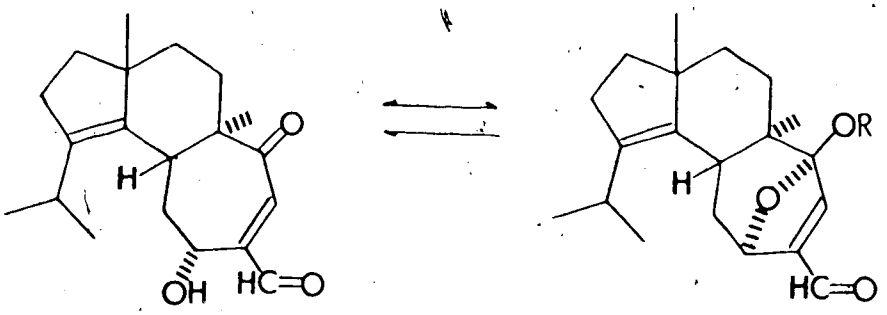
1a 1b



2a 2b

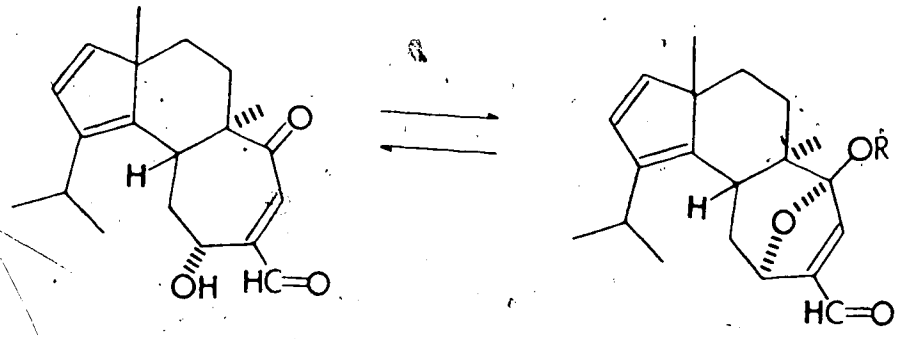
of cyathin A_3 (1a) has confirmed its structure.[8]

Recently the structures of three more cyathins have been determined by correlation with cyathin A_3 and allocyathin B_3. The aldehydes cyathin B_3 (3a, b) and cyathin C_3 (4a, b) co-crystallize and could only be separated as their methyl ketals (3c, 4c).[9,10] Wide-range biological testing of a cyathin B_3/C_3 mixture has revealed potent anti-fungal properties. Neoallocyathin A_4 (5) was found to be an epoxide[11] by correlation with cyathin A_3 (1a, 1b).



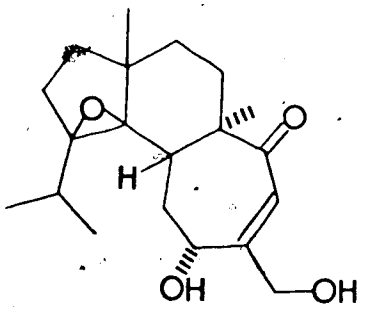
3a

3b R = H
3c R = CH_3



4a

4b R = H
4c R = CH_3



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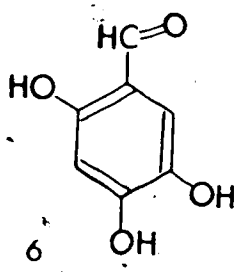
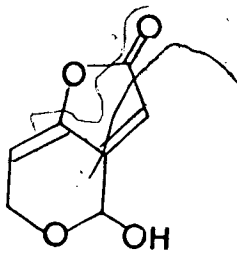
At present seven other diterpenes isolated from Cyathus helenae cultures, and probably sharing the same tricyclic skeleton, have not been fully characterized. A major difficulty here has been the inconsistent production of several of the metabolites by different growths of the fungus. Although the effect of varying conditions on the

growth of C. helenae has been studied,[12] this gradual change in secondary metabolism is difficult to explain.

In addition to the above diterpenes, Cyathus helenae also produces palmitic acid, 2,4,5 - trihydroxybenzaldehyde (6) and patulin (7).



6



7

2,4,5 - Trihydroxybenzaldehyde is thought to be a biogenetic precursor of patulin.

It has been noted[2] that closely related species of fungi often produce the same antibiotics or groups of antibiotics. However this observation does not agree well with results of biological testing of the Nidulariaceae family. For instance, antibacterial activity against B. coli and S. aureus was weakly positive[13] for Cyathus striatus but negative for Cyathus olla and Crucibulum vulgare. Olchowecki[3] reports that Cyathus helenae, C. striatus, C. limbatus, C. poeppigii showed similar bacteristatic activity whereas C. pallidus, C. bulleri, C. berkeleyanus and C. stercoreus did not.

It was decided to culture a variety of strains of Nidulariaceae in these laboratories, since the composition of metabolites produced by any fungus will be dependent on

growth medium and extraction techniques. Mycelium growths on liquid culture medium were obtained in several steps from the fruiting bodies by standard techniques. The medium developed by Brodie[14] (Brodie medium) was used throughout. After a growth period of four weeks the medium was filtered and extracted with ethyl acetate. Evaporation of the solvent gave a solid residue which was assayed for biological activity by a paper-disc agar-plate method[15] using Staphylococcus aureus as the test organism. Of the 18 species tested in this manner, 12 gave a significant zone of inhibition.

One of these, Cyathus bulleri, was chosen for further studies and is the subject of this thesis. This choice was based on factors in addition to the biological activity. The yield of ethyl acetate soluble metabolites (~ 100 mg per litre of Brodie medium) and their apparently unchanging production from growth to growth (as judged by thin-layer chromatography and gas chromatography) were other contributing factors.

Cyathus bulleri Brodie was first discovered in the West Indies[16] in 1967 and has subsequently been found in the islands of Oahu, Kanai and Hawaii.[17] An account of the fruiting body[17] describes it as a pale, strongly plicate species having an epiphragm beset with vertical tufts of red-brown hyphae. The cultures grown for this research produced no fruiting bodies. Instead a thick mass of floating mycelium, very white in color when compared

with other Nidulariceae growths, was obtained. Studies on Cyathus stercoreus[18] have shown that fruiting body growth on Brodie liquid medium is sensitive to calcium ion concentration. Brodie has reported fruiting of C. bulleri in cultures grown on nutrient agar.[16]

The initial culturing and large scale production of C. bulleri used a procedure developed for C. helenae by Johri[4] and modified by Taube,[6] Carstens[9] and Mercer.[11] The original culture was obtained from the collection of Prof. H. J. Brodie. A stock culture of the fungus was kept at 5°C in petri plates and slant tubes of agar. Approximately 200 ml of Brodie liquid medium (for composition see Experimental Section) in a 500 ml flask was innoculated under sterile conditions with discs of mycelium from the petri plate cultures. After a 30 day growth period this became the innoculum flask. The contents of the flask, when broken up with a sterile Waring blender, were then used to innoculate up to 20 Fernbach flasks containing Brodie liquid medium. In this manner, and as described in more detail in the Experimental Section, 'still surface' cultures of C. bulleri could be used to produce quantities of metabolites necessary for identification.

The objective of the work undertaken in this thesis was the isolation, separation and characterization of the mixture of substances produced by Cyathus bulleri. Of initial interest was a comparison of the metabolites with those of Cyathus helenae and the identification of biologically active components.

II RESULTS AND DISCUSSION

1) Characterization of the Crude Cyathus Bulleri Extract

A light brown non-crystalline residue was obtained by ethyl acetate extraction of aqueous media containing the C. bulleri metabolites. This residue, hereafter referred to as the crude extract, weighed between 50 and 100 mg per litre of medium extracted. It was found to be soluble in methanol, ethanol and acetone, partially soluble in chloroform, methylene chloride and ether, and insoluble in water and Skelly B (a mixture of saturated hydrocarbons b.pt. ~ 65°C).

Routine spectroscopic measurements on a crude extract gave the following information. The mass spectrum (ms), using a direct probe and source temperature of 100°C to 200°C, contains m/e 348 as the highest mass number. Other significant peaks appear at m/e 256, 232, 218 and 59, with the base peak at m/e 41. The infra-red (ir) spectrum ($CHCl_3$ solution) shows broad hydroxyl and carbonyl absorption. The ultraviolet (uv) spectrum (methanol solution) contains a shoulder at 243 nm. A sensitive ferric chloride test[19] was negative indicating that no phenolic or enolic functions are present.

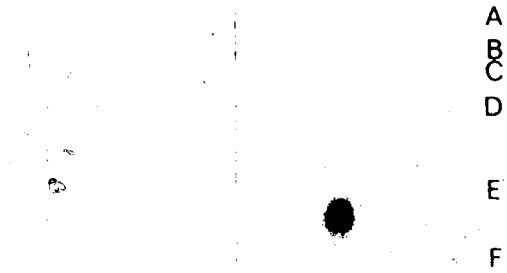
Thin layer chromatography (tlc) using silica gel as adsorbent and a solvent system of methylene chloride/methanol (10:1) resulted in a reasonable separation of the crude

extract. Numerous other tlc systems investigated gave poorer separation. The plates were visualized by color reactions of many of the spots on spraying with 30% aqueous sulfuric acid followed by careful heating with a heat gun. These color reactions, together with the Rf values measured from the plate, were invaluable in identifying compounds later isolated from the crude extract. They were also used to check the constancy of composition of crude extracts from different C. bulleri growths and to compare the crude extracts with those from C. helenae.

Figure 1 shows a visualized analytical tlc plate spotted with three crude extracts. Two of these are from different growths of C. bulleri and the third from the 1500 strain of C. helenae. The plate shows that few, if any, of the metabolites of C. helenae are to be found in extracts from C. bulleri.

A preliminary cataloging of the main compounds produced by C. bulleri was necessary at this time. The compounds are named alphabetically in decreasing Rf order - as in Table 1.

Although tlc is a useful and facile technique for monitoring the crude extracts, it does not give a quantitative measure of components in the extract. Volatile mixtures of compounds can be analyzed using gas chromatography (gc). A flame ionization detector allows detection of nanogram quantities. Thus separation of crude extract samples was attempted on a variety of gc analytical columns.



C. bulleri C. helenae C. bulleri

Figure 1: Silica gel G tlc plate of C. bulleri and C. helenae crude extracts

NAMING OF CYATHUS BULLERI METABOLITES

Compound	Rf[a]	Color reaction[b]
A	0.59	Dark yellow (brown)
B	0.56	Yellow (grey)
C	0.55	Yellow (grey)
D	0.49	Red/brown (brown)
E	0.42	Red (purple)
F	0.35	Dark blue (black)

(a) = $\frac{\text{distance from origin of spot}}{\text{distance from origin of solvent front}}$ using silica gel G (Merck) plates; methylene chloride/methanol (10/1) solvent system. Value dependent on plate activity.

(b) Initial color when sprayed with 30% aq. H_2SO_4 solution and heated to ~ 100°C. Bracketed color produced on cooling.

Table 1

The choice of an appropriate stationary phase for separation of unknown mixtures was largely a trial and error process. The polar stationary phases gave a better separation of the crude extract than those of a non-polar nature in particular, as shown in Figure 2, 3% OV-225 liquid phase on 100/120 GasChrom Q (Applied Sciences) produced a separation of C. bulleri crude extract. In general, the crude extracts gave up to 30 peaks on a chromatograph produced in this manner, depending on the sensitivity range used. It is possible that not all compounds in the extract are eluted by the gc column due to low volatility, high polarity, etc.

An increase in volatility of hydroxylated compounds can be attained by formation of trimethylsilyl ether derivatives. A sample of crude extract in pyridine was heated under anhydrous conditions with hexamethyldisilazane and trimethylchlorosilane for 3 hours at 80°C . Gc analysis of the product showed a change in retention time for many peaks when compared with a chromatogram of unsilylated material, but no significant increase in the number of peaks occurred. This observation does not rule out the possibility that compounds are retained on the column due to their high molecular weight.

When a crude extract of C. helenae was chromatographed under the same conditions as above, only one significant peak was observed. C. helenae extracts contain a number of hydroxylated diterpenes - thus polar higher molecular weight compounds are indeed retained on the column. The one peak

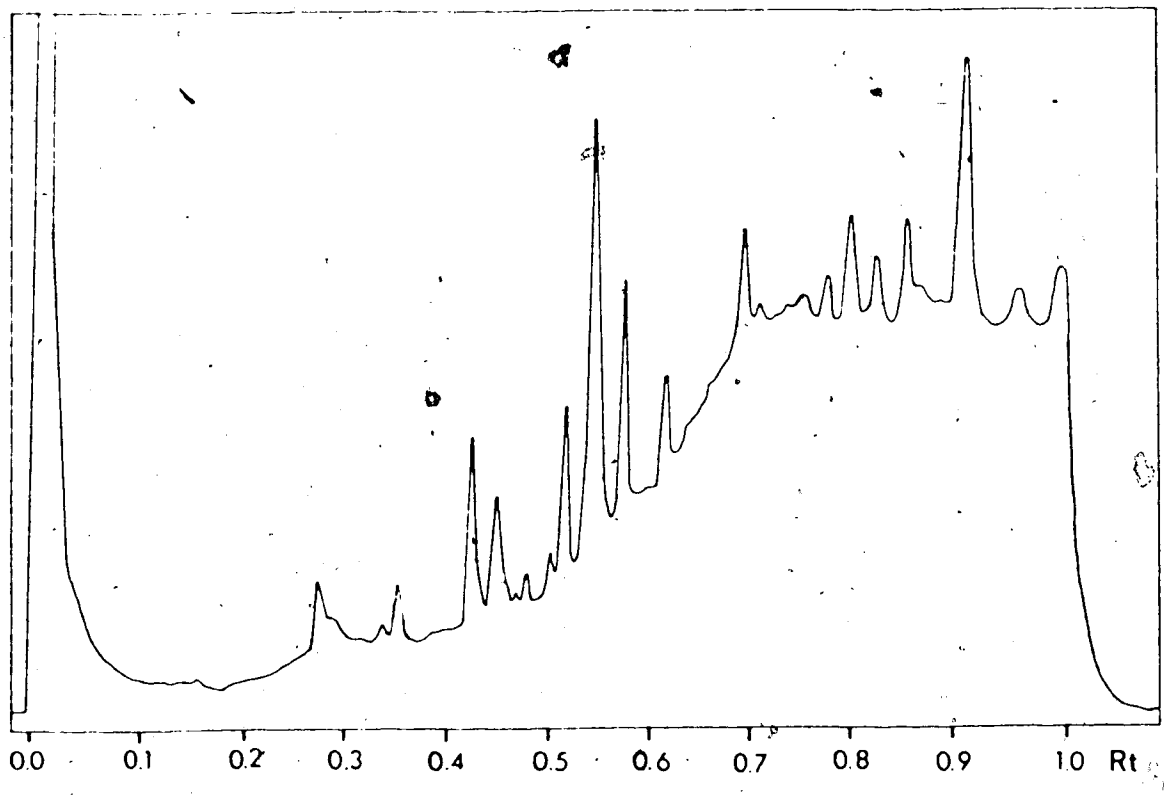


Figure 2: Gas Chromatogram of C. bulleri Extract

10' glass column containing 3%OV-225 on 100/120 Gas-Chrom Q.
Temperature program: 140°C (held for 4 min.), 8°C per min. increase to 240°C (held for 8 min.).
Gas flow: 60 ml N_2/min. Inj., det. ports: 250°C.

observed did not have the same relative retention time (Rt; see page 110 for definition) as any of the peaks in the chromatograph of C. bulleri extracts.

Gas chromatography shows that the crude extract of C. bulleri is a complicated mixture of compounds, many of which form silyl derivatives, and that none of the compounds detectable by this method are present in C. helenae extracts. Comparison of extracts from different growths of C. bulleri by gc showed consistent production of the major metabolites, though not always in precisely the same relative proportions.

The non-identity by gc and tlc of C. bulleri extracts and an ethyl acetate extract of Brodie medium containing no fungal growth, showed that none of the major C. bulleri metabolites were artifacts from the medium.

2) Separation of the Crude Cyathus Bulleri Extract

Simple acid-base extraction of the crude extract was initially attempted. This proved unsatisfactory in selectively removing any component of the extract, in spite of the original aqueous medium having a pH of approximately six. Similarly, separation on the basis of limited solubility in a particular solvent was not useful.

Chromatographic methods (tlc and gc) were useful as identification procedures, as mentioned in the previous section. Since gas chromatography was impractical for large scale separation of complicated mixtures, various liquid-solid chromatography techniques were investigated.

Silica gel G adsorbent was found to give better separation of the extract than alumina or magnesium silicate. During the course of these experiments, three different silica gel chromatography methods were utilized at various times; preparative thin-layer (ptlc), dry column and elution column chromatography.

Ptlc proved laborious for separation of total crude extracts, even using large 20 x 100 cm plates and a mechanized applicator. However, the method was found to be very useful for chromatography of small amounts of material partially purified by column chromatography. The plates were impregnated with 1% inorganic phosphor. This allowed non-destructive location of many bands since irradiation of the plate with uv

light (254 nm) produces a green glow, with dark areas where compounds absorb the light.

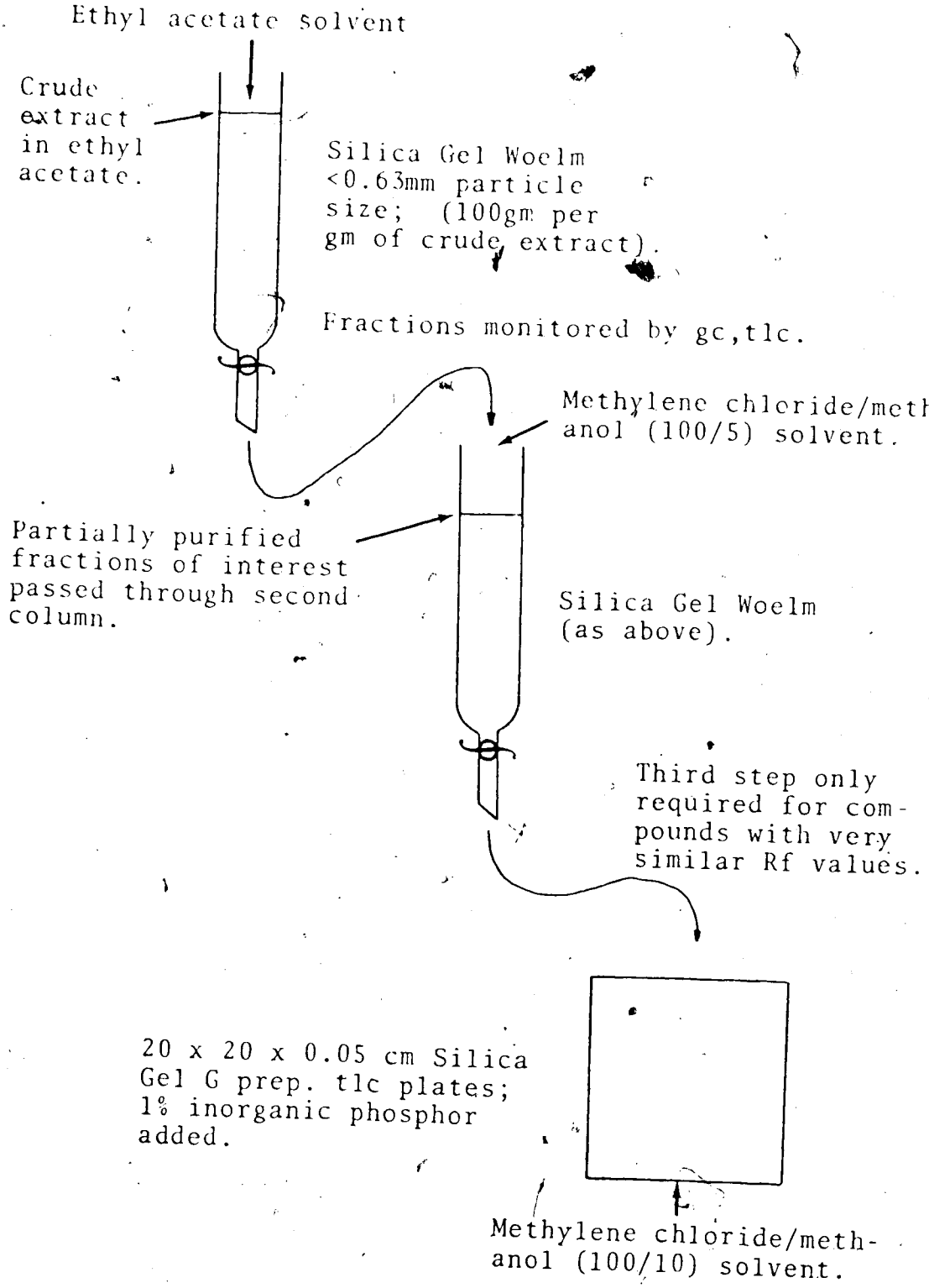
Dry column chromatography is a recent development[20] intermediate between ptlc and conventional wet column methods. Deactivated adsorbents and nylon columns used for dry column chromatography are commercially available. The method is described more fully in the experimental section (pg.107), but several difficulties encountered should be mentioned here. The eluting solvent was limited to a one solvent system, since binary systems resulted in production of air bubbles while the column was being developed, in spite of pre-equilibration of the silica gel adsorbent, as recommended by Loev.[20] Ethyl acetate solvent, with a polarity intermediate between methylene chloride and methanol was used.

Location of separated compounds on the dry column by measurement, using Rf values from an identical tlc system, proved to be inaccurate. Similarly, uv light absorption could not be used to monitor columns - lack of absorption probably being caused by the low concentration of compounds relative to adsorbent and lack of chromophores in many of the compounds. Columns were therefore arbitrarily cut into small segments and extracted, with a resultant loss in resolution.

Eventually elution chromatography was found to be as convenient and efficient as dry column chromatography. A combination of elution chromatography and ptlc was used routinely to separate the crude extracts, as outlined in

Scheme 1.

In this manner, two of the compounds present in the crude extract have been isolated as crystalline materials. These are compounds E and F in Table 1. Compound E has been isolated in sufficient quantity (approximately 100 mg during the course of this work) to allow full structure elucidation, whereas only a tentative structure for compound F can be proposed. In addition, a small amount of compound B has been isolated by dry columm chromatography. The structural features of this compound will also be discussed.



SCHEME I - Chromatographic Separation of Crude Cyathus Bulleri Extract

3) Structure Elucidation of Cybullol (Compound E)

a) Physical and Spectral Data Analysis

Compound E (Rf 0.42, see Chart 1) has been assigned the trivial name cybullol to reflect its origin from Cyathus bulleri and the fact that it is an alcohol. It is easily recognized by its characteristic color reaction when a developed tlc plate is sprayed with 30% aqueous sulfuric acid: the initial red coloration turns purple on cooling and, eventually, light blue.

Gas chromatography, using the same column and oven conditions as described in Figure 2, identified cybullol as the largest component of the crude extract (as determined by peak areas of the chromatograph). Cybullol has a relative retention time - as defined in the general experimental section, page 110, of 0.54.

In a typical chromatographic sequence, 1.5 gm of Cyathus bulleri crude extract, after initial separation as in Scheme I, gave six fractions (total weight 320 mg) containing cybullol. These fractions, on further separation using a second silica gel column and solvent system 2, gave 26 mg of pure cybullol (as judged by tlc, gc). Preparative tlc of other fractions from this column gave an additional 10 mg of pure cybullol. Thus 36 mg or 2.4% of the total crude extract was isolated as cybullol.

A sample of cybullol in methylene chloride which had

been left in the fridge for several days was found to have crystallized. The colorless crystals had a melting point, after recrystallization from methylene chloride/Skelly B, of 125-126°C. The compound is optically active; $[\alpha]_D^{25}$ = -16° (c, 0.4, methanol).

High resolution mass spectrometry (hrms) of cybullol gave an apparent parent peak with m/e 198.1614. This indicated a molecular formula of $C_{12}H_{22}O_2$ (calc. 198.1620). The possibility of a higher molecular weight parent peak which, due to facile fragmentation, is absent from the spectrum, could not be ruled out at this stage. An elemental analysis was not carried out because of the small quantity of material available.

The mass spectrum of cybullol is shown in Figure 3. The indicated molecular formulae of fragment ions were determined by hrms. Loss of a water molecule from the parent ion indicates the presence of an alcohol function. The low intensity peak at m/e 162 is possibly due to the loss of a second molecule of water. The peak at m/e 165 is due to loss of both water and a methyl group.

The infra-red (ir) spectrum (KBr disc) of crystalline cybullol is shown in Figure 4. Important features include the hydroxyl absorption around 3400 cm^{-1} and the lack of carbonyl absorption in the 1600 to 1750 cm^{-1} region. The oxygen functionalities are thus limited to either hydroxyl and/or ether functions. Solution spectra ($CHCl_3$) also show

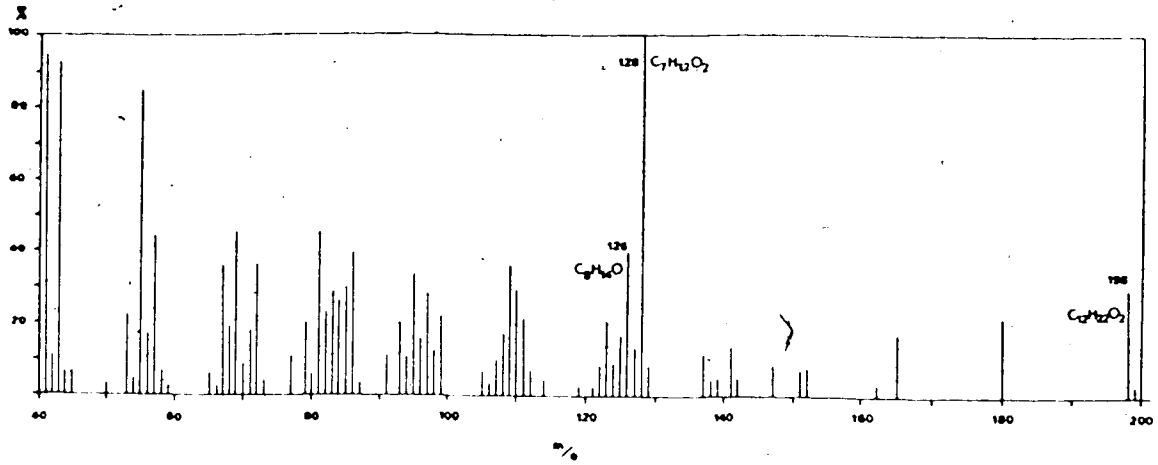


Figure 3: Mass spectrum of cybullol

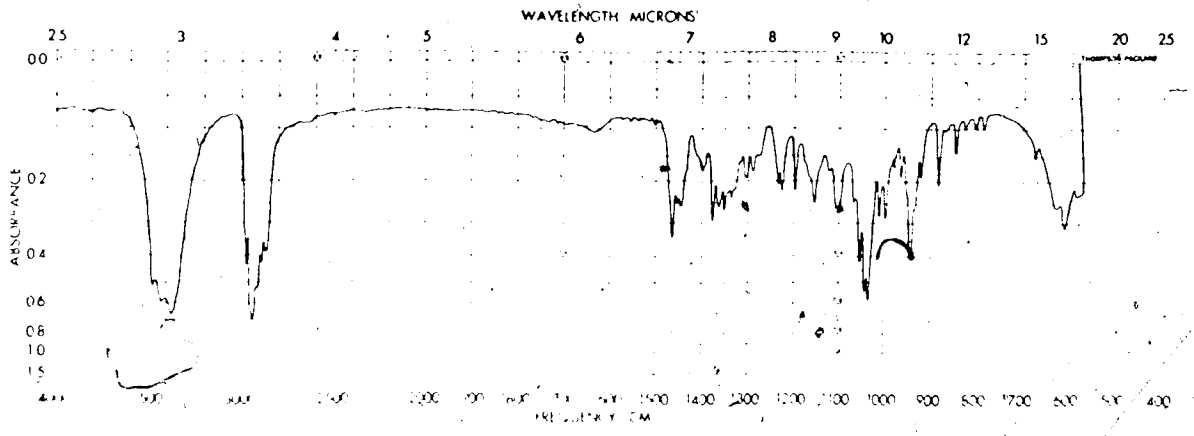


Figure 4: Ir spectrum of cybullol (KBr disc)

hydroxyl absorption (3600 cm^{-1}) and no carbonyl absorption.

Cybullol shows no uv absorption maximum in the region 210 to 350 nm.

The 100 MHz proton magnetic resonance (pmr) spectrum of cybullol (Figure 5) shows secondary and tertiary methyl signals - a doublet at δ0.81 and a singlet at δ1.03. The one proton multiplet signal at δ3.9 is assigned to a hydrogen geminal to a hydroxyl group.

At least one of the oxygen functionalities is an hydroxyl group as indicated by the ir spectrum. The identity of the other was proved as follows. A sample of cybullol was dissolved in deuterated methanol (CH_3OD). The methanol was evaporated and the residue taken up in two drops of deuterated methanol. A ms of the sample was then recorded, using a direct probe which had been dipped in the solution. The ms (Figure 6) had a parent peak at m/e 200. Thus there are two exchangeable protons in cybullol and the remaining oxygen functionality can only be an hydroxyl group. The base peak at m/e 128 in cybullol, which has been shown by hrms to contain both oxygens, is shifted to m/e 130 in cybullol-d_2.

Thus cybullol, with a molecular formula of $C_{12}H_{22}O_2$, contains two methyl and two hydroxyl groups. From the pmr spectrum, there is only one proton adjacent to a hydroxyl group. The compound must therefore contain one secondary and one tertiary hydroxyl group. The molecular formula indicates two sites of unsaturation. Only a fully substituted double bond

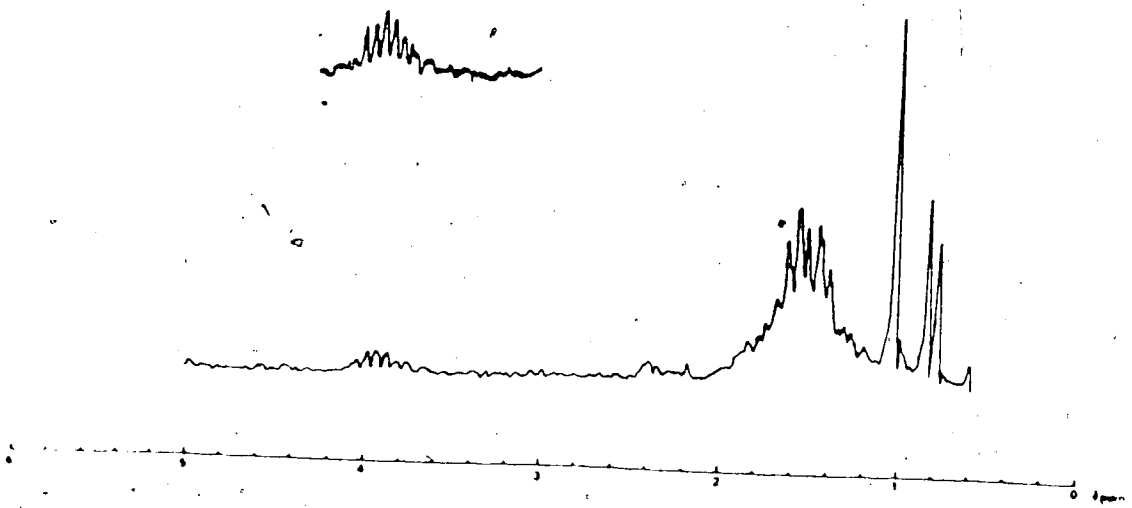


Figure 5: 100MHz pmr spectrum of cybullol ($CDCl_3$)

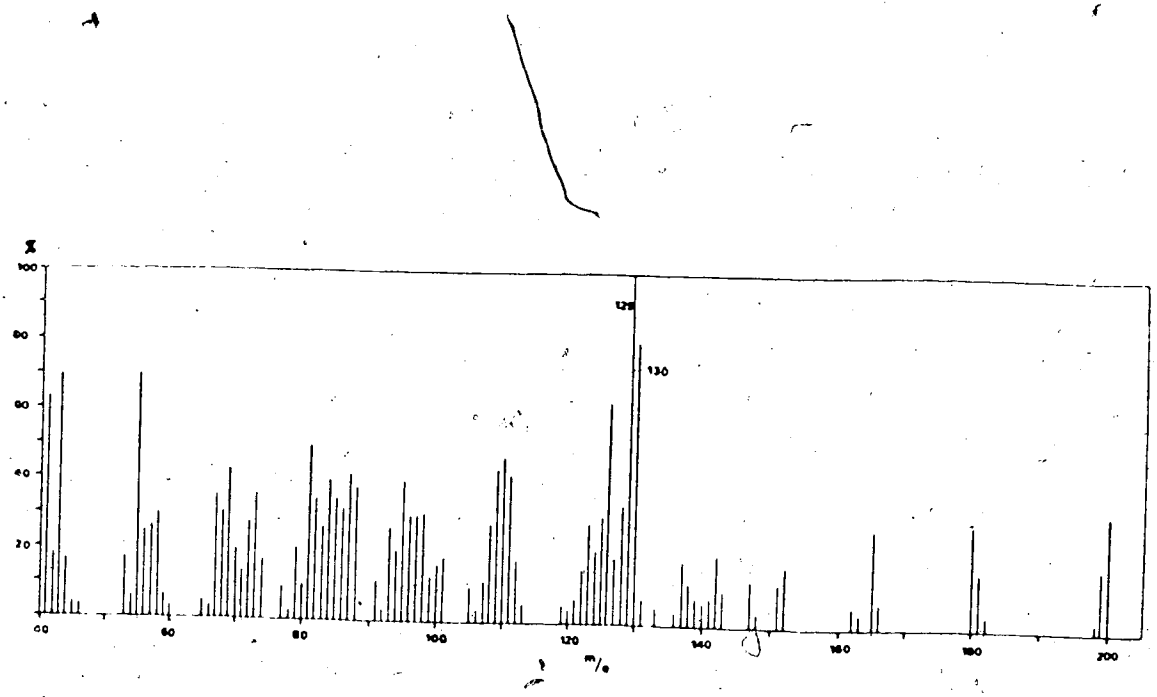
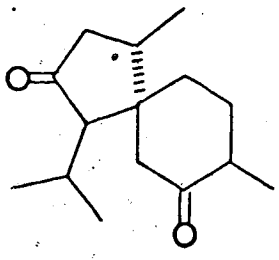


Figure 6: Mass spectrum of cybullol-d_2

could be present since the pmr spectrum shows no vinylic or allylic protons and the ir spectrum shows no olefinic or acetylenic absorption. Attempted hydrogenation of cybullol using platinum oxide as catalyst gave only recovered starting material. As discussed later, cybullol shows no sp^2 carbon in the ^{13}C magnetic resonance (cmr) spectrum. Therefore, cybullol must be bicyclic.

Having accounted for two of the twelve carbons as methyl groups, the most common ten membered bicyclic systems encountered in nature are the decalin and perhydroazulene systems. Spiro ring systems are also found in natural products. For instance, acorone (8)[21a] and α-vetispirene (9)[21b] are ten membered bicyclic ring systems. However, in this spiro system, it is not possible to find a tertiary methyl group unless it is adjacent to a hydroxyl group, as in 10. The tertiary methyl signal in the pmr of cybullol is at too high field to be geminal to an hydroxyl group. The spiro structure is therefore discounted. Other ring systems such as 11 and 12 are not normally encountered among natural products.



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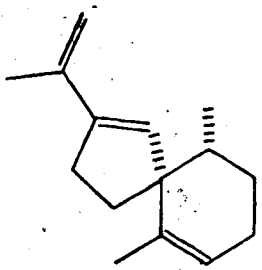

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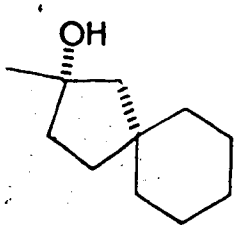

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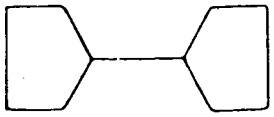

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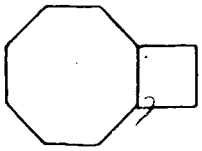

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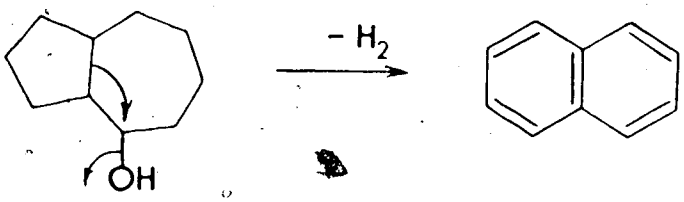
b) Dehydrogenation of Cybullol

Saturated bicyclic ring systems are often identified by the characteristic uv absorption of their aromatic dehydrogenation products. A small quantity (8 mg) of cybullol was dehydrogenated using 5% palladium on charcoal in a sealed tube at 250°C. A mixture of four major products was obtained. One of these has a retention time identical with that of 1-methylnaphthalene, using a gc system that separates 1- and 2-methylnaphthalene. Uv and mass spectral data indicated that two of the products were mono- and dimethylnaphthols. The fourth product appeared to be a dimethylnaphthalene. Its gc retention time was of the same order as that of a mixture of dimethylnaphthalene isomers (available from Eastman Kodak). As pure samples of all isomers of dimethylnaphthalene were not readily available, it was not possible to determine, by comparison of gc retention times, which isomer of dimethylnaphthalene had been produced by dehydrogenation. The mass spectra were consistent with the above assignments (see Experimental Section).

Substituted azulenes are blue and give different uv spectra to the above products. The dehydrogenation, therefore, indicates that cybullol is probably a substituted

decalin although perhydroazulenes such as 13 may possibly rearrange during dehydrogenation, as shown below.



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The tertiary methyl and hydroxyl groups in cybullol must be situated at bridgehead positions since, as mentioned, they cannot be geminal. The formation of a dimethylnaphthalene and dimethylnaphthol must necessarily be accompanied by a 1,2 methyl shift. This is a common occurrence in dehydrogenation reactions. For instance, cis-9-methyldecalin gives a mixture of naphthalene (64%) and 1-methylnaphthalene (46%)[22] when dehydrogenated with palladised charcoal at 325°C.

The secondary methyl group in cybullol must be located on a ring carbon adjacent to a bridgehead position, since otherwise 2-methylnaphthalene should have been present in the dehydrogenation mixture.

c) Acetylation of Cybullol

A monoacetyl derivative was obtained when cybullol was acetylated under a variety of conditions:

1) methylene chloride as solvent; an excess of acetic anhydride with a catalytic amount of pyridine; eight hours at 25°C

2) acetic anhydride as solvent containing a catalytic amount of pyridine; four hours at 25°C

3) same as 2), but solution refluxed for one hour

4) benzene as solvent; a threefold excess of isopropenyl acetate; p-toluenesulfonic acid catalyst; refluxed for one hour.

Under condition 1), only partial acetylation occurs. Even under condition 4) no diacetate was produced - only the monoacetate and a minor amount of dehydration product could be detected. The difficulty in forming a diacetate of cybullol is in agreement with assignment of one hydroxyl group to a tertiary position.

The mass spectrum, ir spectrum and pmr spectrum of the monoacetate are shown in Figures 7,8 and 9 respectively. The m/e 162 fragment in the ms, caused by loss of acetic acid and water from the parent ion, indicates that the monoacetate contains an alcohol function. This is confirmed by the weak OeH absorption in the ir spectrum (Figure 8).

The pmr spectrum of the monoacetate (Figure 9) contains a multiplet at δ5.0. This is caused by the proton geminal to the acetoxyl group. The proton geminal to a hydroxyl group in cybullol produces a multiplet at δ3.9. The shift of the signal on acetylation - approximately 1.1 ppm downfield - further confirms the secondary position of this hydroxyl group. If the δ3.9 signal was caused by methylene protons (i.e. a primary alcohol) then the corresponding signal in the acetate would only have been shifted by approximately 0.5 ppm.

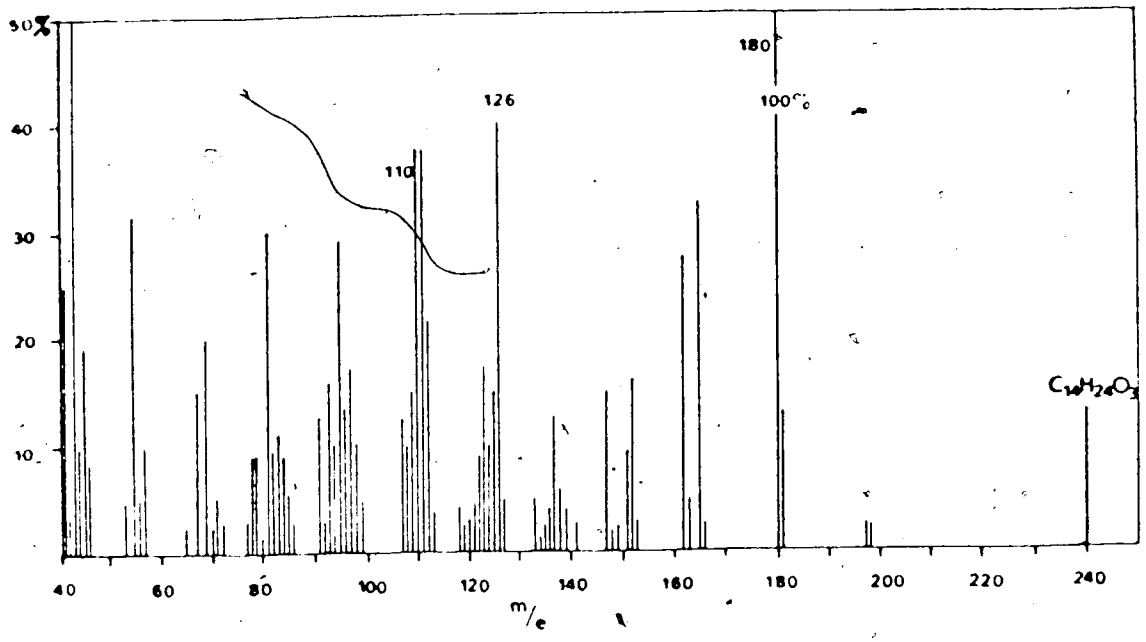


Figure 7: Mass spectrum of cybullol monoacetate

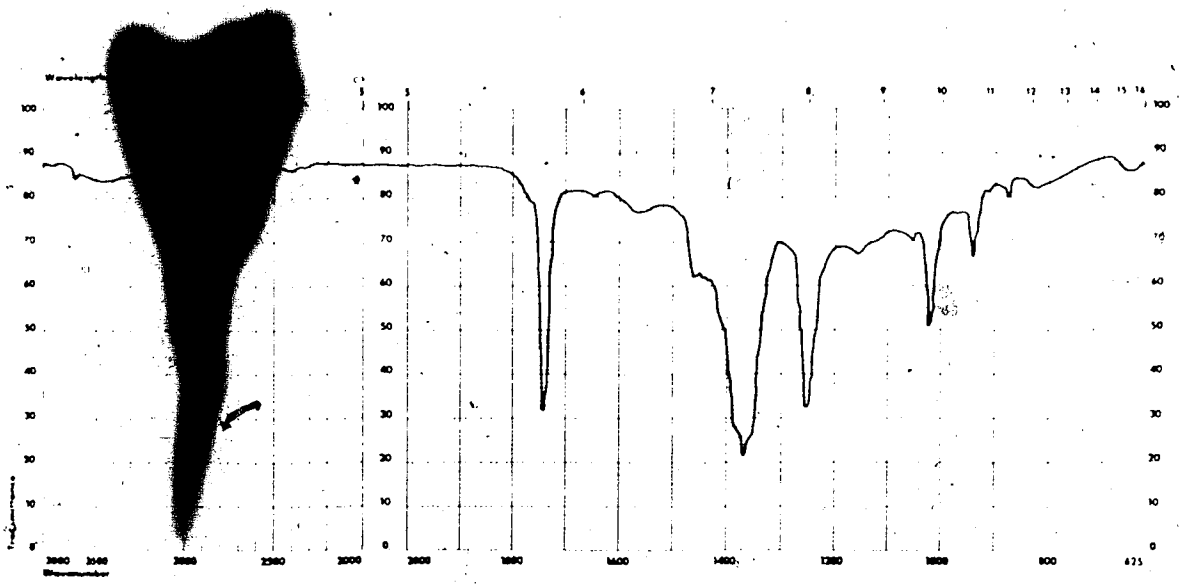


Figure 8: Ir spectrum of cybullol monoacetate (CCl_4)

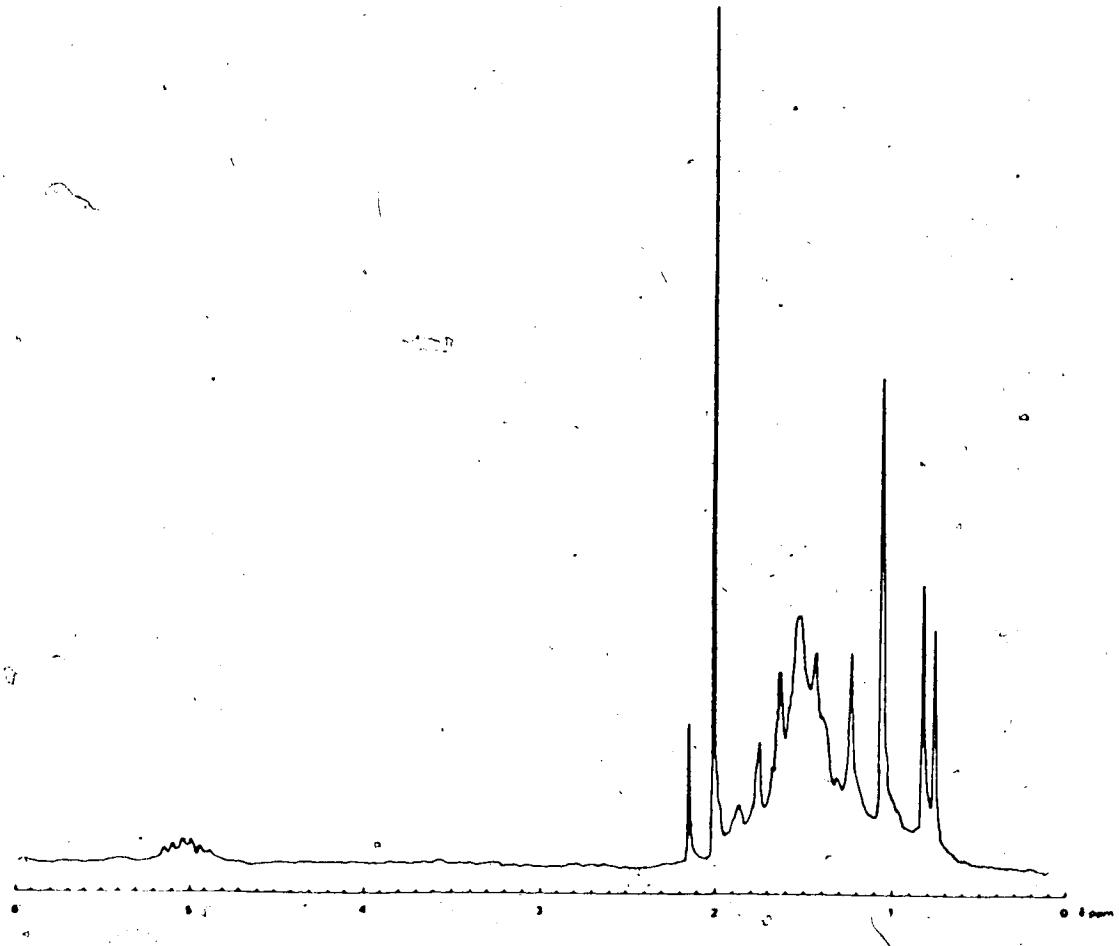


Figure 9: 100MHz Fourier transform (FT) pmr spectrum of cybullol monoacetate ($CDCl_3$)

d) Location and Orientation of the Secondary Hydroxyl Group

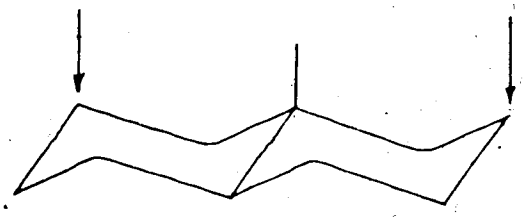
The secondary hydroxyl group in cybullol is equatorial, and the ring carbon to which it is bonded is flanked by methylene groups. This can be deduced from the width at half height ($W_{1/2}$) and multiplicity of the signal at δ3.9 in the cybullol spectrum (Figure 5) and at δ5.0 in the monoacetate spectrum (Figure 9). The signal, caused by the proton geminal to the hydroxyl or acetate function, is a symmetrical heptet. This pattern can be caused by coupling to six equivalent adjacent protons. Alternatively two sets of two equivalent adjacent protons produce a triplet of triplets which may overlap and appear as a heptet. Clearly the first alternative is not possible since the proton causing this signal is not geminal to two methyl groups. The heptet in the pmr spectrum of cybullol can therefore only be caused by a proton on a carbinol carbon flanked by two methylene groups.

The Hassner rule,[23] applicable to protons geminal to an hydroxyl group, states that the $W_{1/2}$ of the bands due to equatorial protons is 5-10Hz, while that for axial protons is 15-30Hz. The $W_{1/2}$ of the signal in the pmr spectra of cybullol and its acetate is approximately 25Hz. The proton geminal to the secondary hydroxyl in cybullol must be axial and the hydroxyl group is therefore equatorial.

The equatorial orientation of the secondary hydroxyl group was confirmed by epimerization of cybullol in the following manner. Cybullol was oxidized to a keto-alcohol using

Jones' reagent. Details of the keto-alcohol are given in the next section. The keto-alcohol was reduced with sodium borohydride to give a mixture of cybullol and epicybullol (1:4 by gc). The epimers were separated by ptlc; epicybullol was less polar than cybullol. The 100MHz spectrum of epicybullol (Figure 10) shows a multiplet at δ4.1 having a $W_{1/2}$ of approximately 8Hz. The downfield shift and decrease in $W_{1/2}$ of this signal compared to the corresponding signal for cybullol are both indicative of an equatorial proton[24] and thus epicybullol has an axial secondary hydroxyl group.

The pmr spectrum of epicybullol also shows an informative downfield shift of the tertiary methyl signal when compared with the same signal from cybullol. This shift, from δ1.03 to δ1.26, is typical of the shift produced by the introduction of a 1,3 diaxial hydroxyl-methyl interaction in a cyclohexane ring.[25a,b] Thus in the decalin systems shown below, the new secondary hydroxyl group can only be in one of the arrowed positions.



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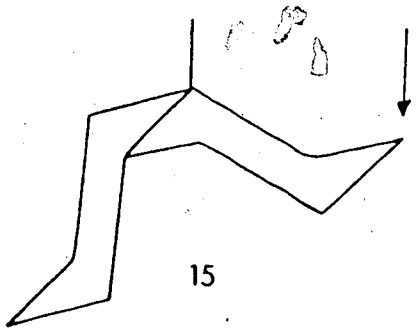

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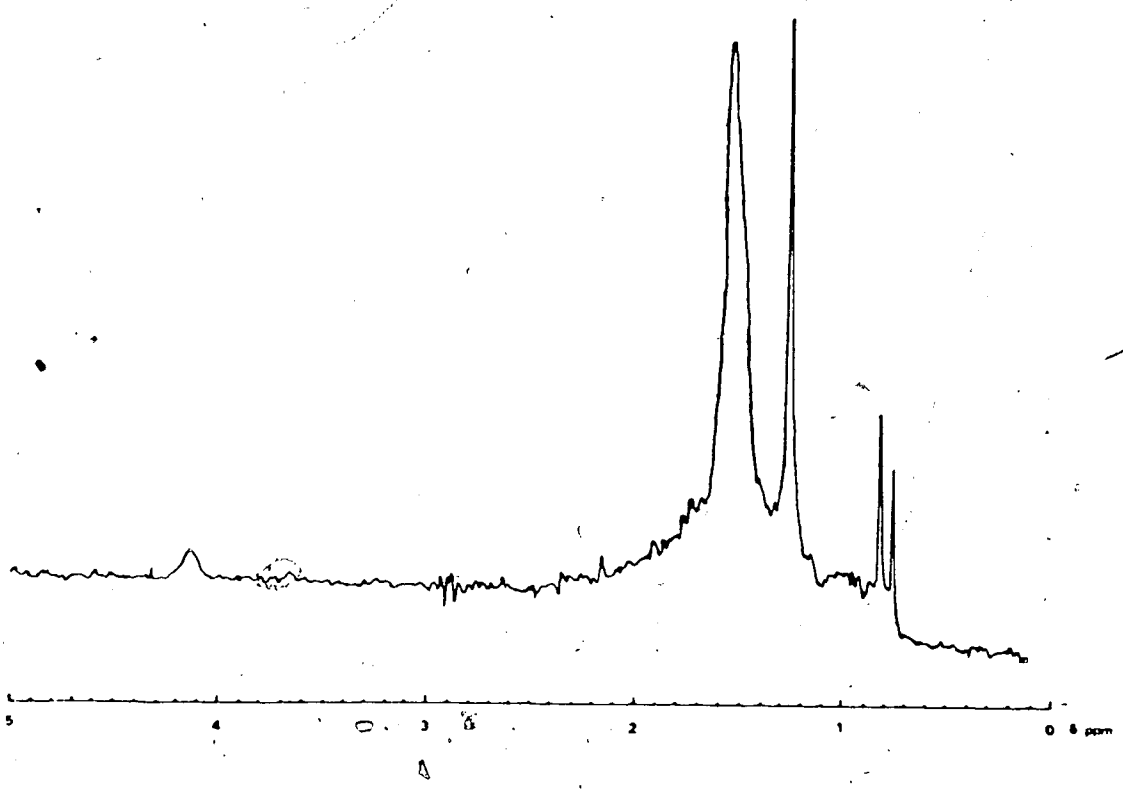
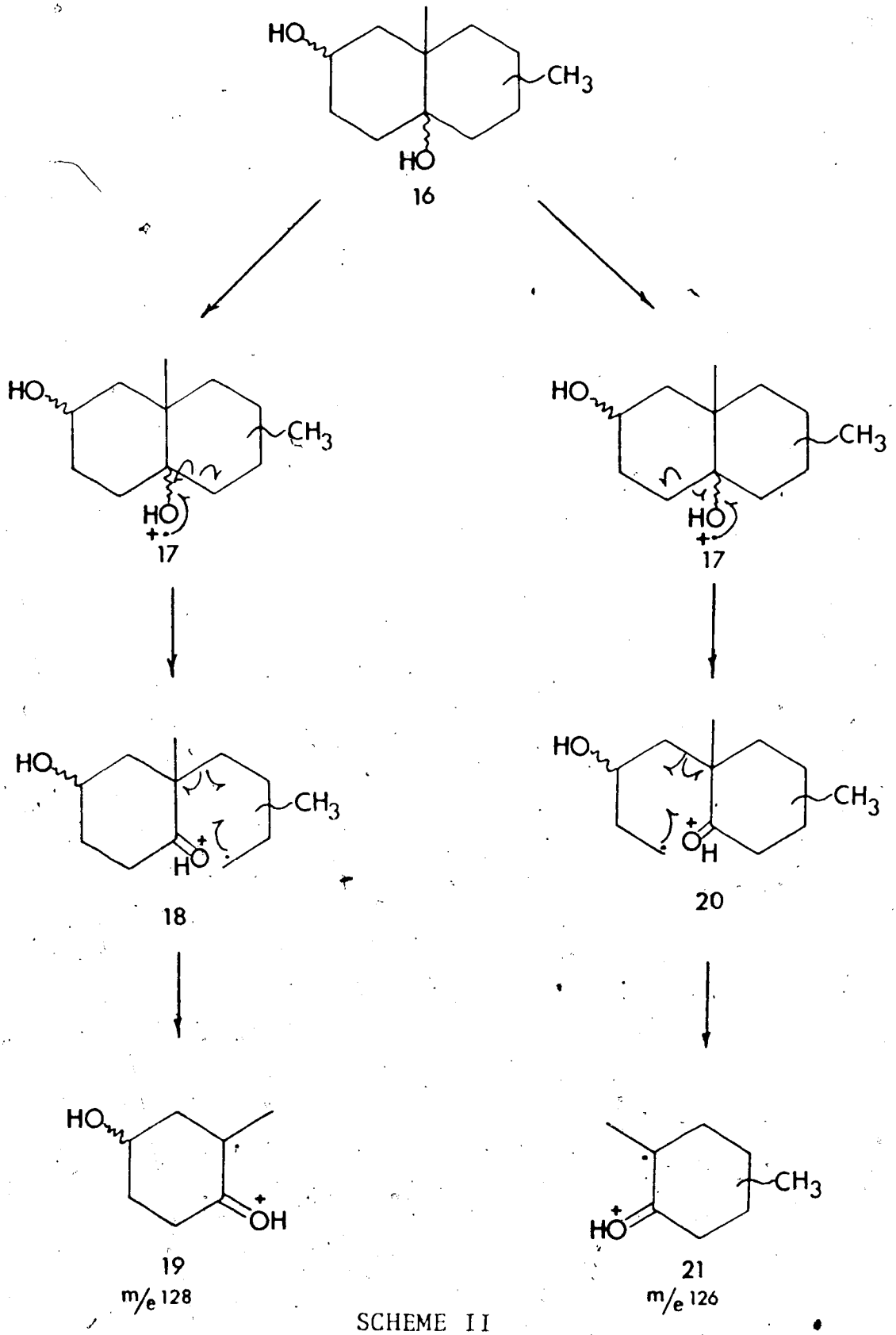


Figure 10: 100MHz (FT) pmr spectrum of epicybullol ($CDCl_3$)

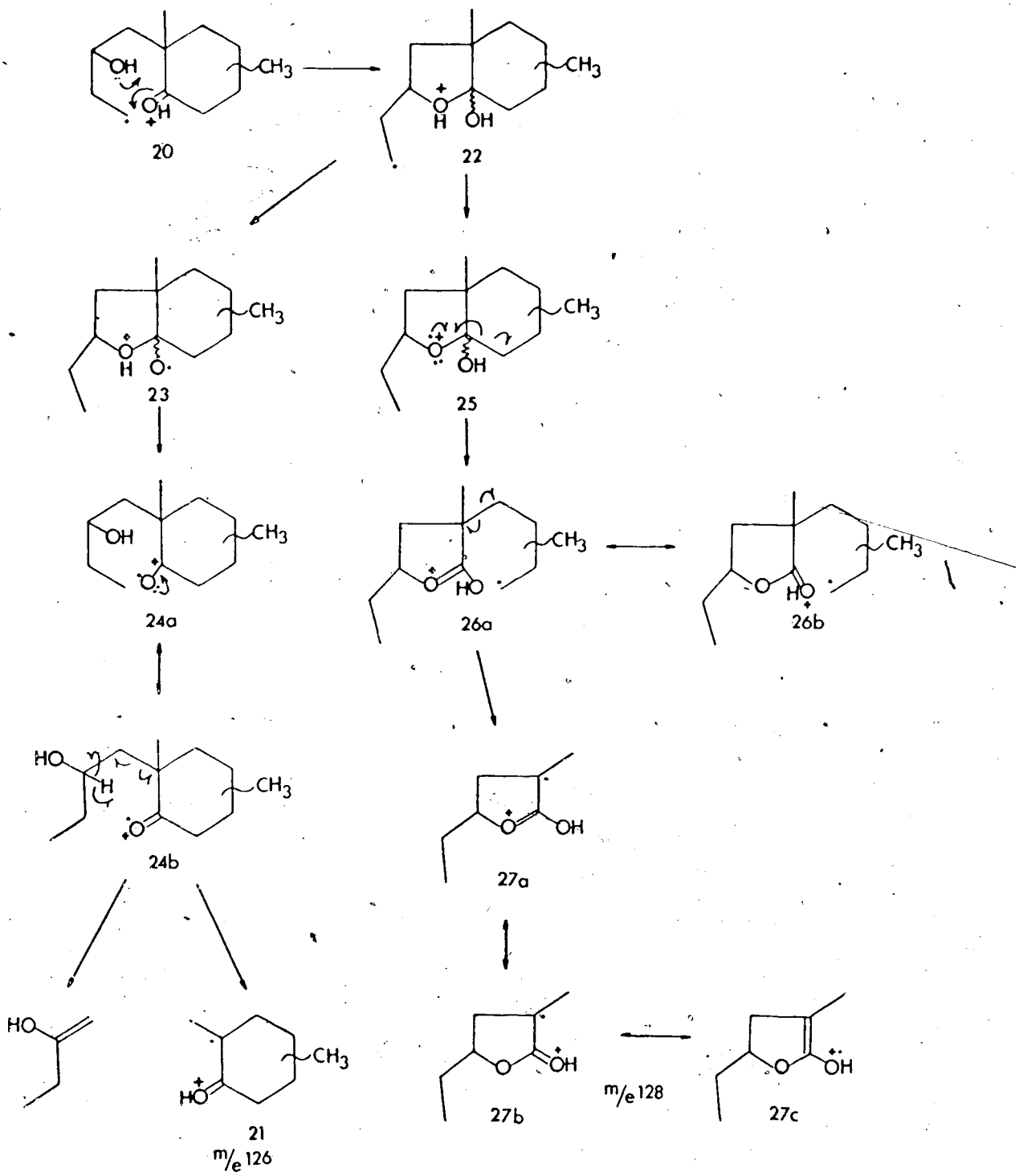
At this stage it is useful to further analyze the mass spectrum of cybullol (Figure 3). Fragments with mass numbers 128 (base peak) and 126 have the formulae $C_7H_{12}O_2$ (meas. 128.0801, calc. 128.0837) and $C_8H_{14}O$ (meas. 126.1020, calc. 126.1044) respectively. They can be explained by the mechanism shown in Scheme II if the secondary methyl and hydroxyl groups are not in the same ring.

A mechanism analagous to Scheme II has been proposed for the mass spectral fragmentation of a number of 5-hydroxysteroids.[26a] The ms of deuterated cybullol (Figure 6) contains peaks at m/e 129 and 130 confirming the identity of the m/e 128 fragment. However m/e 126 is not shifted to m/e 127 in the deuterated compound. This can be explained by an internal hydrogen exchange during fragmentation. An alternate scheme, accounting for this exchange, is shown below (Scheme III). This scheme is more plausible than Scheme II since it predicts both the retention of m/e 126 and the appearance of m/e 130 in the ms of cybullol-d_2. Possibly Scheme II represents a generalized mechanism for fragmentation of decalins with a ring junction hydroxyl group, while Scheme III becomes more energetically favorable when a secondary hydroxyl group is suitably orientated to form a five membered ring.

As expected, in the mass spectrum of the monoacetate (Figure 7) the m/e 128 peak is not present. If the same fragmentation mechanism as in Scheme II were involved, peaks at m/e 170 and m/e 126 would be predicted. In fact, there


HO
CH3
HO
16
HO
CH3
HO
17
HO
CH3
HO
17
HO
CH3
H
O
18
HO
CH3
O
H
20
HO
OH
19
m/e 128
CH3
HO
21
m/e 126

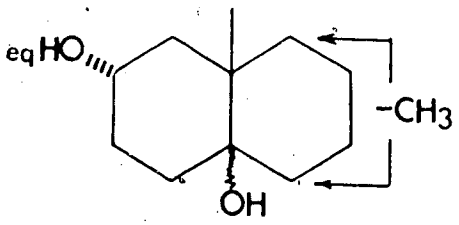
SCHEME II



SCHEME III

are prominent peaks at m/e 126 (40%) and m/e 110 (37%). If the fragment of mass 170 loses a molecule of acetic acid, then a mechanism analogous to Scheme II does explain the major fragmentation of the monoacetate. In Scheme III, replacement of the secondary hydroxyl group by an acetyl group blocks the initial ring formation. This mechanism could not operate in the case of the acetate. However, an alternative mechanism to Scheme II is still possible. Initial loss of acetic acid, followed by low energy electron transfers, as in Scheme IV, may well represent a more energetically favorable route to fragments of mass 126 and 110. No metastable transition has been detected for either of the possible processes.

The partial structure of cybullol, deduced from the information discussed so far is summarized in 33.



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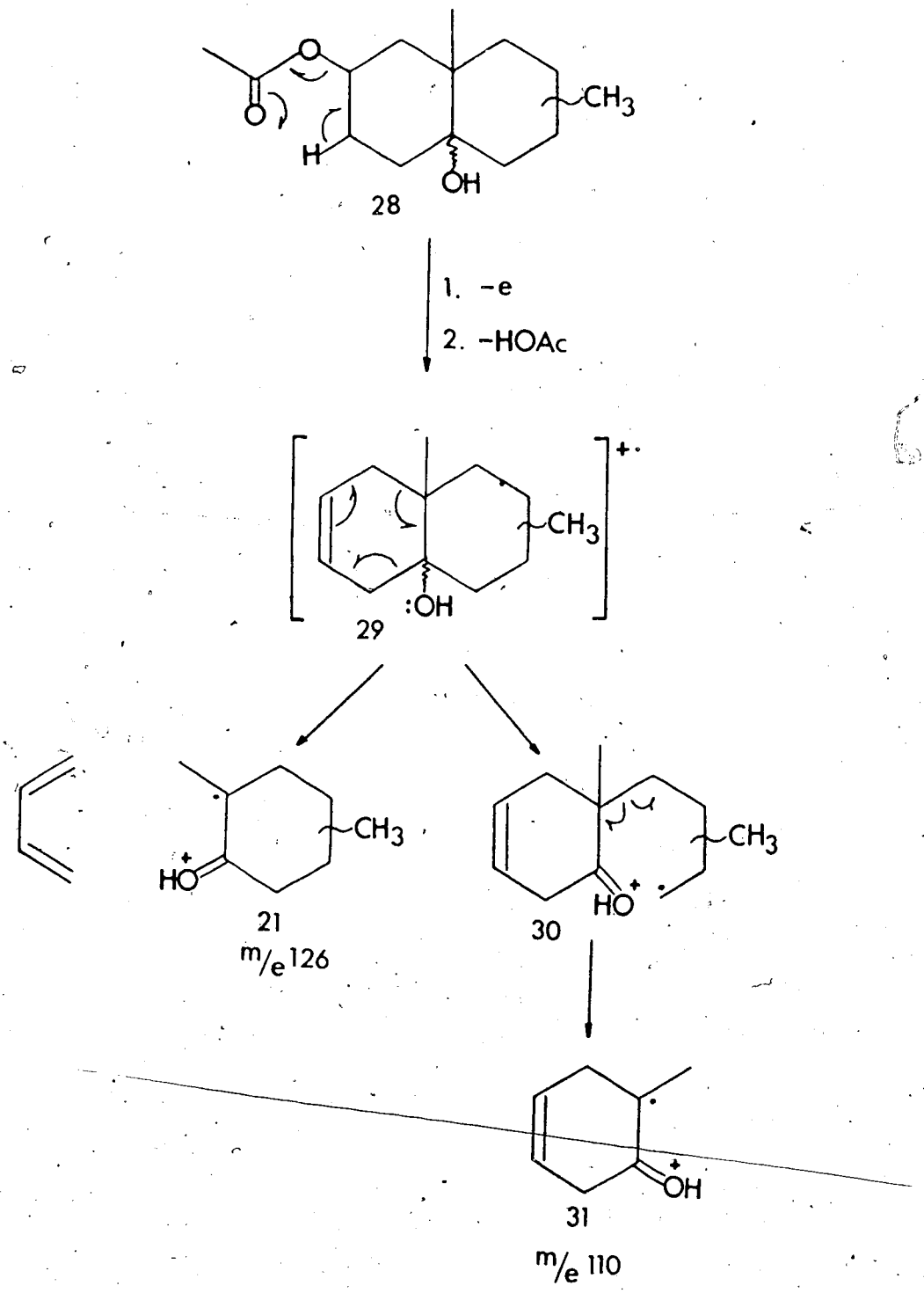
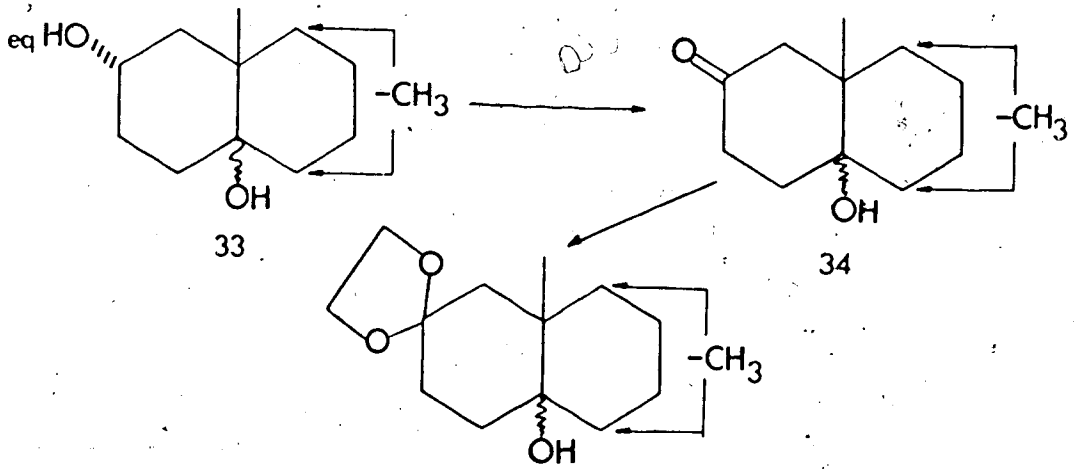
e) Location of the Secondary Methyl Group

To determine which position the secondary methyl group occupies in the decalin system (structure 33), it was necessary to prepare the ethylene ketal 35 via the keto-alcohol 34. Mass spectral fragmentation patterns of ethylene ketals,


CH3
H
OH
28
1. -e
2. -HOAc
CH3
:OH
29
CH3
HO
21
m/e 126
CH3
HO
30
OH
31
m/e 110

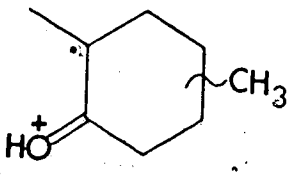
SCHEME IV

extensively studied by Djerassi [26b] provide information concerning the structure in the vicinity of the ketal function.



Cybullol was oxidized using Jones' reagent to give a single product (by gc, tlc) in over 70% yield. The ms (Figure 11) contains the expected parent peak of m/e 196. The lack of a m/e 128 peak and the increase in intensity of the peak at m/e 126 confirm the earlier analysis of fragmentations in the mass spectrum of cybullol.

The peak at m/e 126 was found to be a doublet by hrms, caused by the radical ions $C_8H_{14}O$ (75%; meas. 126.1042, calc. 126.1045) and $C_7H_{10}O_2$ (25%; meas. 126.0678, calc. 126.0681.) By analogy with Scheme II, the radical ions are structures 21 and 36 respectively.



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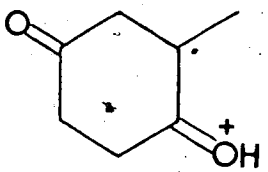

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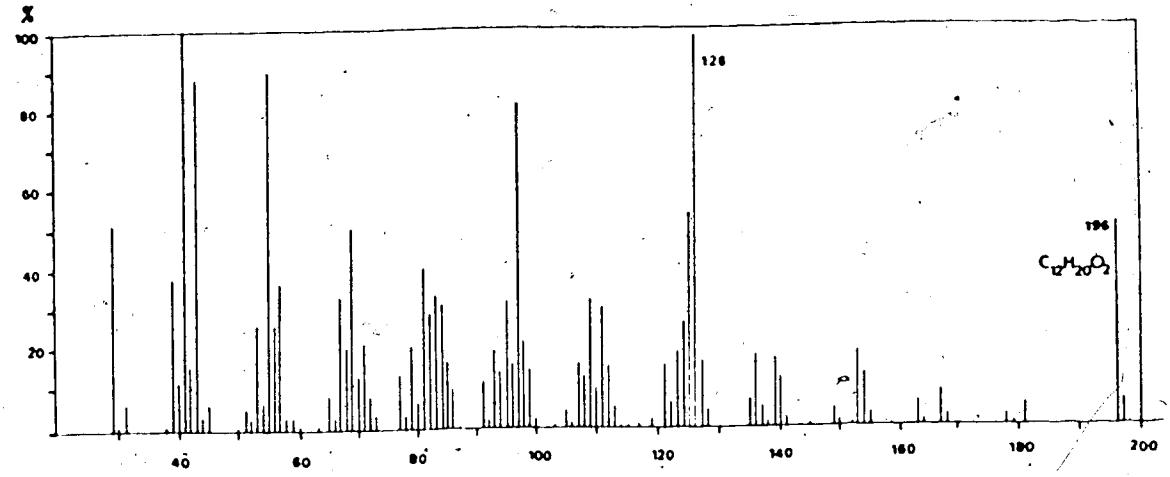


Figure 11: Mass spectrum of keto-alcohol 80

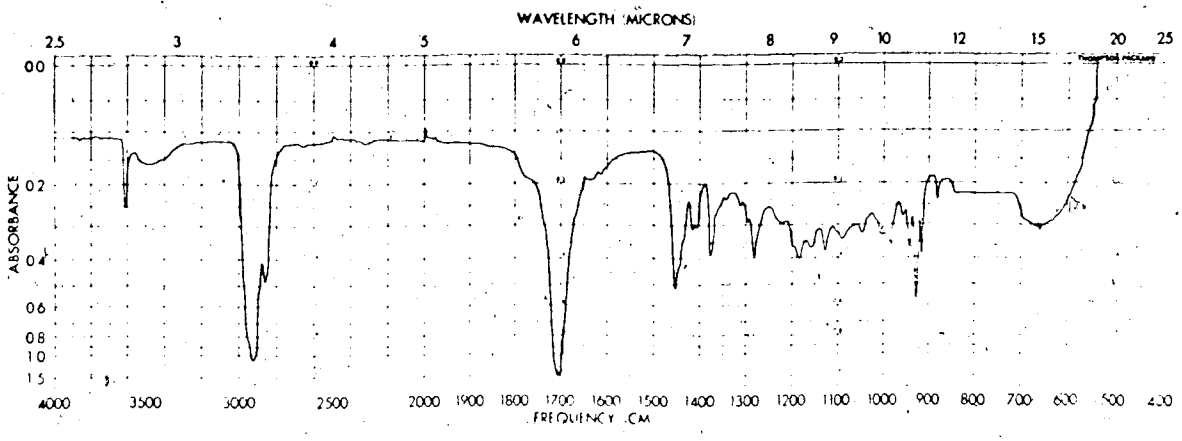


Figure 12: Ir spectrum of keto-alcohol 80 (CCl_4).

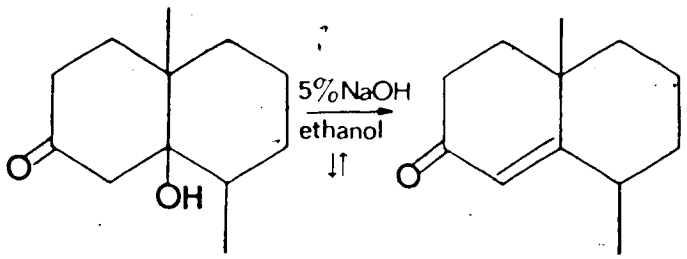
However, carrying the analogy with Scheme II further, one would expect 21 to carry a higher percentage of the ion current than 19 in the fragmentation of cybullol (cf. 21 (75%) and 36 (25%) in the fragmentation of the keto-alcohol 34). This again indicates that the fragmentation of cybullol may take place by the alternative mechanism proposed in Scheme III.

The ir spectrum (Figure 12) shows both carbonyl and hydroxyl absorptions. The tertiary hydroxyl group therefore did not undergo acid-catalyzed dehydration during Jones' oxidation.

The circular dichroism (cd) and optical rotatory dispersion (ord) spectra of the keto-alcohol show a positive Cotton effect at 288nm. The cd spectrum has a molar coefficient of dichroic absorption ($\Delta\varepsilon$) of 1.73. The amplitude (a) of the ord spectrum was 64. These measurements proved useful in determining the ring junction stereochemistry and absolute stereochemistry of cybullol - to be discussed in later sections.

A further proof of the secondary hydroxyl group position in cybullol is provided by the failure of the ketol to undergo base-catalyzed dehydration. Marshall has utilized base dehydration of 37 to give the α,β unsaturated ketone 38.[27]

If the hydroxyl groups in cybullol were in a 1,3 rela-

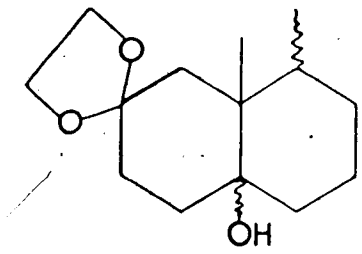


37 38

tionship, base-catalyzed dehydration of its keto-alcohol would be expected to occur. When the keto-alcohol was heated under reflux in 2% ethanolic sodium hydroxide only starting material was recovered, in agreement with partial structure 33 for cybullol.

Ketalization of the keto-alcohol 34 was effected on a small scale by refluxing a benzene solution of 34 with ethylene glycol and p-toluenesulfonic acid. Water was continuously removed from the system by allowing the refluxing benzene to pass over 4Å molecular sieves. Spectral analysis showed that the expected product had been formed. The mass spectrum (Figure 13) contained a parent peak at m/e 240; the ir spectrum (CCl_4 solution) showed an intense band at 1090cm^{-1} (C-O stretch), hydroxyl absorption at 3600 cm^{-1} and only weak carbonyl absorption (1700cm^{-1}).

The major fragments to be seen in the mass spectrum of structures 35a and 35b can be predicted by analogy with Djerassi's proven mechanisms for fragmentation of steroidal ketals.[26b]

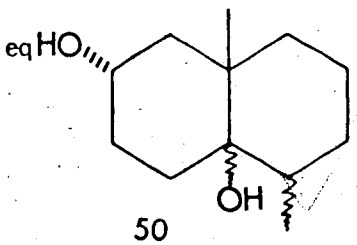


35b

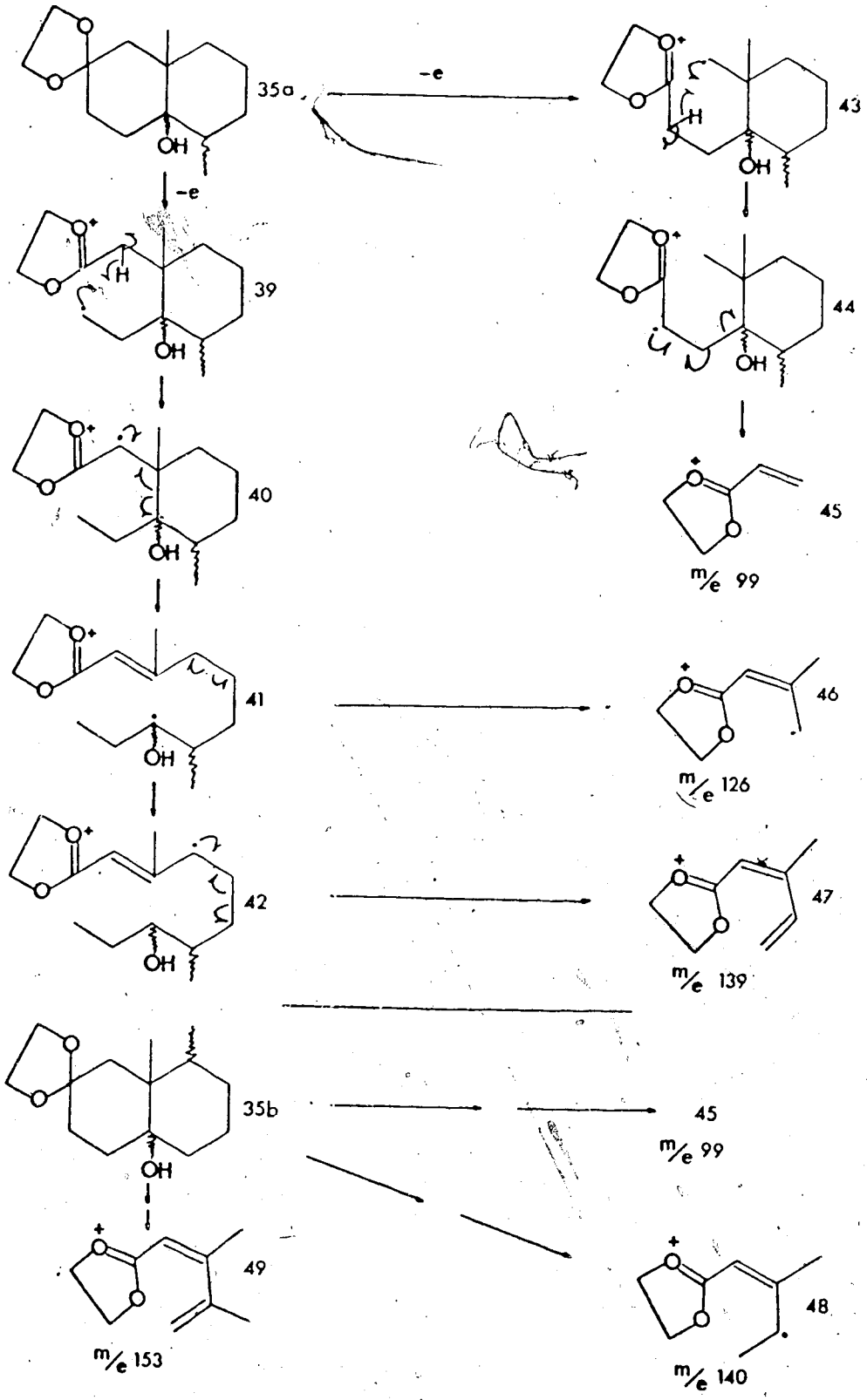
The ethylene ketals 35a and 35b will produce different mass number fragments, (Scheme V); m/e 139,126 and 99 from structure 35a; m/e 153,140 and 99 from structure 35b. The relevant peak heights from the mass spectrum (Figure 13) of the ethylene ketal are shown below.

m/e	% of base peak
153	1.2
140	1.5
139	5.4
126	11.1
113	39
100	100
99	47

Thus the ethylene ketal produced in this reaction must be 35a, and the partial structure of cybullol can now be represented by 50.



50


35a
-e
43
OH
-e
39
44
40
45
m/e 99
41
46
m/e 126
42
47
m/e 139
35b
45
m/e 99
49
48
m/e 153
m/e 140

SCHEME V

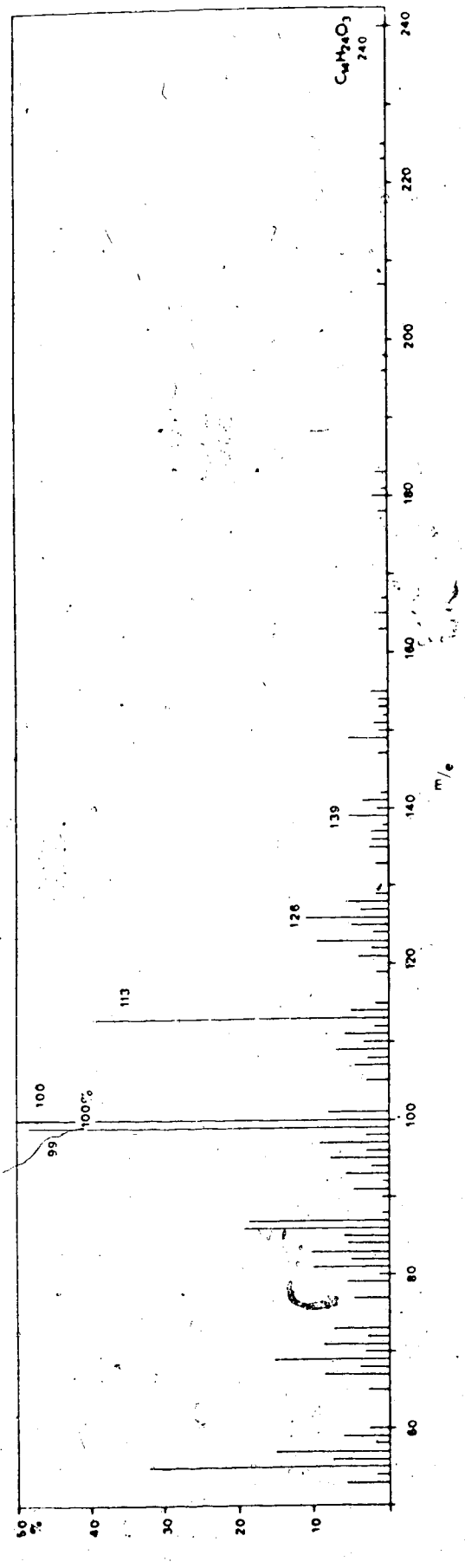
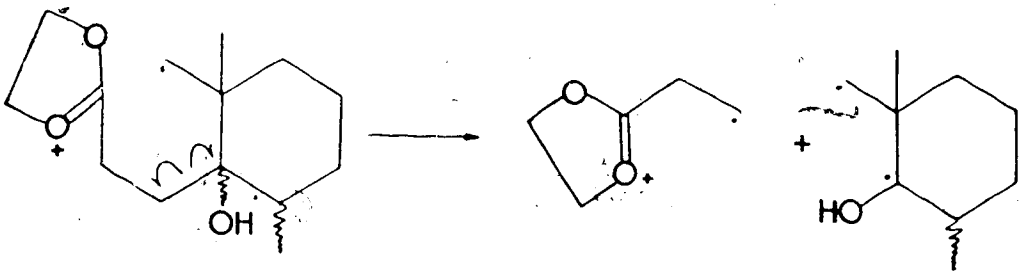


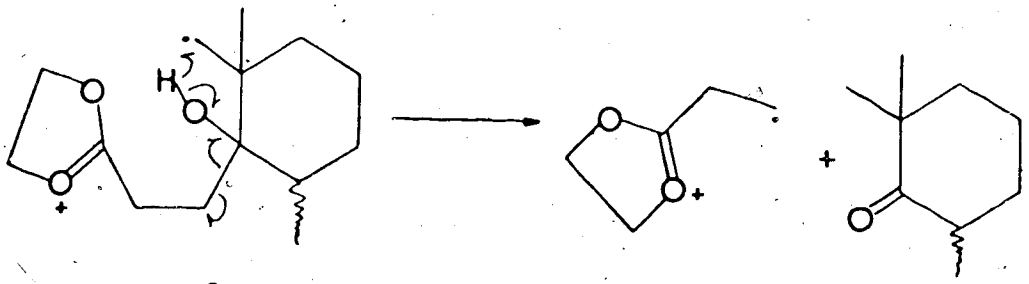
Figure 13: Mass spectrum of ketal 35a

Mass spectra of ethylene ketals of this type usually show a base peak at m/e 99, formed as illustrated in Scheme V. The base peak at mass number 100 in Figure 13 can be rationalized by a fragmentation of the radical ion 43 not involving hydrogen transfer.



43 51 (m/e 100) 52

Alternatively and perhaps more likely, hydrogen transfer from the hydroxyl group to the primary radical through a five-membered transition state and concurrent or subsequent bond cleavage as shown below would lead to the ion radical 51 and 2,2,6-trimethylcyclohexanone (53).

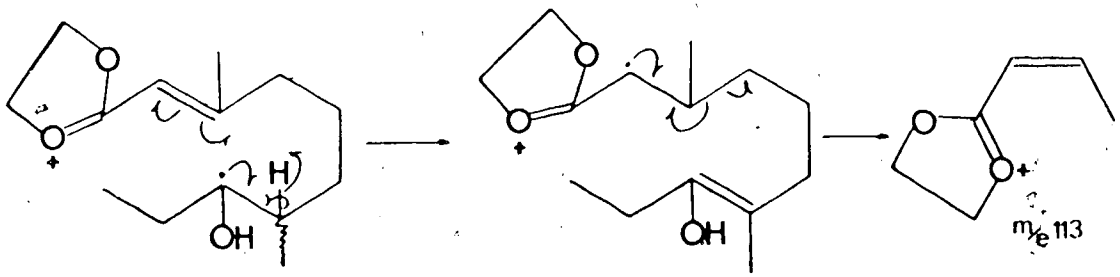


43 51 (m/e 100) 53

It would be interesting to know if the mass spectra of 2-(ethylene ketal)-5-hydroxysteroids also show this anomalous base peak.

Djerassi mentions that steroidal ketals may yield the m/e 99 ion through a six-membered cyclic transfer (ref. 26b,

pg. 37). Applying this mechanism to radical 41 results in a m/e 113 fragment.



41 54 55

The mass spectrum of ethylene ketal 35a (Figure 13) shows a strong peak (38%) of mass number 113.

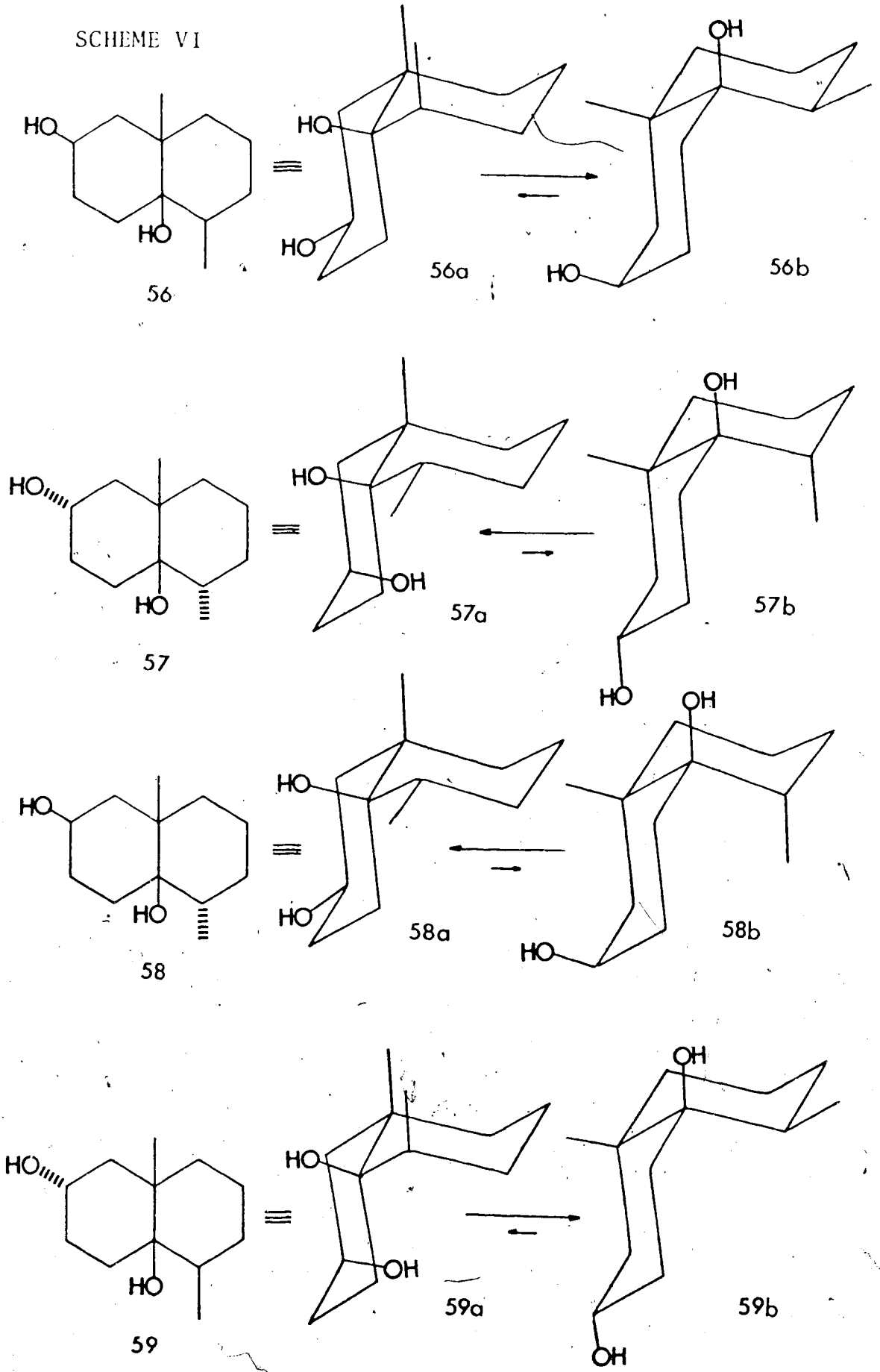
Molecular formulae of fragments with mass numbers 99,100,113,126 and 139, determined from hrms, are all in agreement with the assigned structures.

f) The Ring Junction Stereochemistry

Is cybullol a cis- or trans- decalin system? It is useful to analyze the configurational isomers possible. At this point absolute structures will not be considered, i.e. every structure drawn in this section represents an enantiomeric pair.

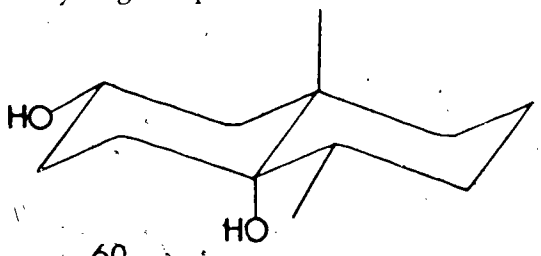
The cis-decalins are conformationally mobile. The four possible stereoisomers (56,57,58 and 59) are shown in Scheme VI. Scheme VI also shows both possible conformations for each configuration. For stereoisomer 56, conformation 56b should be the favored conformation (serious 1,3-diaxial methyl-methyl interaction in 56a) and since this carries an axial secondary hydroxyl group, 56 may be eliminated. Similar reasoning (serious non-bonded interactions in 57b) eli-

SCHEME VI

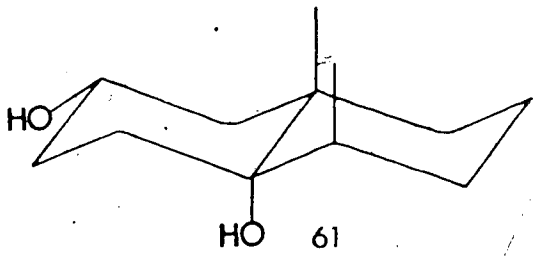

HO
HO
56
56a
OH
HO
56b
HO
HO
57
57a
OH
OH
57b
HO
HO
58
58a
OH
HO
58b
HO
HO
59
59a
OH
OH
59b

minates 57. Thus if cybullol is a cis-decalin it must be either 58 (conformation 58a) or 59 (conformation 59b).

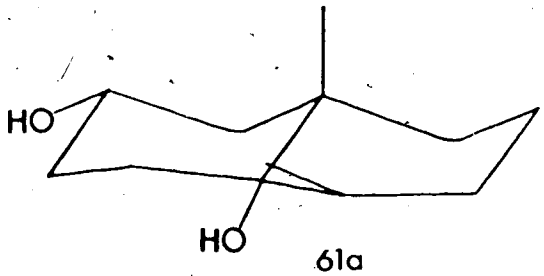
The trans-decalins are conformationally rigid and an axial secondary methyl group must therefore be considered as a possibility. Since trans-decalins have higher symmetry than their cis counterparts, only two structures, 60 and 61, are possible. Conformationally, structure 61 is expected to be distorted from the usual chair-chair arrangement (as shown in 61a), due to severe steric interactions between the two methyl groups.



60



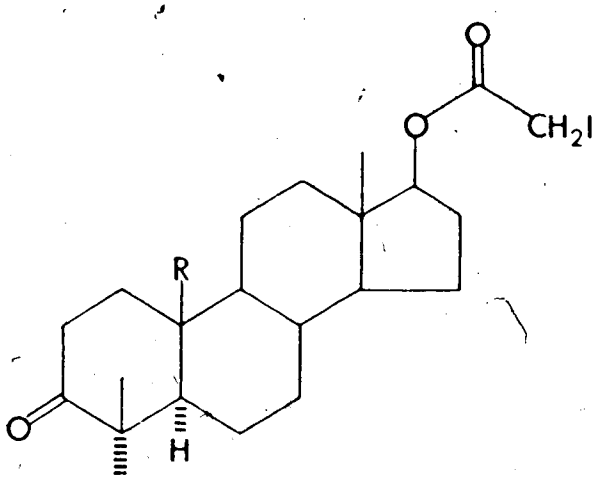
61



61a

An indication that 1,3 diaxial methyl interactions do produce ring distortions is provided from an X-ray analysis of the steroid 62.[28] The A ring is considerably flattened when $R=CH_3$ compared with the normal chair conformation when R=H.

Of the four possible cybullol structures (58a, 59b, 60, 61a), structure 58a can be ruled out by the following evid-



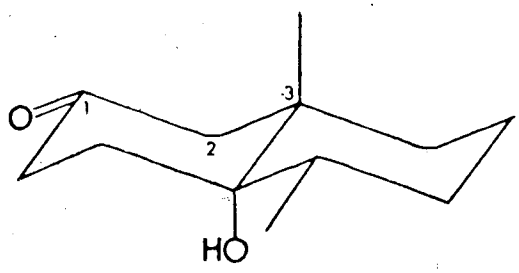
62 R=H, CH_3

ence. The pmr spectrum of the hydroxyl epimer of cybullol (Figure 10), as previously discussed, shows that the secondary hydroxyl group and the bridgehead methyl group are in a 1,3 diaxial relationship. However, epimerization of structure 58a would not produce this geometry.

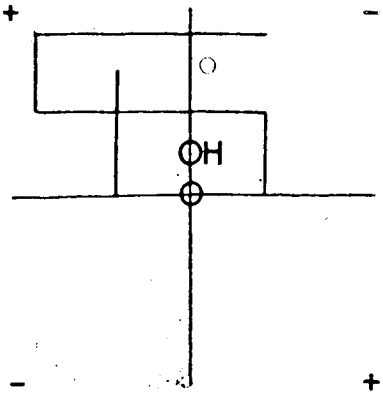
Kirk and Klyne have recently published an empirical analysis of the circular dichroism of decalones.[29] The paper attempts to relate the magnitude of the Cotton effect ($\Delta\varepsilon$) for the $n \rightarrow \pi^*$ transition in ketones to certain structural features. The keto-alcohols (63→66) expected from oxidation of compounds 58→61 are shown in Scheme VII, together with their octant diagrams.

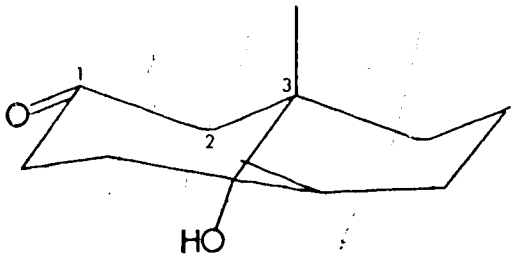
The cd spectrum of the keto-alcohol prepared by oxidation of cybullol contains a positive Cotton effect at 286nm with a differential dichroic absorption ($\Delta\varepsilon$) of +1.73 (methanol solvent). The structural classification of 63, 65 and

POSSIBLE KETO-ALCOHOLS FROM CYBULLOL & THEIR OCTANT DIAGRAMS

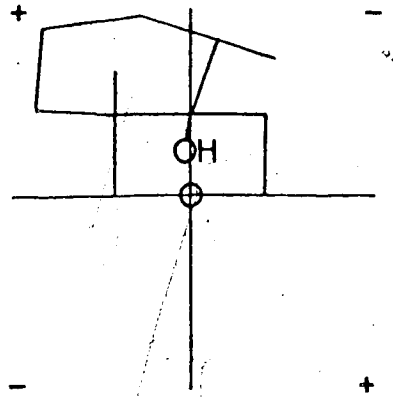


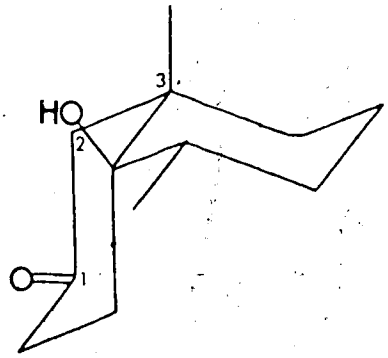
63



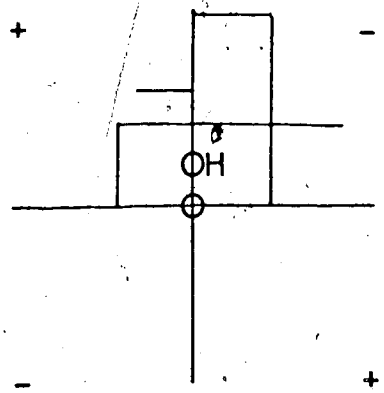


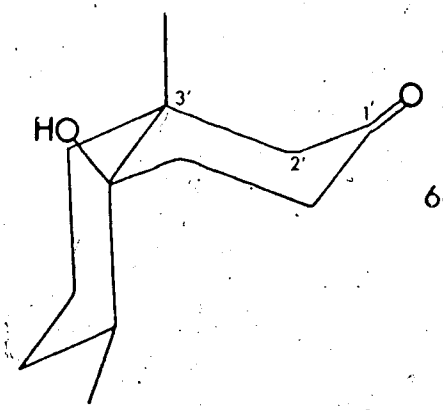
64



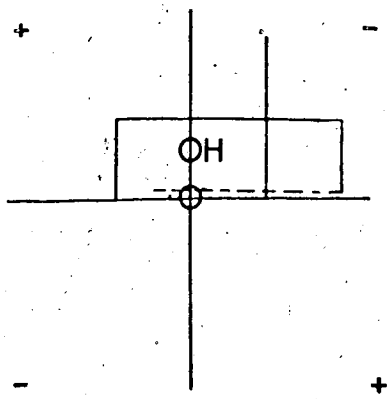


65





66



SCHEME VII

66 and their calculated Δε values are summarised in Table II. The parameters are not applicable to distorted structures, such as 64.

Table II EMPIRICAL CALCULATIONS OF Δε FOR STRUCTURES 63, 65 and 66

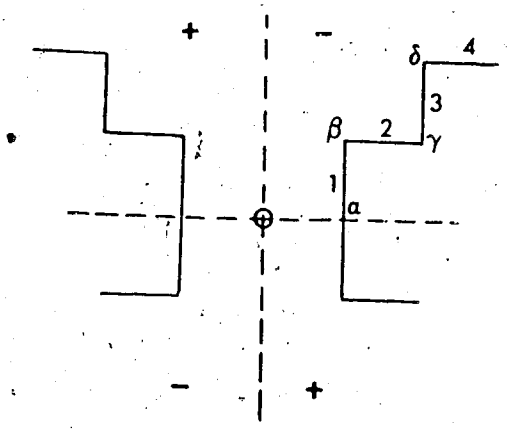
Structure	Ring classification	Δε	Methyl classification	δΔε	Total Δε
63	t3	+1.4	βax(Class 1)	+0.6	+2.0
65	c3ax	+0.1	βeq	-0.6	-0.5
66	c3'eq	-0.5	βax(Class2)	+0.1	-0.4

The structures are analyzed using Kirk and Klyne's method, as follows. The basic decalone ring system is first identified. The trans-decalone 63 is t3 (t=trans, 3=position of ring junction nearest to ketone; see Scheme VII). The cis rings have to be further classified according to the conformation of the particular bond in the second ring located at the numbered bridgehead (ax=axial, eq=equatorial). Structure 65 is therefore c3ax because the bond in the second ring at position 3 is axial to the first ring. Primed numbers are used if the nearest ring junction to the ketone occurs by counting in a clockwise manner. Structure 66 thus becomes c3'eq. This simply allows enantiomers to be distinguished in the classification scheme.

Significantly, of the numerous unstrained c3ax and c3eq systems listed (ref. 29) none has a $\Delta\varepsilon$ value larger than 1.0. The majority of t3 systems have $\Delta\varepsilon$ greater than 1.0. On a comparitive basis the cd measurement is therefore indicative of a trans ring junction in cybullol.

Each decalone ring system has been assigned a $\Delta\varepsilon$ parameter. The parameters are either consignate or dissignate. Consignate terms are positive when the structural feature they describe is in a positive octant and vice versa. Dissignate terms describe features formerly described as showing anti-octant behavior. For example, in 63 the t3 ring system has a consignate $\Delta\varepsilon$ of 1.4. Most of the structure is in a positive octant, so $\Delta\varepsilon$=+1.4. In 65, the c3ax ring system is dissignate; $\Delta\varepsilon$=0.1. As the structure lies mainly in a negative octant, $\Delta\varepsilon$=+0.1.

The $\Delta\varepsilon$ values are modified by methyl groups if present in certain positions of the molecule. These positions, known as 'primary zig-zags', are defined by reference to the octant diagram below.



Only methyl groups attached to these 'primary zig-zags' modify the $\Delta\varepsilon$ values. For example, structure 63 has a 3-bond 'primary zig-zag' in the upper left (rear) octant. The axial methyl group in a β position is classified as βax (class 1) and adds 0.6 (consignate) to the value of $\Delta\varepsilon$. The class 1 designation indicates a 'primary zig-zag' of more than two bonds. In 66, the 'primary zig-zag' is only two bonds in length; the β axial methyl group is defined as βax (class 2). A 0.1 (dissignate) change in $\Delta\varepsilon$ occurs with this class. Other positions of the methyl group on the 'primary zig-zag' are assigned parameters that are not dependant on the length of the 'primary zig-zag'. The βeq methyl group in structure 65 is therefore not further divided into classes.

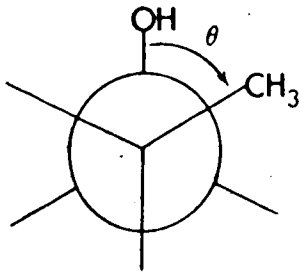
The negative sign of $\Delta\varepsilon$ for the cis-decalones simply indicates that cybullol, with a positive $\Delta\varepsilon$, would be the opposite enantiomer to the two structures drawn. The total $\Delta\varepsilon$, obtained by summing the two contributions (Table II) is much larger for the trans-decalone 63 than for either of the cis-decalones. The distorted structure 64 is expected to have a similar $\Delta\varepsilon$ value to 63, from the octant diagram. The measured value of $\Delta\varepsilon$ (=1.73) is within 0.3 units of the calculated value for the trans-decalone 50. The accuracy of the predicted $\Delta\varepsilon$ values is $\pm$ 0.2 according to the authors,[29] except in cases of strained structures, where ring distortion might be expected to occur. However,

no structures containing a hydroxyl group are mentioned in the paper. The dissignate effects of certain methyl groups may result from an inductive withdrawal of electrons from the 'primary zig-zag' (ref. 29, pg. 1102). The hydroxyl group can be expected to produce such an inductive effect in structures 63,65 and 66, even though it is not located directly on a primary zig-zag. This could increase the error limit in predicted $\Delta\varepsilon$ values.

The difference in the predicted value for the trans-decalone and the measured value of $\Delta\varepsilon$ (~15%) may also be due to experimental error. A 4.32mg sample of the keto-alcohol in 2ml of methanol was used to measure the spectrum. The weighing and volume measurement errors are less than 5%. Impurities in the sample, however, could introduce a larger error. Generally one would expect any impurity to produce a lower $\Delta\varepsilon$ than the true value, since it is extremely unlikely that the impurity will also produce a positive Cotton effect at 286nm. These facts indicate that cybullol has a trans-decalone ring system as in 63 and 64.

The trans ring junction stereochemistry is confirmed by the pyridine-induced chemical shift in the pmr spectrum of cybullol. Proton chemical shifts of numerous hydroxylic compounds have been measured in deuteriochloroform and pyridine-d_5.[30] In compounds containing vicinal methyl and hydroxyl groups, solvent shifts $\Delta(=\delta CDCl_3 - \delta C_5D_5N)$ caused by

vicinal deshielding in the solute-solvent complex are related to the dihedral angle, θ, subtended between the methyl group and the hydroxyl function.



When the dihedral angle is 180°, Δ is approximately -0.03ppm. As θ decreases in magnitude, Δ increases. At approximately 60°, Δ is in the range -0.20 → -0.27ppm. These values were used to predict the Δ values in structures 58 → 61 (Table III). The secondary hydroxyl group in these structures has little effect on the Δ value of the ring junction methyl group signal (cf. 5α-androstan-2β-ol and 5α-androstan-2α-ol; Δ for protons at C-19 is +0.01 and +0.02 ppm respectively).[30]

The measured values of Δ for the ring junction methyl signal in cybullol and trans-1,10-dimethyl-cis-9-decalol (67, sample kindly supplied by Prof. J. A. Marshall) are -0.03 and -0.18 ppm respectively (Table IV). Thus, by comparison of Tables III and IV, cybullol has a trans ring junction. It is not possible to distinguish between the trans-decalols 60 and 61 by this method, since the degree of ring flattening in 61 and hence the dihedral angle θ, is

Table III

PREDICTED Δ VALUES† IN CIS- AND TRANS- DECALOLS

Structure	Tertiary Methyl		Secondary Methyl	
	≃ θ	Δ(ppm)	≃ θ	Δ(ppm)
Cis-decalols 58 and 59	60°	-0.20 → -0.27	60°	-0.20 → -0.27
Trans-decalol 60	180°	-0.03	60°	-0.20 → -0.27
Trans-decalol 61	180°	-0.03	60° → 180°*	-0.03 → -0.27

* Angle cannot be accurately predicted

† = $\delta(CDCl_3) - \delta(C_5D_5N)$

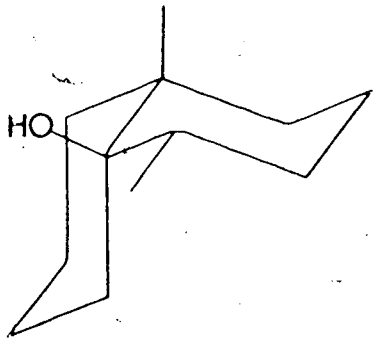
Table IV

MEASURED Δ VALUES*

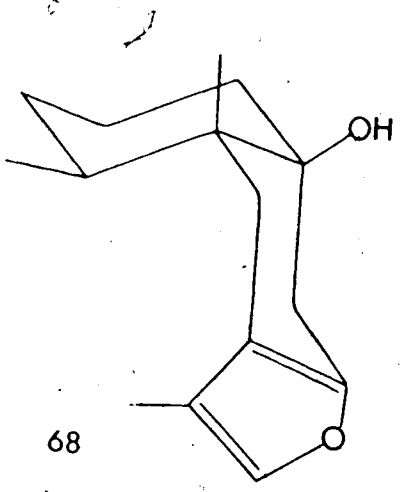
	$CDCl_3$ (δ)	C_5D_5N (δ)	Δ(ppm)
a) cybullol			
Tertiary methyl	1.03	1.06	-0.03
Secondary methyl	0.81	0.95	-0.14
b) trans-1,10-dimethyl-cis-9-decalol (67).			
Tertiary methyl	0.96	1.14	-0.18
Secondary methyl	0.84	0.98	-0.14

* = $(\delta CDCl_3 - \delta C_5D_5N)$

difficult to assess.



67



68

Tetradymol (68) has a structure, determined by X-ray analysis,[31] that compares quite closely with the cis-decalols 58 and 59. The pyridine-induced solvent shift Δ of -0.18 ppm for the tertiary methyl signal in 68 confirms that a cis-decalol system of this type produces a much larger Δ than that observed in cybullol.

The cmr spectrum of cybullol (kindly determined by Prof. J. B. Stothers) shows twelve carbon resonances (Table V). Off-resonance decoupling distinguished the methyl, methylene, methine and quaternary carbons. Specific off-resonance decoupling was used to determine which methyl carbon signal was caused by the ring-junction methyl group. The assignments in Table V were made using the above information.

Table V

CMR CHEMICAL SHIFTS OF CYBULLOL

(ppm)*	assignment
73.6	$-\underline{C}-OH$
38.8	$-\underline{C}-CH_3$
66.8	$-\underline{C}H-OH$
33.9	$-\underline{C}H-CH_3$
45.0	$-\underline{C}H_2-$
34.7	$-\underline{C}H_2-$
31.2	$-\underline{C}H_2-$
30.3	$-\underline{C}H_2-$
29.8	$-\underline{C}H_2-$
20.3	$-\underline{C}H_2-$
21.1	$-\underline{C}-\underline{C}H_3$
15.2	$-\underline{C}H-\underline{C}H_3$

* relative to TMSi, $CDCl_3$ solution

There are no signals present that can be assigned to sp^2 (90→170 ppm) or sp (70→90 ppm) carbons, confirming the assertion made earlier that cybullol contains no unsaturations.

It is possible to predict cmr chemical shifts of cis- and trans-decalin systems. The accuracy of these predictions depends on two factors; a) the availability of chemical shift data for compounds with similar structures to those being studied and b) the accuracy and additivity of parameters used to modify these chemical shifts.

An indication of the ring stereochemistry of cybullol is obtained by comparison of the predicted chemical shifts of structures 58,59 and 60 with the assigned measured values. Parameters and model compounds for 61, a strained structure, do not exist but the chemical shifts predicted for 60 can be compared qualitatively with those of 61.

cis-9-Methyldecalin (69) and trans-2-hydroxy-9-methyldecalin (70) were used as model compounds. Their cmr chemical shifts[32,33] are listed in Table VI. The parameters used to predict the chemical shifts of 58,59 and 60 are shown in Table VII. In general these parameters are an average of effects observed in a number of cyclohexanes[34] or decalins.[32,33] In certain cases (noted in Table VII) the parameter is based on a single observation and must be viewed with some reservation.

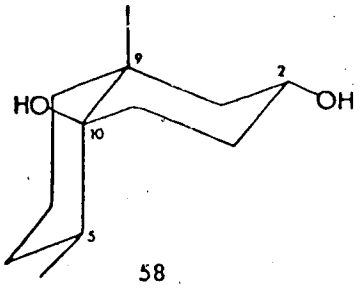
Parameters for the effect of adding a ring junction

Table VI

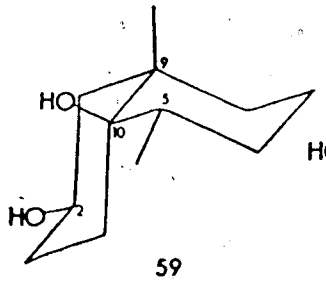
^{13}C SHIELDINGS[a] OF DECALIN SYSTEMS

Structure	69	70	58		59		60	
	obs.	obs.	calc.	obs.	calc.	obs.	calc.	obs.
C-1	30.4	50.9	36.9	45.0	44.0	45.0	44.0	45.0
C-2	22.8	66.9	63.5	66.8	64.1	66.8	66.5	66.8
C-3	21.8	36.4	28.0	29.8	29.5	34.7	29.5	30.3
C-4	28.4	27.9	29.7	30.3	29.4	31.2	31.3	31.2
C-5	28.1	28.1	35.8	33.9	34.3	33.9	35.1	33.9
C-6	27.8	26.9	29.8	31.2	28.6	30.3	28.9	29.8
C-7	22.8	21.2	22.4	20.3	21.8	20.3	20.8	20.3
C-8	42.3	41.6	35.1	34.7	28.0	29.8	34.4	34.7
C-9	33.1	34.7	31.3	38.8	31.3	38.8	35.0	38.8
C-10	41.7	44.9	78.2	73.6	77.4	73.6	82.4	73.6
3°-Me	28.3	16.6	23.8	21.1	23.8	21.1	17.7	21.1
2°-Me		-	15.2	15.2	15.2	15.2	15.2	15.2

[a] in ppm from TMSi $CDCl_3$ solution



58



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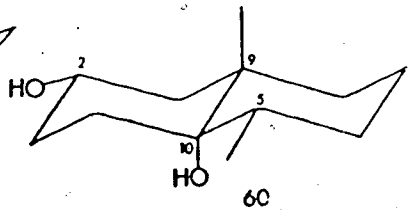

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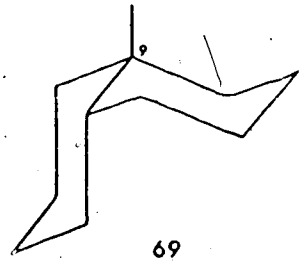
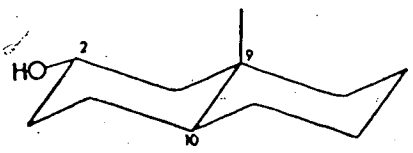


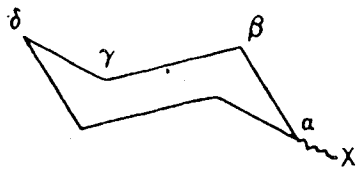
69



70

Table VII

PARAMETERS [32,33,34] (ppm) USED IN CALCULATION OF CHEMICAL SHIFTS OF STRUCTURES 45, 46 AND 47

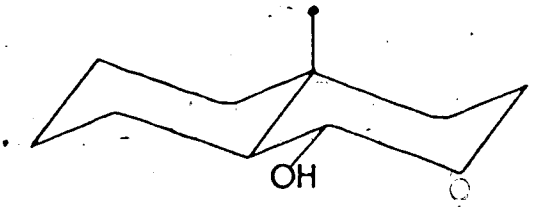


X	α	β	γ	δ
eqMe	5.6	8.9	0.0	-0.3
eqOH	41.7	8.6	-2.1	-1.0[c]
axOH	39.4	6.6	-6.9	-0.4
Ring junction OH[b]	30.9	0.3[a]/3.4		

a) If β position is at ring junction

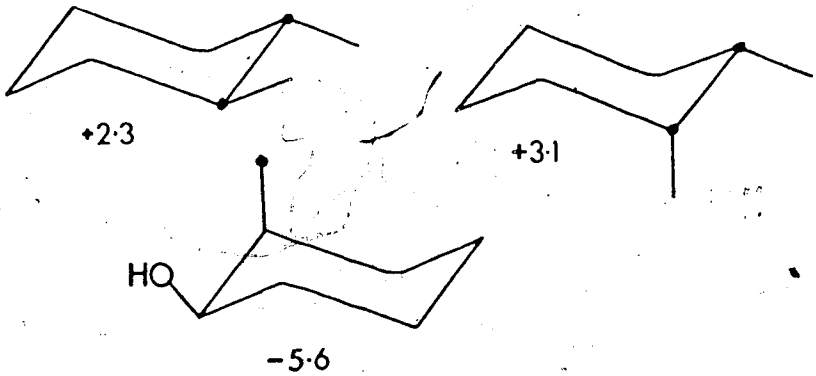
b) Based on one observation (these laboratories)

c) Unless δ carbon = Me:-

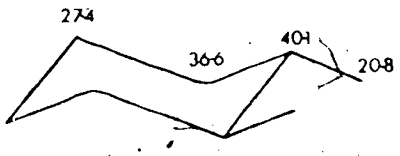


+1·1[35a]

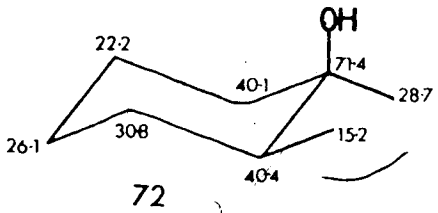
Additional parameters



hydroxyl group to a cis- or trans-decalin structure are not available in the literature. The parameters listed (Table VII) for this substituent were obtained by comparison of the spectra of trans-1,2-dimethylcyclohexane (71) and trans-1,2-dimethylcyclohexan-1-ol (72). The cmr chemical shifts (ppm, relative to TMS) are shown in 71 and 72.



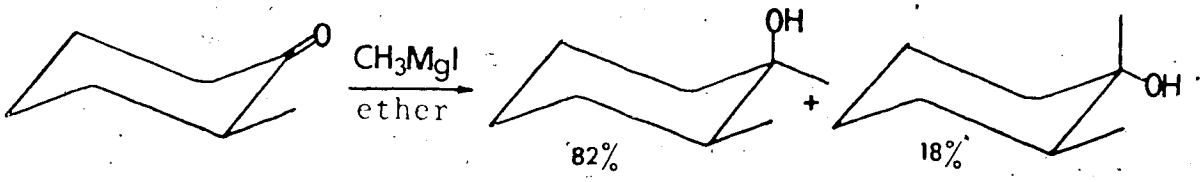
71



72

The parameters derived from these two structures indicate that the ring junction hydroxyl group has less effect on α- and β- carbon shift positions than either an axial or an equatorial hydroxyl substituent.

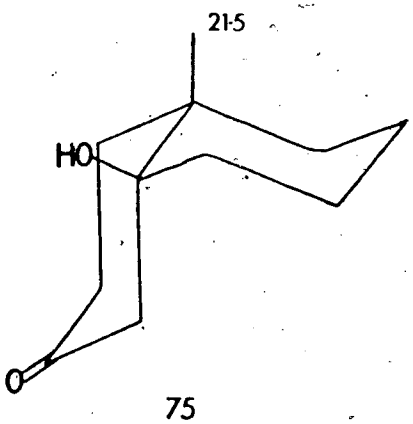
trans-1,2-Dimethylcyclohexan-1-ol (72) was synthesized from 2-methylcyclohexanone (73).



73 72 74

Treatment of 73 with methylmagnesium iodide gave a mixture of isomers 72 and 74 in 71% yield. The major isomer, 72, (82% by gc) was separated by dry column chromatography and identified by comparing chromatographic data with literature values.[35b] The cmr spectrum of 72 was measured.

The calculated and observed cmr shifts of 58, 59 and 60 (Table VI) will be analyzed further. The tertiary methyl signal, measured at 21.1 ppm, seems to indicate from its low field position that cybullol has a cis ring junction. The calculated value of this signal for 58 and 59 (23.8 ppm) is closer to the measured value than the calculated value for 60 (17.7 ppm). Also, the measured value for cybullol is remarkably close to the 21.5 ppm chemical shift of the angular methyl group in cis-10-hydroxy-5-methylcyclohexan-3-one (75).[36]



75

However, the estimated 17.7 ppm chemical shift in 60 must be treated with caution. In calculating this figure, no account was taken of the trans ring junction hydroxyl group, since no model is available from which to calculate a useful parameter for this effect. In general, antiperiplanar heteroatoms at a γ position to carbon produce an upfield shift in that carbon,[37] although exceptions have been noted when the heteroatom is located at the bridgehead of a bicyclic system. From this information one can only conclude that a trans ring junction methyl group could possibly give a signal at a low field position of 21.1 ppm.

One of the methylene signals in cybullol resonates at 45.0 ppm. In calculating the methylene carbon shifts for structure 58, the lowest field signal can only be at approximately 36.9 ppm (Table VI). Significantly, in structures 59 and 60 one of the calculated methylene carbon shifts is very close to the measured low field signal.

A quantitative estimate of the 'best fit' of calculated carbon shifts was obtained by summing the individual differences between calculated and observed values in Table VI The totalled differences Δ for structures 58,59 and 60 are 34.4, 30.1 and 21.1 ppm respectively. The low Δ value for 60 occurs in spite of a large error in the C-10 signal calculation. This error can be attributed to the ring junction hydroxyl parameter which is based on only one observation.

The calculations for 60 started from model 70. Only two substituent effects had to be taken into account, compared with three in the calculation of the cis-decalin shifts. Any error in parameters would increase the Δ value of 58 and 59 more than that of 60. In fact, using trans-1-methyl-decalin as a model for 60 the Δ value increases to 28.1 ppm. This is still lower than either of the cis-decalin Δ values, indicating that cybullol is most probably a trans-decalin system.

trans-1,10-Dimethyl-cis-9-decalol (67) is an excellent model from which to calculate the cmr chemical shifts of 59. The cmr spectrum of 67 was recently determined. Cmr chemical shifts of 59 were then calculated (Table VIII) using the equatorial hydroxyl parameters. The totalled differences, Δ , between measured and calculated chemical shifts of 59 was 23.3 ppm. This high value compared with Δ for 60 (21.1 ppm) again indicates that cybullol is a trans-decalin system.

Structure 61 is expected to show ^{13}C chemical shifts similar to 60, except that both methyl groups would be more shielded due to steric interactions. The extent of this shielding would depend on the amount of ring flattening in the structure. Structure 61 is therefore less likely than 60 because the calculated ring junction methyl signal would be even further away from the observed value of 21.1 ppm.

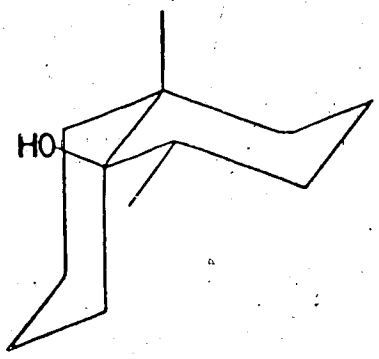
Table VIII

^{13}C SHIELDINGS[a] OF DECALIN SYSTEMS

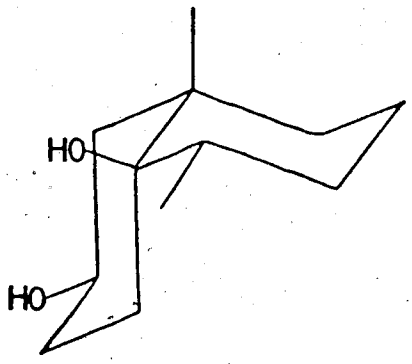
Structure	67	59	
	meas.[b]	meas.	calc. (from 67)
C-1	37.0	45.6	45.0
C-2	21.5	63.2	66.8
C-3	21.2	29.8	30.3
C-4	34.2	32.1	31.2
C-5	37.5	37.5	33.9
C-6	32.5	32.5	34.7
C-7	21.6	21.6	20.3
C-8	26.0	26.0	29.8
C-9	38.0	35.9	38.8
C-10	75.2	73.1	73.6
3°Me	22.3	23.4	21.1
2°Me	14.9	14.9	15.2

a) in ppm from TMSi, $CDCl_3$ solution.

b) determined by Prof. J. B. Stothers on a sample supplied by Prof. J. A. Marshall.



67



59

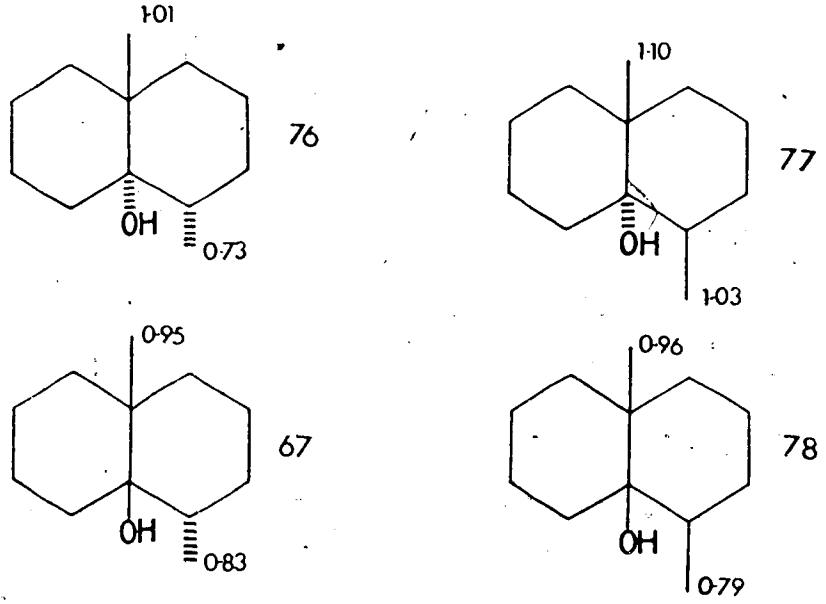
In summary, all of the spectral data, except the cmr ring junction methyl signal, can be explained if cybullol has a trans-decalin ring system. Even the anomalous methyl signal is not conclusively negative evidence, since model compounds are unavailable for the trans-9-methyl-10-hydroxy decalin system.

g) Correlation of Cybullol with Geosmin

Geosmin (from the Greek "ge" = earth, "osme" = odor) is an earthy-smelling substance first isolated from Actinomycetes by Gerber.[38,39] Its structure was proven to be trans-1,10-dimethyl-9-decalol (76).[40,41] Since its initial isolation in 1965, geosmin has been found in cultures of the algae Symploca muscorum[42] and Oscillatoria tenuis,[43] as well as in other Actinomycetes cultures.[44] The compound has been shown to be a causative agent of musty drinking water in Ohio.[45]

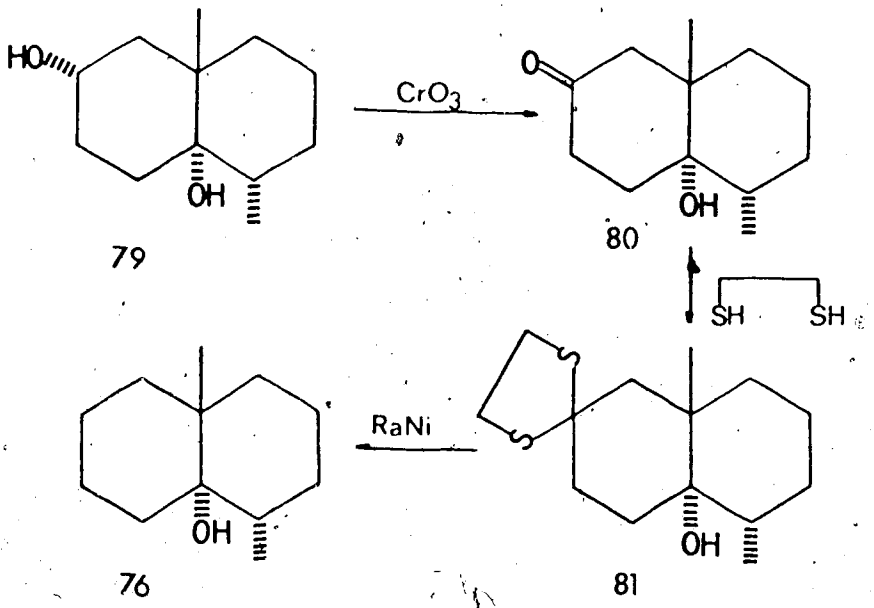
In proving the structure of geosmin, Marshall has synthesized[41] all four geometric isomers of 1,10 dimethyl-9-decalol (67,76,77,78; Scheme VIII). The structure of cybullol (79) has been conclusively proven by removal of the secondary hydroxyl group and comparison of the product with the above isomers. The conversion required three steps (Scheme IX): Jones' oxidation of cybullol, thioketalization of the resulting keto-alcohol and Raney nickel hydrogenolysis of the thioketal.

Pmr (CCl_4) CHEMICAL SHIFT OF METHYL SIGNALS (δ ppm)



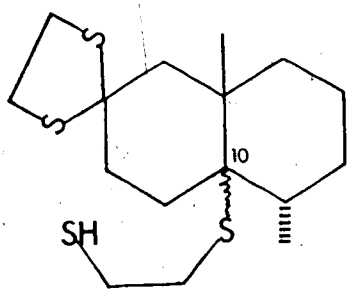
SCHEME VIII

CONVERSION OF CYBULLOL TO GEOSMIN



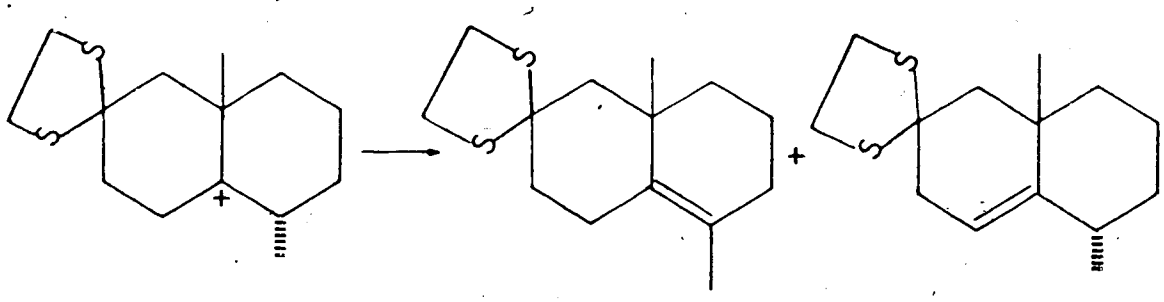
SCHEME IX

Jones' oxidation of cybullol gave the keto-alcohol 80, as previously described (pg.37). Various reaction conditions were investigated in order to obtain the thioketal (81). Initially the keto-alcohol (6.5mg) was dissolved in 1,2-ethanedithiol (0.5ml) containing boron trifluoride etherate (25µl). After stirring for one hour at 25°C, followed by work-up under basic conditions, a mixture of four products (by gc, relative retention times 0.60→0.65, cf. starting material, 0.57) was obtained. A mass spectrum of the mixture has a parent peak at m/e 348, which indicates that one of the epimers of 82 is a component of the reaction mixture.



82

One can envisage 82 being formed through the carbonium ion intermediate 83. Proton abstraction from this intermediate would give either 84 or 85. The four products are thus tentatively assigned structures 84,85 and the two C-10 epimers of 82. After separation of the mixture by prep. tlc a mass spectrum of one of the fractions did show the parent peak expected for 84 or 85 (m/e 254).



83 84 85

In order to moderate the reaction conditions, the milder Fieser method[46] was investigated. The keto-alcohol (80) dissolved in glacial acetic acid was treated with approximately four equivalents each of 1,2-ethanedithiol and boron trifluoride etherate. After the reaction mixture was stirred for one hour at 25°C, only one product was detected along with unreacted starting material. Analysis of the product by gc indicated 64% starting material and 36% product (relative retention time 0.87). By optimizing reaction conditions (see detailed experimental, pg. 123) the conversion to product was increased to 75%. After separation of the two components by prep. tlc, the less polar component gave a mass spectrum (Figure 14) consistent with structure 81.

It is interesting to compare the mass spectrum of thioketal 81 with that of the ethylene ketal (35a) (Figure 13). As mentioned by Djerassi (ref. 26b, pg. 25), the ethylene ketal function is far superior to the thioketal in directing fragmentation in a predictable manner. This is demonstrated by the relative intensities of the parent peak and the major

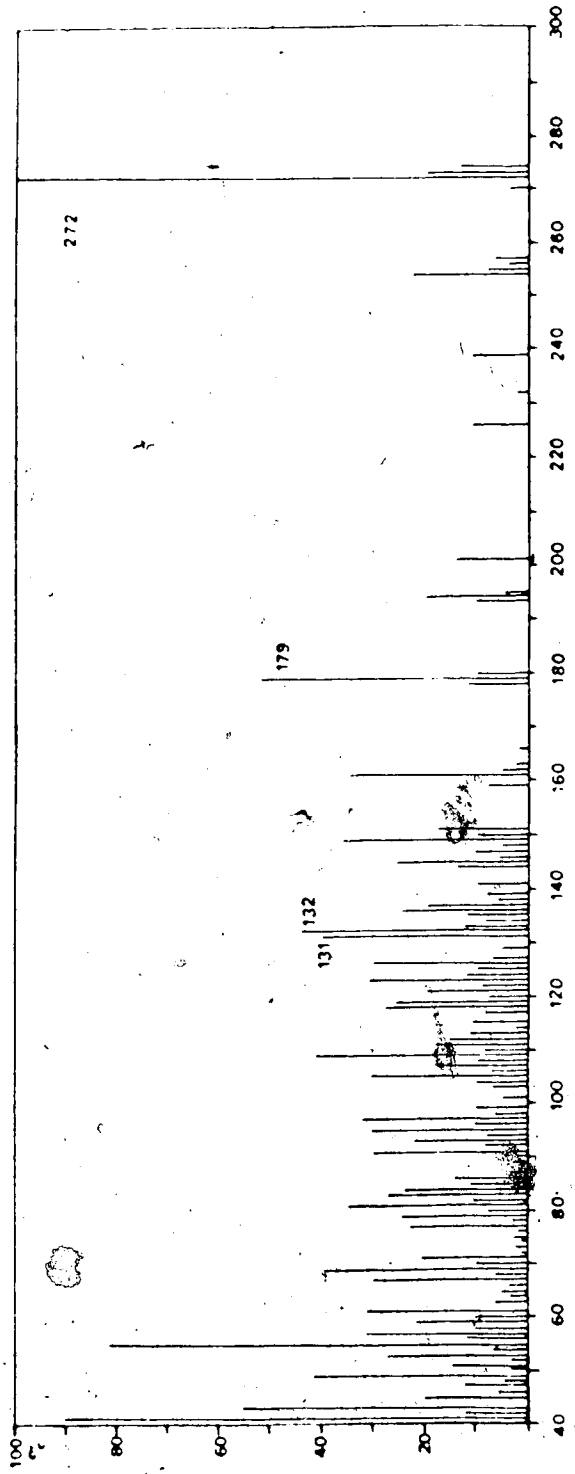
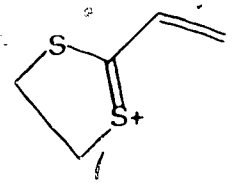
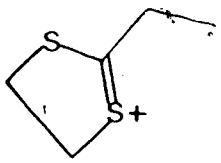


Figure I4: Mass spectrum of thioketal 8I

fragments in the two spectra; in Figure 14 the parent peak is the base peak, while in Figure 13 the parent peak is less than 5% of the base peak. However, there are similarities in the two spectra; the peaks at m/e 131,132 are caused by formation of fragments 86 and 87, by analogy with fragments 45 and 51 from the ethylene ketal.



86

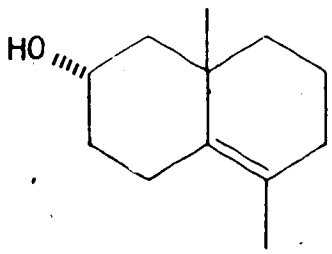


87

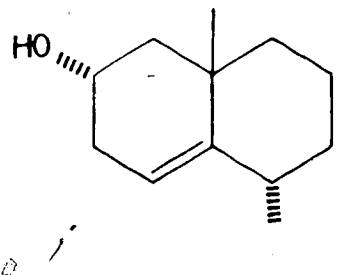
Since prep. tlc of the thioketalization reaction mixture resulted in considerable loss of material, the majority of the product was used without purification in the final hydrogenolysis step. A solution of the thioketal in 95% ethanol was stirred with W-2 Raney nickel[47] at 25°C. The reaction was monitored by gc; after four hours thioketal 81 had been converted to three products with gc relative retention times 0.17 (58%), 0.15 (15%) and 0.08 (27%). Some keto-alcohol present in the starting material was unchanged. The identity of the three hydrogenolysis products was established by gc-ms. The major product (Rt=0.17) displayed an apparent parent peak at m/e 182 and a base peak at m/e 112 (Figure 15), as found in the ms of geosmin.[43]

The other products are tentatively assigned structures

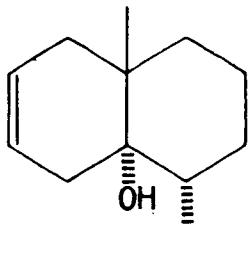
88 or 89 (Rt=0.08) and 90 (Rt=0.15) on the basis of the following evidence. The ms of 90 (Figure 17) contains a small parent peak at m/e 180 and a base peak at m/e 126, in agreement with the expected facile retro-Diels-Alder fragmentation of the molecular ion (Scheme IV, 29→22).



88



89



90

Also, as expected, the gc relative retention time of 90 (Rt=0.15) is very similar to the Rt=0.17 product, later identified as geosmin. The ms of 88 or 89 (Rt=0.08; Figure 16) contains a parent peak at m/e 180, but no predominant ring fragmentation ion. As shown in Schemes II→IV, major ring fragmentations are directed by a stabilized positive charge on the ring junction hydroxyl group. Consequently no such ring fragmentation is observed for 88 or 89, where a ring junction hydroxyl group is not present.

Van Tamelen has noted[48] that deactivated Raney nickel often produces olefins as minor products during hydrogenolysis reactions. As W-2 Raney nickel has a short shelf-life, it was suspected that deactivation might be causing the problem in this case. The reaction conditions were modified to

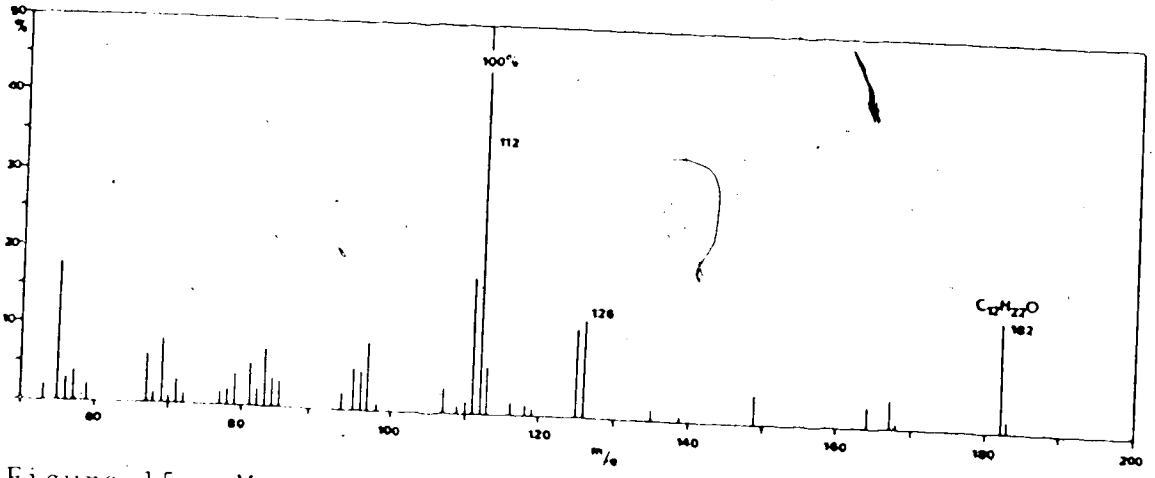


Figure 15: Mass spectrum of hydrogenolysis product 76

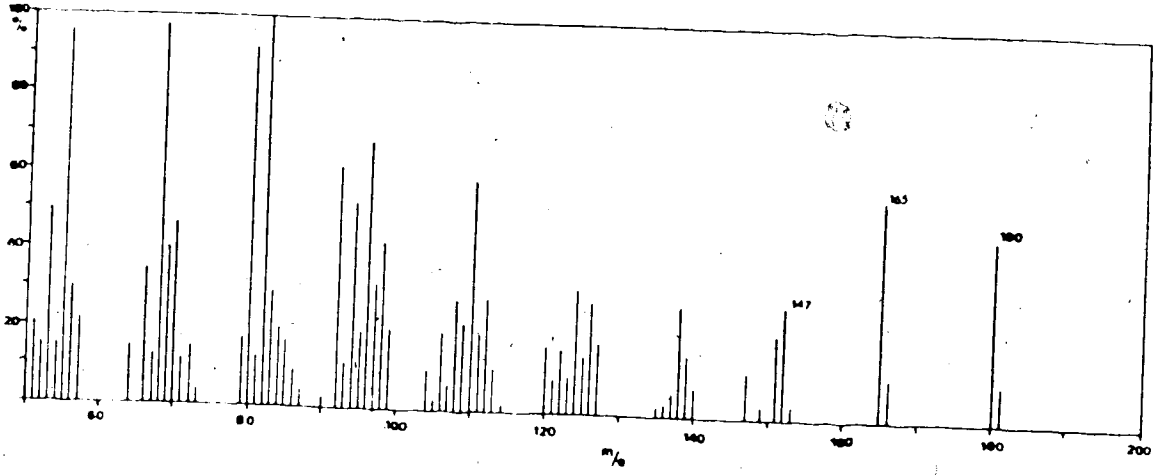


Figure 16: Mass spectrum of hydrogenolysis product 88/89

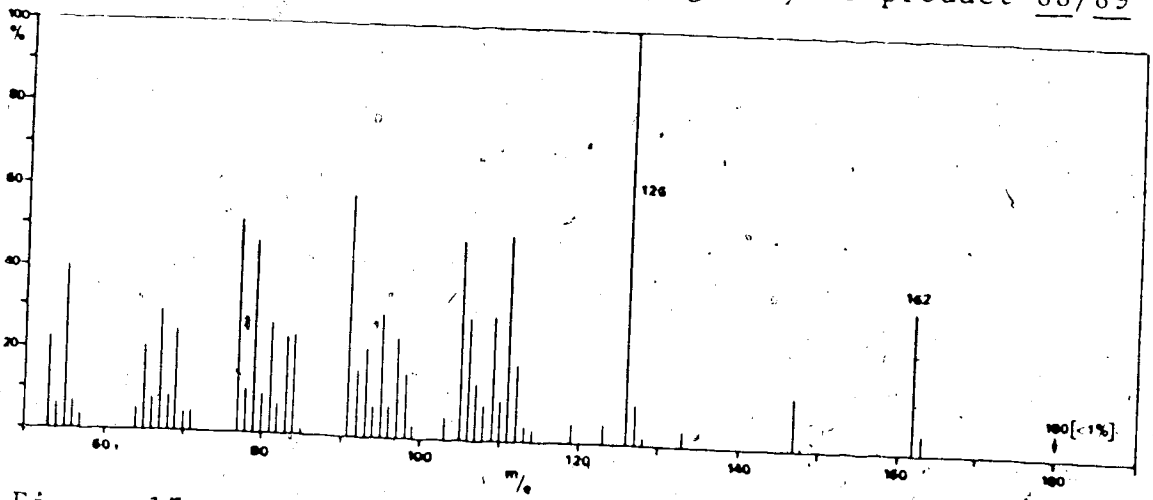


Figure 17: Mass spectrum of hydrogenolysis product 90

increase the reactivity of the Raney nickel. By refluxing an ethanolic solution of the thioketal with W-2 Raney nickel for five hours the percentage of the required product (Rt= 0.17) was increased to 80%, with 88/89(20%) as the only other product.

The two products, 76 and 88/89 together with unreacted keto-alcohol (80) from the starting material, were inseparable by tlc. The products were therefore separated by prep. gc using a 5' glass column containing OV-225 on 100/120 Gas-Chrom Q. A simple fraction collector[49] consisting of a glass capillary tube packed with 20-50 mesh Amberlite XAD-2 resin was used to collect 1 to 2mg samples of pure material.

Isolated fractions of the compound with a gc relative retention time of 0.17 had a very strong earthy odor. A sample of 67, kindly supplied by J. E. Marshall, had a different relative retention time (0.20) and a more camphor-like odor than the above fractions. It is perhaps possible that the nose can be used as an instrument for determining stereochemistry in this case. According to Amoore's stereochemical theory of olfaction,[50a] a globular body of about 80$\AA^3$ in volume is the ideal shape for the perfect camphoraceous odorant. Models of structures 67 and 76 show that the cis-decalin has a much more rounded shape than the relatively planar trans-decalin. The trans-decalin is thus excluded from the globular site of camphoraceous odor detection in the nose. Marshall[41] has also noted the subtle differences in smell bet-

ween isomers 76,77,67 and 78. A reference notes that the odor of geosmin may be detected by the nose "even when a flame-ionization detector does not respond to the substance present".[50b]

The apparent volatility of the odorous fractions from gc necessitated special handling techniques. The solution ir spectrum (Figure 18) of the hydrogenolyzed product with Rt=0.17 was obtained be eluting the resin-filled collection tube with CCl_4 directly into a micro-cell. Comparison of this spectrum with the ir spectrum of geosmin (Figure 19)[43] shows that the two compounds are identical. The Fourier transform pmr spectrum was similarly obtained by eluting a collection tube with deuteriochloroform into an nmr tube. The pmr spectrum (Figure 20) shows a methyl signal at δ0.75. The signal at δ1.25 is caused by a common contaminant. Comparison of the measured methyl chemical shifts with literature values (Scheme VIII) confirms the hydrogenolysis product as 76. The structure of cybullol is therefore conclusively shown to be 79, since none of the reactions in the synthetic sequence 79 through 76 will change the ring junction stereochemistry (cf. ref. 41, where a similar reaction sequence produced no cis,trans-decalin isomerization).

h) Absolute Stereochemistry of Cybullol and Geosmin

As cybullol has now been proven to be one of the enantiomers of 79, the absolute stereochemistry can be determined from the octant diagram for this structure shown in Scheme

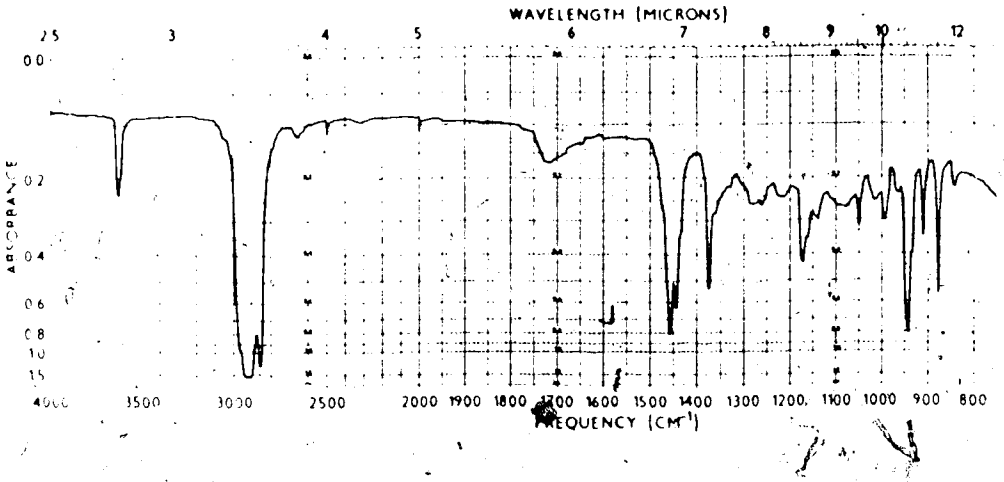


Figure 18. Ir spectrum of hydrogenolysis product 76 ([redacted]$_4$)

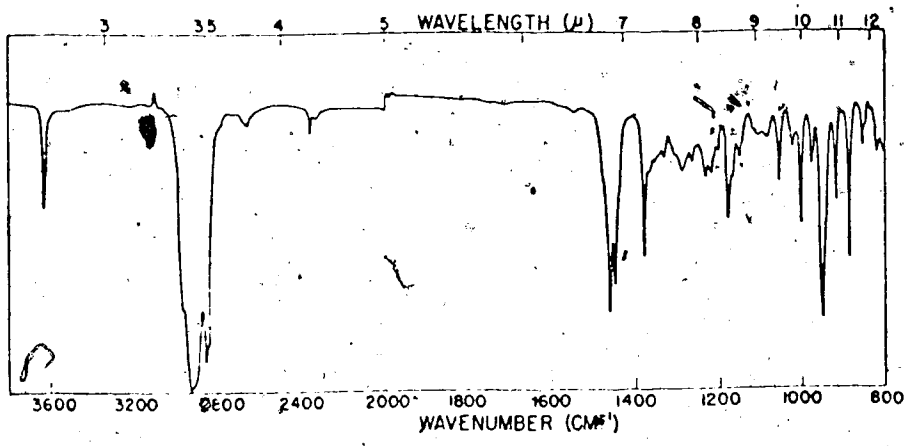


Figure 19: Ir spectrum of geosmin (76, CCl_4)[43]

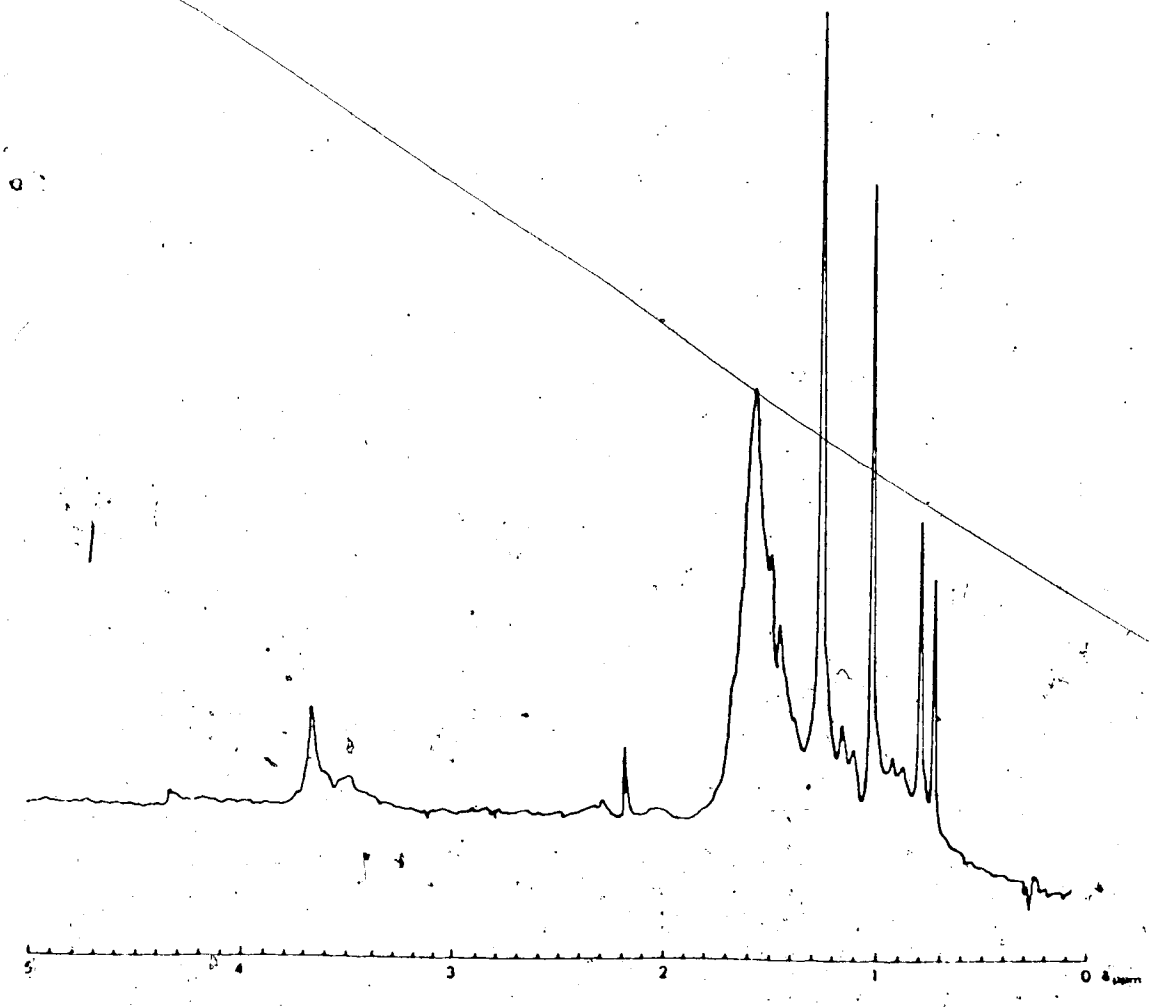
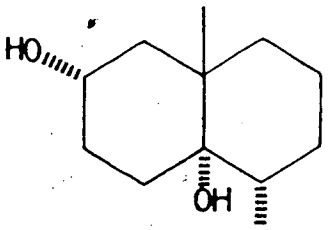
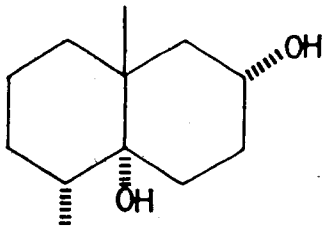


Figure 20: 100MHz (FT) pmr spectrum of hydrogenolysis product 76 ($CDCl_3$)

VII. Cybullol produces a positive Cotton effect at 298nm in the cd/ord spectra. The absolute structure must therefore be as shown in 79 below as opposed to 79e, since 79 has been calculated to give a positive Cotton effect (section II-3e).

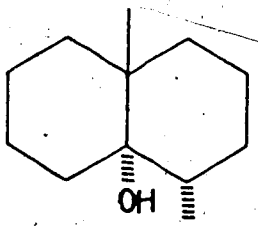


79



79e

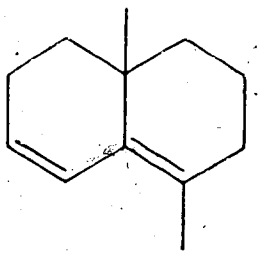
The geosmin synthesized from cybullol shows a negative rotation at the sodium D line. It was not possible to determine accurately the specific rotation value $(\alpha)_D^{25°C}$, since the geosmin could not be completely freed of solvent for accurate weighing. Naturally occurring geosmin also has a negative specific rotation ($[\alpha]_D^{25°C} = -16°$). The absolute stereochemistry of naturally occurring geosmin, not previously determined, is therefore as represented in 76 below.



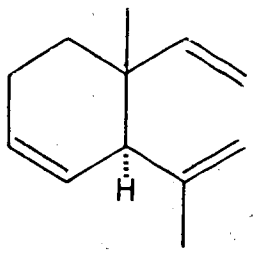
76

i) Biogenetic Considerations

Gerber speculates[40] that biogenetically geosmin is a sesquiterpene which has lost an isopropyl group. Apart from cybullol, cogeijerene (91)[51] is the only other naturally occurring compound with this type of skeleton reported to date. It was isolated from the steam distillate of Geijera parviflora essential oil, together with geijerene (92). Both compounds are racemic.



91



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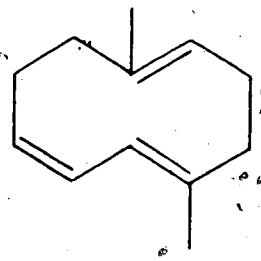
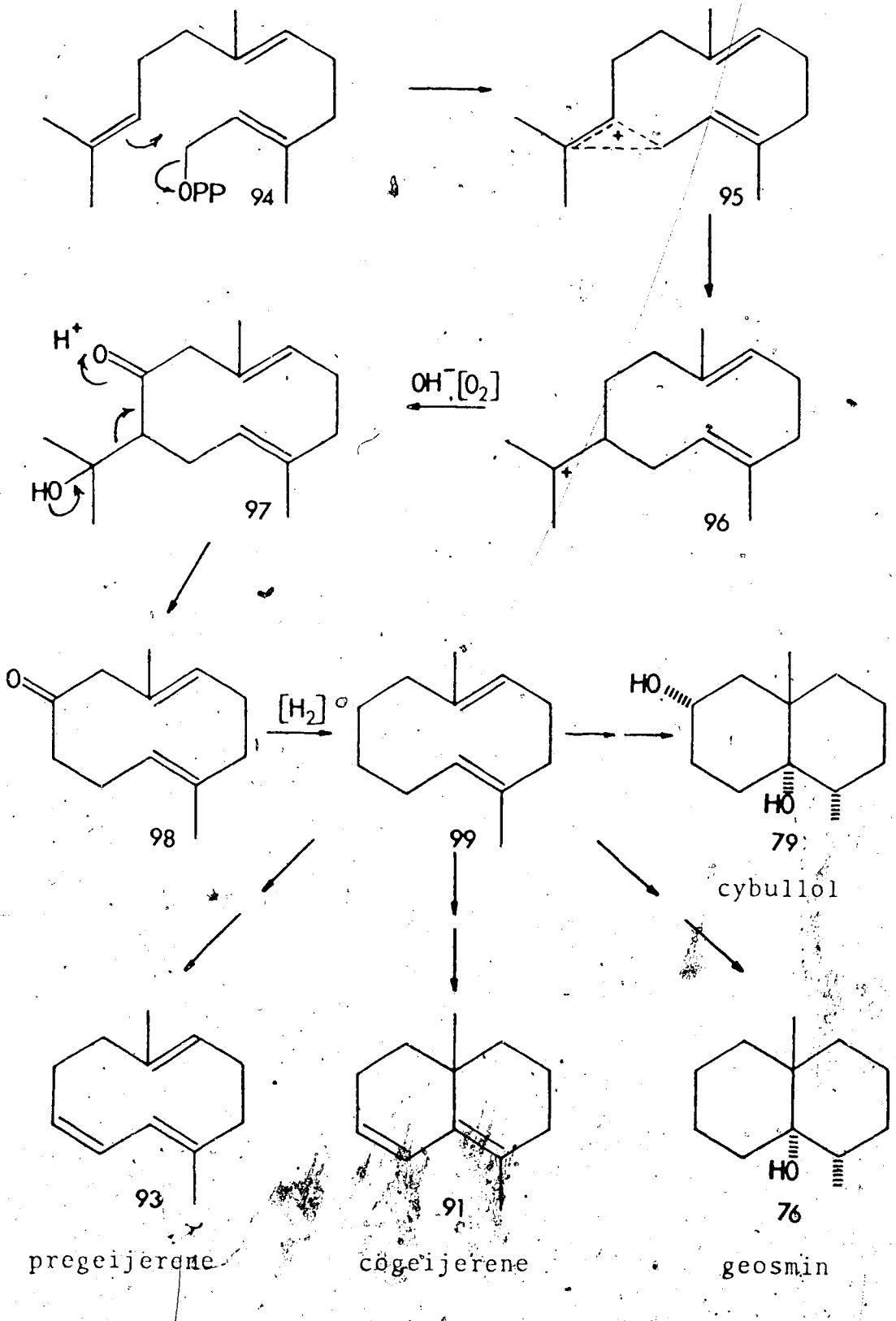


93

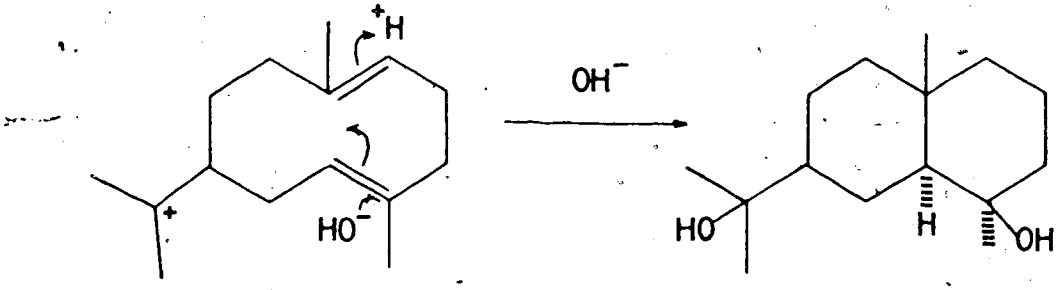
Sutherland subsequently discovered[52] that geijerene (92) is mainly an artefact, produced by steam distillation, from pregeijerene (93). A biosynthetic scheme is proposed that would account for the co-occurrence of 91 and 93, (Scheme X), although cogeijerene should be optically active if produced in this manner. Cybullol and geosmin could also originate from intermediate 99.

The intermediate 96 is a direct precursor of the eudes-


OPP
94
95
H+
O
HO
97
OH⁻[O₂]
96
O
[H₂]°
98
99
HO
HO
79
cybullol
93
91
HO
76
pregeijerene
cogeijerene
geosmin

SCHEME X

mane class of sesquiterpenes[53] (96→100).



96 100

It is perhaps surprising that no sesquiterpenes have been found to co-occur with cogeijerene or geosmin. The next section of this thesis will describe the isolation of a C-15 compound from Cyathus bulleri. All of the available evidence concerning this compound indicates that it has a eudesmane-type skeleton.

4. Partial Structure of Compound F

Compound F (Rf 0.35, see Chart I) was isolated by the same procedure as used for cybullol (Scheme I). It is recognized by a dark blue/black coloration produced when a developed tlc plate is sprayed with 30% aqueous sulfuric acid. Compound F has a gc relative retention time of 0.87.

On a comparative basis, the Cyathus bulleri fungus produces approximately one fifth as much compound F as cybullol (by gc). The low production of compound F and the difficulty in separating it from cybullol by chromatography have resulted in only 18mgs being isolated to date. Compound F is only partially soluble in methylene chloride and crystallizes on standing as colorless needle-like crystals, m.pt. 152°C.

The ms of compound F (Figure 21) shows an apparent parent ion with m/e 256. High resolution ms indicates a molecular formula of $C_{15}H_{28}O_3$ (meas. 256.2046; calc. 256.2039). The fragment ions at m/e 238 (M^+-H_2O), 223 ($M^+-(H_2O + CH_3)$), 220 (M^+-2H_2O) and 205 ($M^+-(2H_2O + CH_3)$) indicate that compound F has at least two alcohol functions.

The ir spectrum of compound F (Figure 22; nujol mull) confirms that an alcohol function is present (strong absorption at 3300cm^{-1}). The low intensity carbonyl absorption at 1700 - 1750cm^{-1} is due, in retrospect, to impurity in the crystalline sample. The crystalline material contained about 20% impurity (mainly cybullol) as indicated by gc.

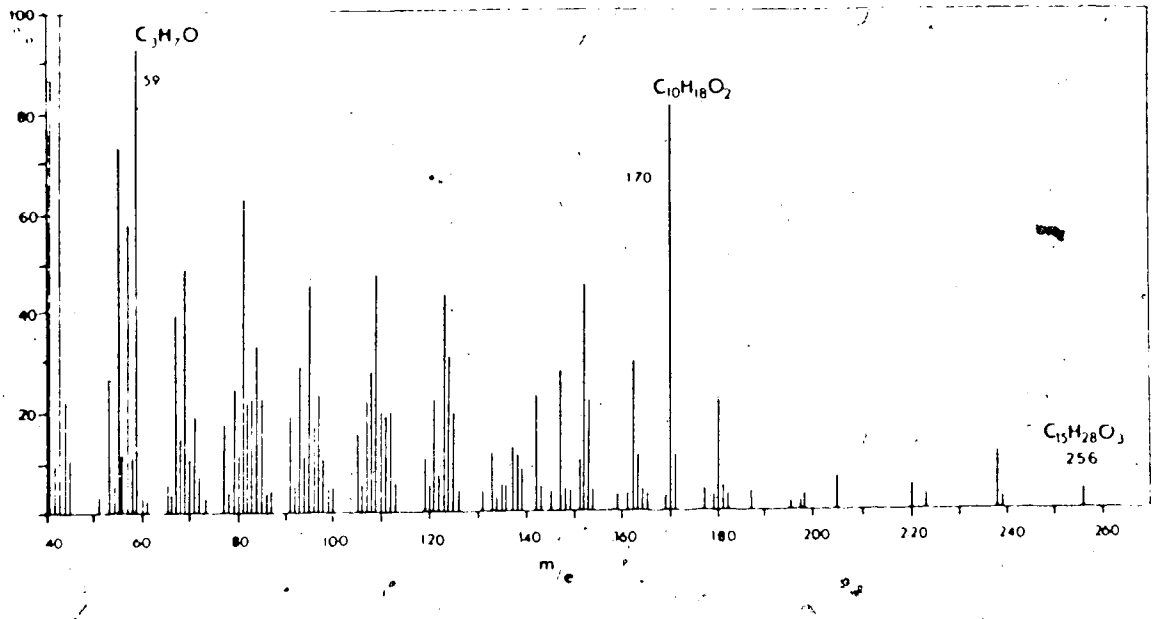


Figure 21. Mass spectrum of compound F

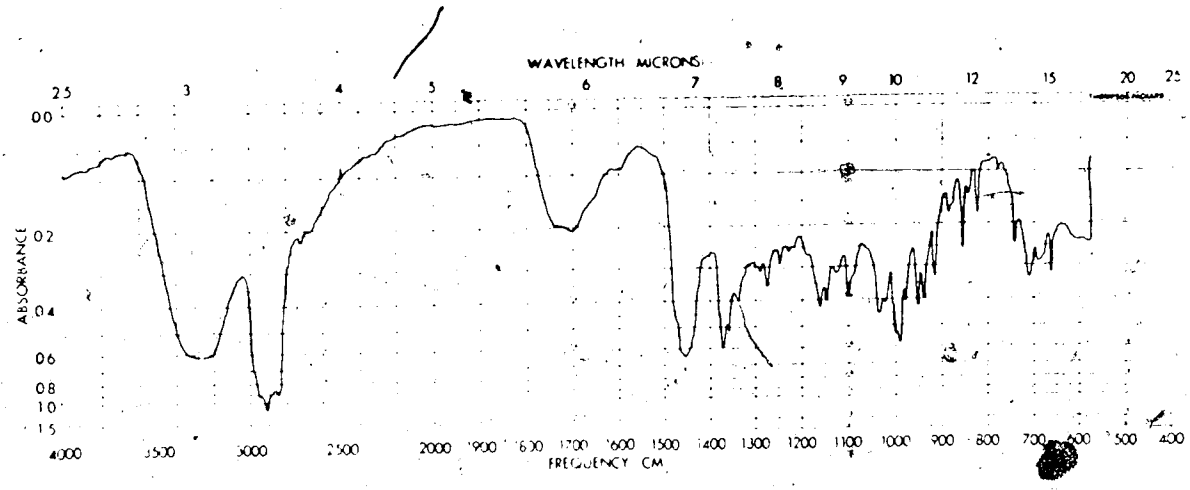
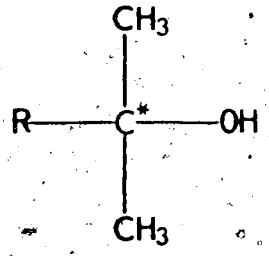


Figure 22: Ir spectrum of compound F, (Nujol mull)

The Fourier transform pmr spectrum (Figure 23; $CDCl_3$ solvent) is informative in the methyl proton region. It contains a doublet (J=7Hz) at δ1.04, a singlet at δ1.05 and a singlet at δ1.30. A common impurity peak at δ1.25 is now thought to be due to silicone grease impurity.

Although Fourier transform pmr allows very small samples (< 1mg) to be analyzed, the method suffers from the disadvantage of providing no meaningful integration. This is because the areas under the peaks are dependant on the T_1 relaxation times of each proton. Fourier transform pmr signals caused by protons in similar environments, however, should be comparable because T_1 values will be similar.

The peaks at δ1.05 and δ1.30 were traced and weighed; the weights were in the ratio 1 to 2. This probably indicates that the low field signal is caused by two equivalent methyl groups since the higher field signal is almost certainly a methyl singlet (three protons). The low field position of the two equivalent methyl groups is consistent with the presence of a geminal hydroxyl group, as in the partial structure 101.



101

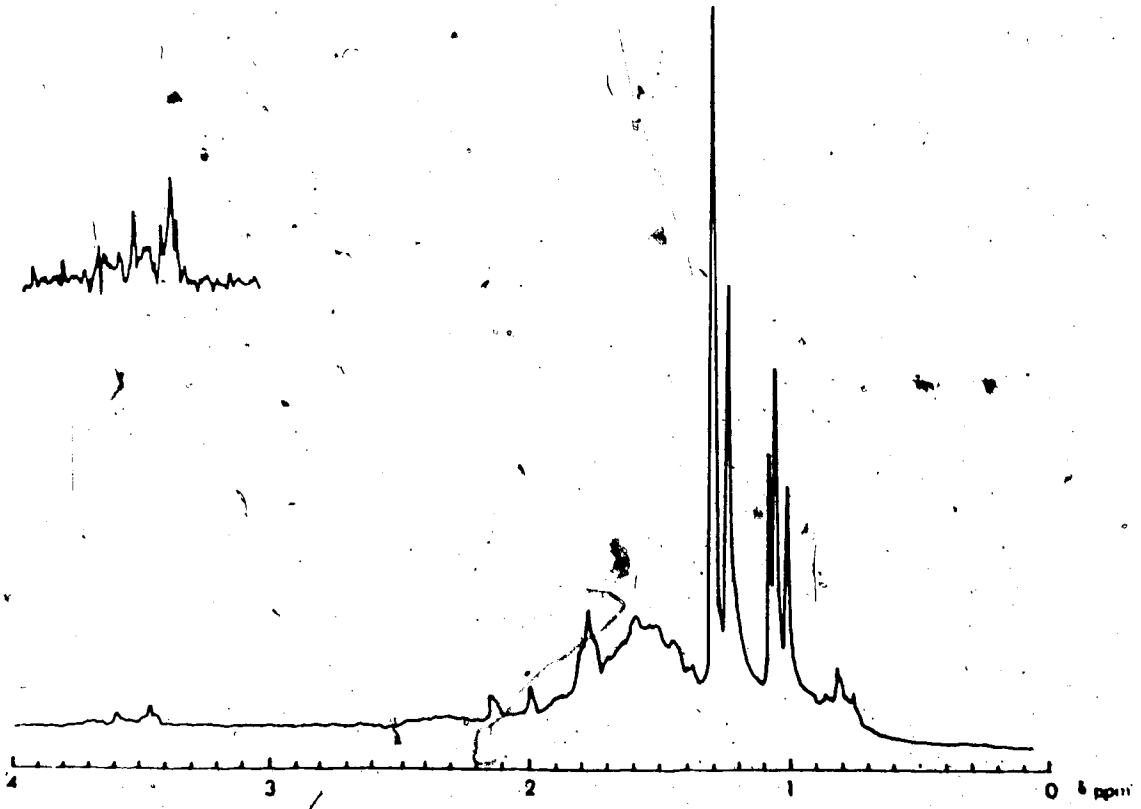


Figure 23: 100MHz (FT) pmr spectrum of compound F ($CDCl_3$)

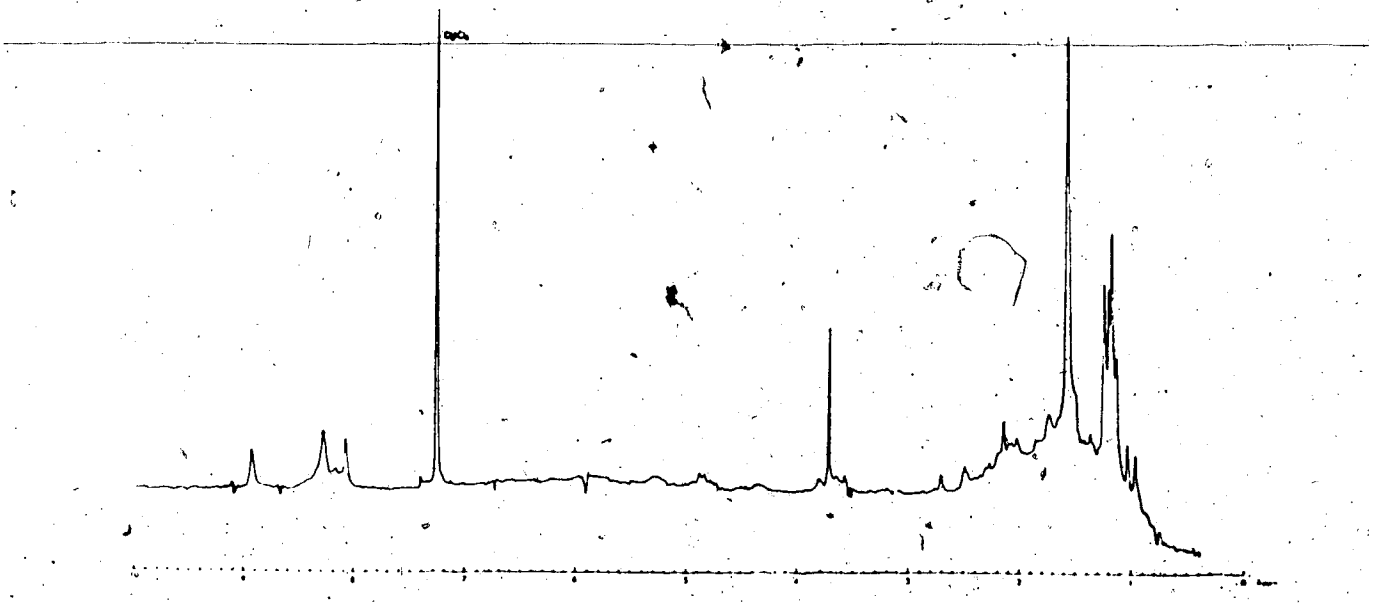
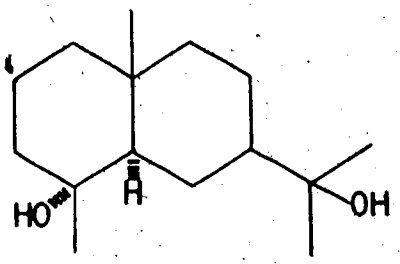
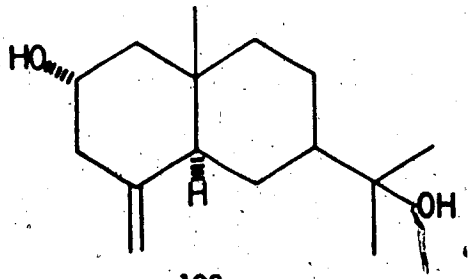


Figure 24: 100MHz (FT) pmr spectrum of compound F plus one drop of trichloroacetyl isocyanate ($CDCl_3$)

Although one might expect the two methyl groups to be non-equivalent if proximate to a chiral centre, there is ample precedent for this sort of structure producing a singlet pmr signal. For example, 102[54] and 103[55a] produce singlets in $CDCl_3$ at δ1.10 and δ1.20 respectively. The type of signal produced is possibly related to the ease of rotation of the



102



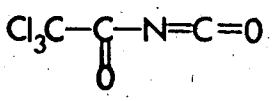
103

R-C* bond. The partial structure 101 is further substantiated by the presence of a large m/e 59 fragment ion in the ms (Figure 21). This is due to a $C_3H_7O^+$ ion (meas. 59.0499; calc. 59.0497) which can be formed by a fragmentation mechanism characteristic of tertiary alcohols.[55b]

Several experiments were performed on compound F to determine the number of hydroxyl groups present. Deuterium exchange of hydroxylic protons was attempted by shaking a solution of compound F in CH_3OD. Using the same procedure as with cybullol, a sample of deuterated material was introduced into the mass spectrometer. The spectrum produced however was inconclusive. The parent peak moved to m/e 257 and the m/e 258 peak was enhanced cf. Figure 21 (61% of the m/e 257

intensity). By analogy with cybullol, either m/e 258 or m/e 259 should be the parent peak since the mass spectrum (Figure 21) has indicated that compound F contains at least two hydroxyl groups. The difficulty in exchanging these hydroxylic protons may be caused by internal hydrogen bonding although, without knowing the full structure of compound F, this must be regarded as hypothesis.

Trichloroacetyl isocyanate (TAI) (104), has been used for in situ pmr determination of the number of hydroxyl groups present in a molecule.[56a] A carbamate (105) is rapidly formed with a hydroxyl group when excess TAI is added at



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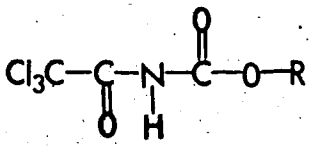


105

room temperature to an nmr tube containing a deuteriochloroform solution of an alcohol. Even highly hindered hydroxyl groups have been functionalized and the excess reagent shows no pmr signal. The carbamate N-H signals usually appear in the δ8-9 region as distinct singlets, permitting determination of the total number of hydroxyl functions.

The method was applied to compound F. The Fourier transform pmr spectrum produced by adding one drop of TAI to a solution of compound F (2mg) in $CDCl_3$ (Figure 24) contains three distinct singlets at δ8.08, 8.28 and 8.92, showing that

the compound contains three hydroxyl groups. The chemical shift of the methyl signals in the carbamate are also informative when compared with the corresponding alcohol signals (see Table IX).

Table IX

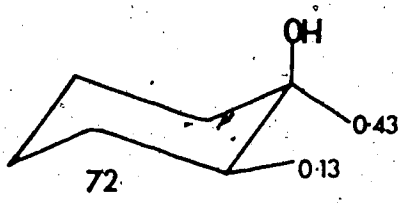
CHEMICAL SHIFT OF METHYL PROTON SIGNALS IN COMPOUND F AND ITS TRICHLOROACETYL CARBAMATE DERIVATIVE

Compound F	Carbamate	Δ *ppm
δppm	δppm	
1.04(d)J=7Hz	1.17(d)J=7Hz	0.13
1.05(s)	1.18(s)	0.13
1.30(s)	1.58(s)	0.28

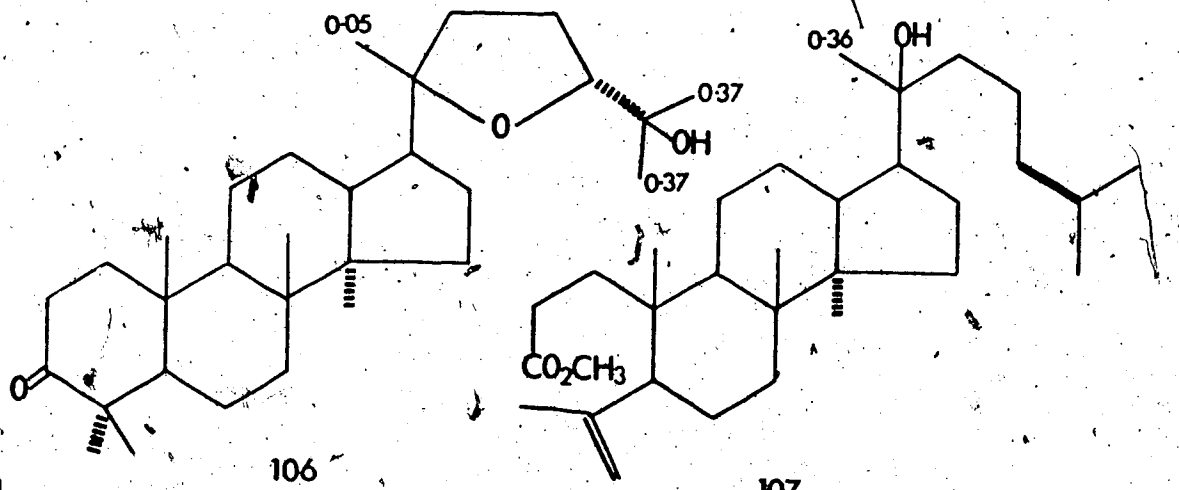
* = δcarbamate - δalcohol

To determine the significance of the Δ(=δcarbamate - δalcohol) values in compound F, it is necessary to compare them with corresponding values in model compounds. In t-butyl alcohol, a shift in the methyl signal (Δ) of 0.35 ppm is observed on addition of TAI; in 72 the Δ value for the methyl alpha to the hydroxyl group is 0.43 ppm. Thus the Δ value of 0.28 ppm for the 'isopropyl' signal of compound F is comparable with the shift of signal produced in a methyl group alpha to a tertiary hydroxyl group, confirming the partial structure 101. Lavie[56b] has noted without comment similar

shifts in methyl signals following reaction of cabraleone (106) and methyl dammarenolate (107) with TAI. The Δ values are shown on the structures below. Δ is negligible for methyl groups not in the proximity of a hydroxyl function.



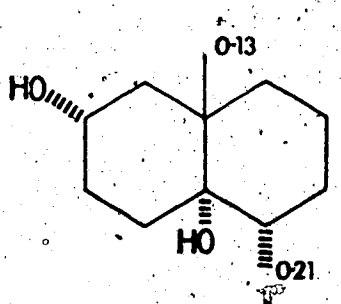
72



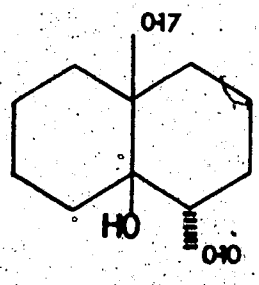
106 107

In isopropyl alcohol, a model for a methyl group situated alpha to a secondary hydroxyl group, Δ for the methyl signal is 0.17, significantly smaller than the above values.

The change in methyl shifts (Δ) of 79 and 67 have also been determined and are shown below.



79



67

The significance of these figures and their application to determining the structure of compound F must await further studies with model compounds.

It should be noted that trichloroacetyl isocyanate also reacts with the epoxide function in epichlorohydrin (108)[57a] and with several enol ethers.[57b] Thus, TAI should be used with caution for structure elucidation of hydroxylated compounds containing these functionalities.

108

Having established that compound F is a triol, acetylation was used to obtain information about the type of hydroxyl functions present. Compound F (5mg) in acetic anhydride (1ml) containing pyridine as catalyst was stirred at 25°C for 5.5 hours. The only product (by tlc) was a monoacetate, as judged from the ms (Figure 25), the ir spectrum (Figure 26) and the pmr spectrum (Figure 27). The formation of a monoacetate strongly suggests that one hydroxyl group is primary or secondary and that the other two are tertiary. To distinguish between primary and secondary hydroxyl groups, it is necessary to compare the pmr spectra of compound F (Figure 23) and its carbamate derivative (Figure 24). In Figure 23 a multiplet at ~δ3.5 is assigned to a proton gemi-

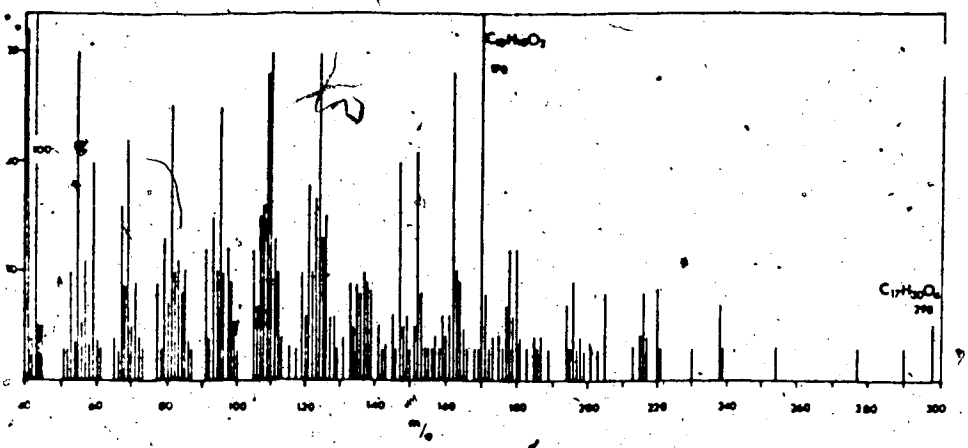


Figure 25: Mass spectrum of compound F monoacetate

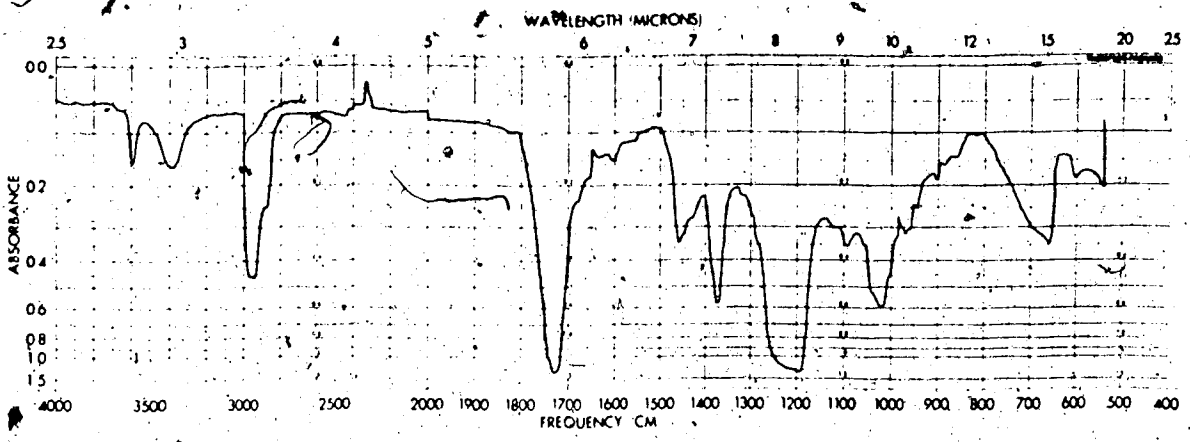


Figure 26: Ir spectrum of compound F monoacetate ($CHCl_3$)

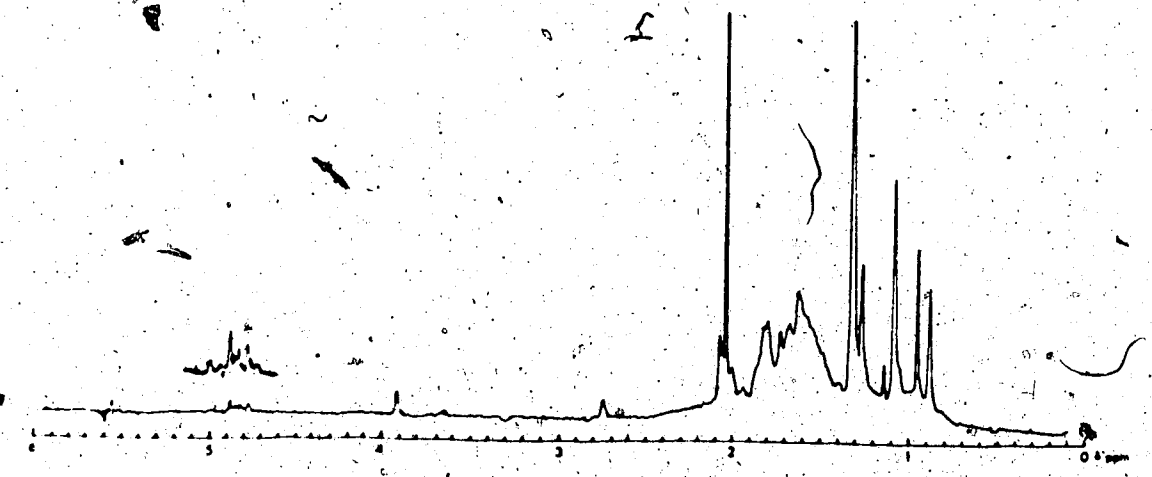
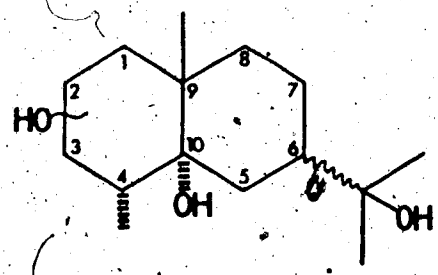


Figure 27: 100MHz (FT) pmr spectrum of compound F monoacetate ($CDCl_3$)

nal to a secondary hydroxyl group since the geminal signal in the carbamate (δ4.9; Figure 24) has shifted downfield by 1.4 ppm, a typical value for secondary alcohols.[56a] The pmr spectrum of the acetate (Figure 27) contains a multiplet at δ4.8; a chemical shift indicative of a proton geminal to a secondary acetate function.

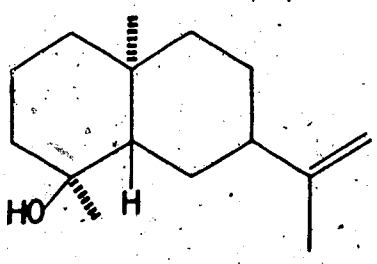
As mentioned in section II-3h, the intermediate 96 proposed in the biogenesis of cybullol is a direct precursor of the eudesmane class of sesquiterpene. By combining the spectral data considered above with the hypothesis that compound F will be related biogenetically to cybullol; it is possible to propose a partial structure for compound F. Partial structure 109 possesses a eudesmane skeleton and is consistent with all spectral data.



109

There is no evidence for the stereochemistry of the ring junction and the secondary methyl group other than the analogy with cybullol. The stereochemistry at C-6 is undefined. In the majority of naturally occurring eudesmane-type sesquiterpenes, the isopropyl group is equatorial.[58]

Although an equatorial group at this position will give a less sterically-hindered structure, it must be realized that, as mentioned by Parker et al,[53] classical chemical concepts of strain and steric interactions need not necessarily be the dominant factors in biosynthesis since, in vivo, the substrate must concur with the conformational requirements of the particular enzyme involved. This is demonstrated by the occurrence in nature of both intermedeol[59] (110) and neo-intermedeol[60] (111) with axial and equatorial isopropenyl groups respectively.



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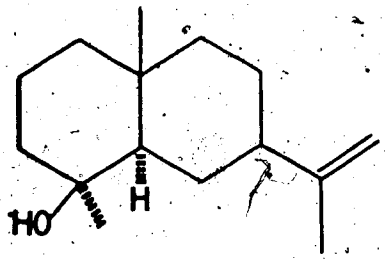
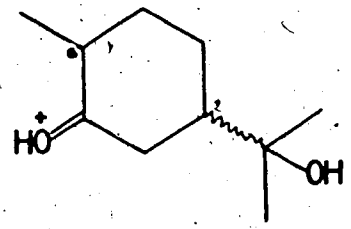


111

The pmr signal of the 'isopropyl' protons in compound F is at slightly lower field (0.1 ppm) than the corresponding signals in structures 102 and 103. Possibly the isopropyl group is axial in structure 109 since then Van der Waals deshielding of the isopropyl protons would occur.

Several features of the mass spectrum of compound F (Figure 21) can only be explained if the secondary hydroxyl group is not in the same ring as the isopropyl group. The peak at m/e 170 (relative intensity 82%) is caused by a

$C_{10}H_{18}O_2^+$ fragment (meas. 170.1301; calc. 170.1307). By analogy with the mass spectral fragmentation of cybullol, a major fragmentation of structure 109 should be the cleavage of the C_1-C_9 and C_4-C_{10} bonds to give the ion radical 112, with mass 170.



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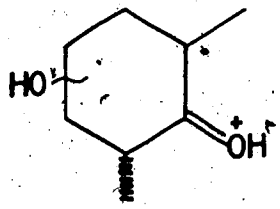
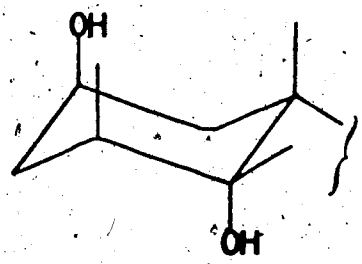


113

If the secondary hydroxyl group had been located in position 5,7 or 8 (Structure 109), a peak at m/e 186 would have been observed since the ion radical 112 would then contain an additional hydroxyl group. The ms of the acetate (Figure 25) confirms this analysis. The m/e 170 peak is still present so the secondary hydroxyl group is not contained in the $C_{10}H_{18}O_2^+$ fragment. Cleavage of the C_9-C_8 and C_{10}-C_5 bonds in structure 109 will produce fragment ion 113 of mass 142. A peak with m/e 142 is present in the ms of compound F (Figure 21) but, as expected, it is absent from the ms of the acetate (Figure 25). The exact position of the secondary hydroxyl group cannot be determined from the information available. Theoretically the coupling of the carbinol proton should distinguish a hydroxyl group at car-

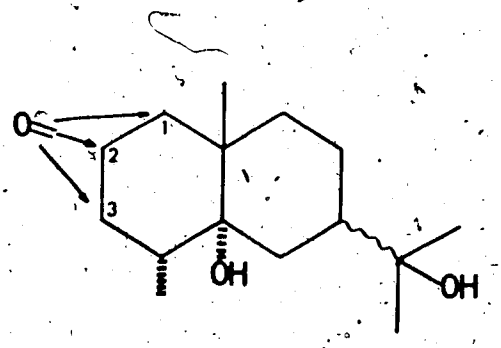
bons 1,2 or 3 (structure 109). However, due to the small quantity of pure material available it has not been possible to obtain a pmr spectrum of sufficient resolution to provide this information.

The pmr of the acetate (Figure 27) contains a methyl doublet at δ0.91 (J=6.5Hz). The corresponding signal in compound F is situated 0.13 ppm downfield from this position. This shift indicates that the secondary hydroxyl group is spatially close to the secondary methyl group. A 1,3-diaxial relationship, as in 114 is unlikely however since the tertiary methyl signal is not shifted significantly on formation of the acetate.



114

In order to determine the exact structure of compound F, it will be necessary to isolate further material and perform several experiments. Notably, dehydrogenation should confirm the eudesmane skeleton and cd data from the keto-alcohol expected on mild oxidation will allow the absolute structure to be determined. Reactions of this keto-alcohol (115) should allow location of the position of the secondary hydroxyl.



115

Acid-catalyzed deuterium exchange would allow distinction of C-2 from C-1 and C-3. If C-3 is involved, base catalyzed elimination of the C-10 hydroxyl should be possible.

5. Some Information Concerning Compound B

Another metabolite of C. bulleri - compound B - was isolated on one occasion. A total of 3mgs of crystalline material (mpt 244-248°C, from ether) was obtained. By tlc, the compound has an Rf of 0.56 (see Chart I) and is recognized by the yellow coloration produced when a developed plate is sprayed with 30% aqueous sulfuric acid and heated. Compound B has a gc relative retention time of 0.75.

The ms of compound B (Figure 28) contains a base peak at m/e 232, which appears to be the parent peak. The intensity of this peak suggests either that the molecular ion is very stable or that the peak represents the major fragmentation product of a low abundance parent peak. The peaks at m/e 217 and 214 are evidence that the parent peak is m/e 232, because they then represent M^+-CH_3 and M^+-H_2O respectively. A molecular formula of $C_{13}H_{12}O_4$ is indicated by hrms (meas. 232.0736; calc. 232.0736), The compound therefore contains eight sights of unsaturation.

The ir spectrum of crystalline compound B (Figure 29; nujol mull) contains both hydroxyl and carbonyl absorption. There are two or possibly three absorptions in the carbonyl region at 1761, 1752 (shoulder) and 1710cm^{-1}. These absorptions indicate that compound B contains an ester, lactone or carboxyl function (1761cm^{-1}) as well as a ketone or aldehyde (1710cm^{-1}). A carboxyl function is however inconsistent

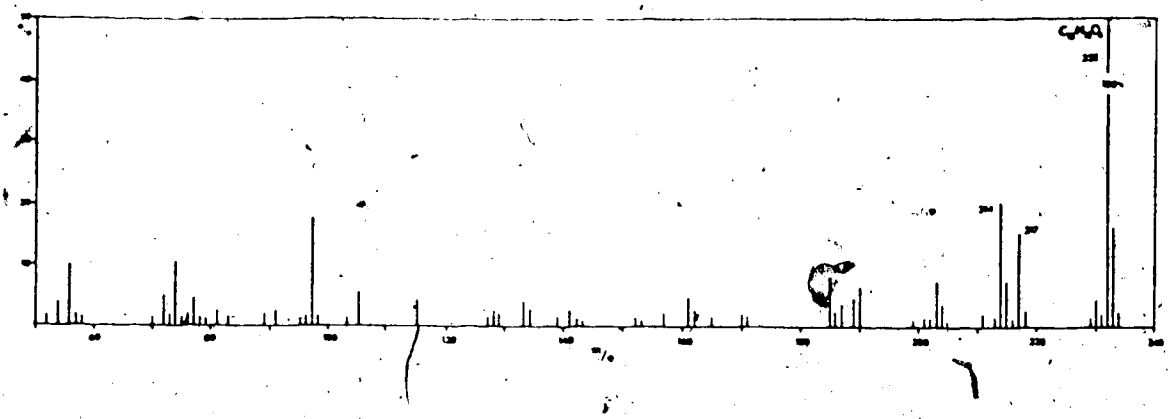


Figure 28: Mass spectrum of compound B

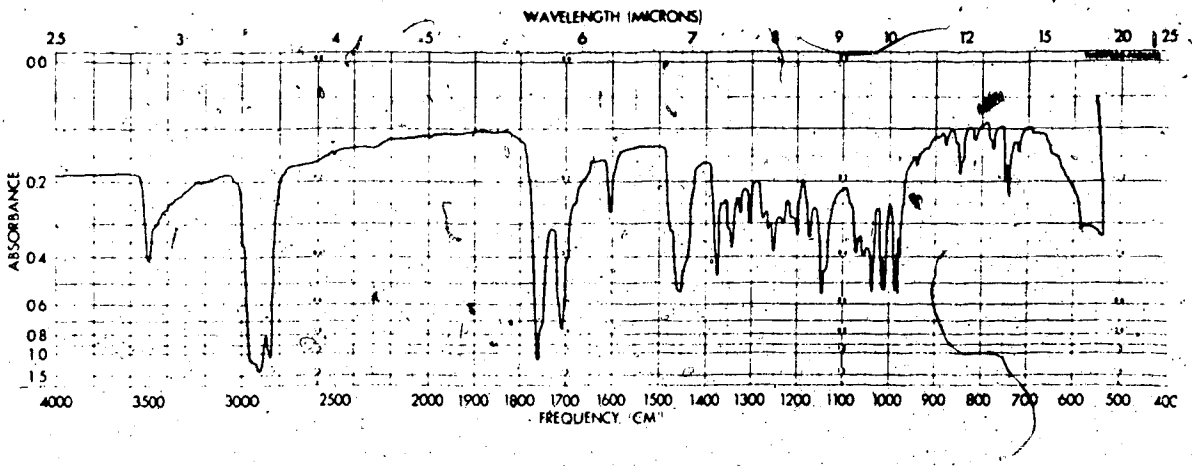


Figure 29: Ir spectrum of compound B (nujol mull)

with the low polarity tlc characteristics of compound B. The high degree of unsaturation of compound B leads one to expect an aromatic molecule. A peak at 1605cm^{-1} is consistent with this expectation, although usually there are at least two peaks in the 1500-1600cm^{-1} region for aromatic compounds.

The aromatic proton signals observed in the Fourier transform pmr spectrum of compound B (Figure 30; δ7.8) confirm the above assignment. The number and intensity of peaks in the methyl region are difficult to reconcile with a formula containing only twelve protons, and suggests that an impurity may be present.

The uv spectrum of compound B (methanol solution) contains three low-intensity absorption maxima at 238 ($\varepsilon \simeq 400$), 276 ($\varepsilon \simeq 100$) and 285 ($\varepsilon \simeq 100$)nm. Most aromatic compounds display stronger absorption maxima than this.

No further work has been attempted on this compound. From the information available, it is not possible to deduce a structure for compound B.

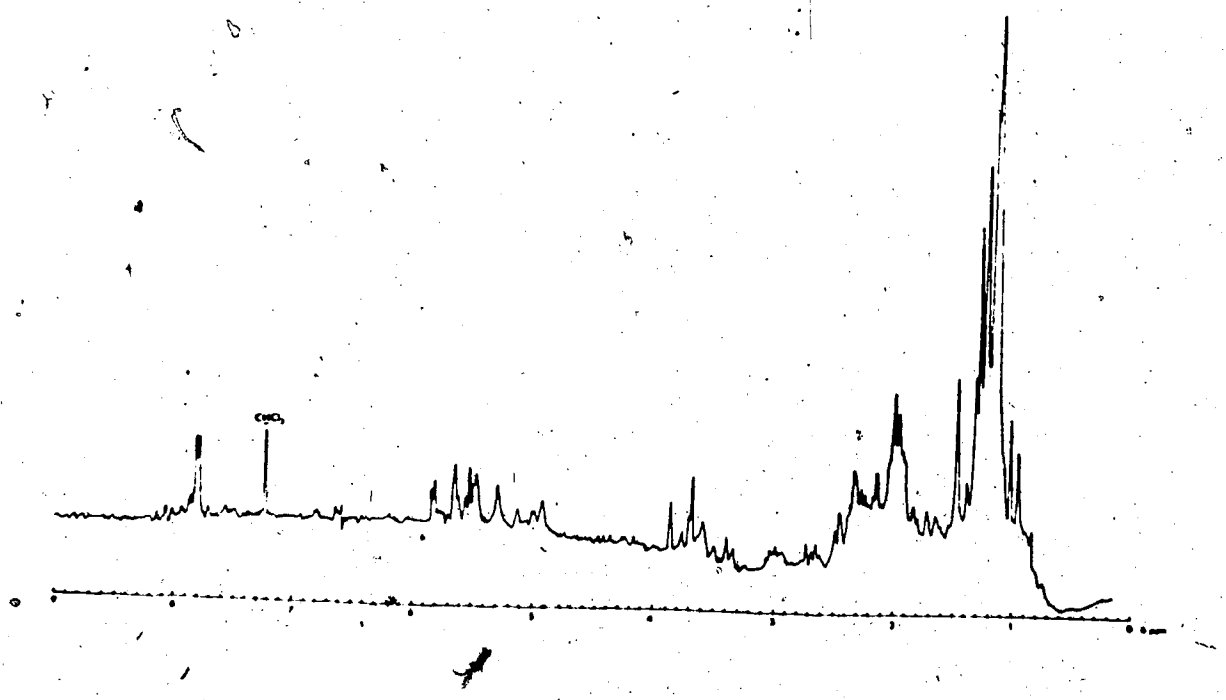


Figure 30: 100MHz (FT) pmr spectrum of compound B ($CDCl_3$)

6. Biological Activity of C. Bulleri Metabolites

Cybullol inhibited the growth of penicillin-resistant Staph. aureus at a 250µg level. At a similar concentration compounds B and F showed no inhibition.

A sample of crude extract from C. bulleri was tested for biological activity against a wide range of bacteria by Smith, Kline and French Laboratories. The results (Appendix I) show that the crude extract has no useful activity at 250µg/ml, the highest concentration tested.

III EXPERIMENTAL

Growth of C. Bulleri Cultures; Extraction of Metabolites

Cultures of C. bulleri have been grown using procedures developed for C. helenae by Johri[4] and modified by Taube,[6] Carstens[9] and Mercer.[11] Fruiting bodies of C. bulleri, obtained from Prof. H. J. Brodie, were sectioned after sterilization of their surfaces. The sections were incubated on sterile nutrient agar. After a mycelial growth had become established, blocks of agar containing the growth were transferred to fresh nutrient agar. Repetition of this process produced a homogeneous growth of mycelium. The cultures were stored at 5°C in petri plates and slant tubes containing nutrient agar.

Brodie liquid medium was used for the large scale growth of C. bulleri cultures. The aqueous medium contains the following nutrients per litre: maltose, 5.0gm; dextrose, 2.0gm; yeast extract, 2.0gm; KH_2PO_4, 0.5gm; $Ca(NO_3)_2.4H_2O$, 0.5 gm; $MgSO_4$.(anhyd), 0.24 gm; peptone, 0.2 gm; dl-asparagine, 0.2gm; $Fe_2(SO_4)_3$, trace; glycerol, 6 ml.

To initiate large scale cultures of C. bulleri, discs of agar containing the mycelium were transferred to 500ml Erlenmeyer flasks containing sterile Brodie medium (200ml).

The cultures were incubated at room temperature for a minimum of 30 days. The Erlenmeyer flasks then became innoculum flasks for up to 20 2 litre Fernbach flasks. Mycelium in the innoculum flasks have been left for a period of up to two years with no loss of ability to produce cultures on fresh medium. The mycelium appear to reach a maximum growth at about six months, presumably due to exhaustion of food supplies in the medium.

Large scale 'still-surface' cultures of C. bulleri were grown as follows. Up to 20 2 litre Fernbach flasks were each filled with 1 litre of Brodie medium. The stoppered flasks were autoclaved at 127°C, 15 lbs pressure for 20 minutes. Meanwhile the contents of one innoculum flask were broken up in a sterile Waring blender. When the Fernbach flasks had cooled to room temperature, 10ml of the blended suspension was pipetted into each of the flasks. The cultures were then left at room temperature for 30 days after which the medium, now containing the fungal metabolites, was drained from the flasks and the mycelium reflooded with sterilized Brodie medium. After a second growth of 30 days the medium was again drained. In order to prevent the Fernbach flasks from becoming overcrowded, the mycelium was then discarded and a new innoculum was used to introduce the third growth. During the course of this work, approximately 20 growths have been produced in this way.

The medium containing the fungal metabolites was filtered through cheesecloth to give a clear brown solution; pH~6. The solution was extracted with an equal volume of ethyl acetate (extracted twice with half volumes of solvent). The yellow extract was dried (Na_2SO_4), filtered and evaporated to dryness, giving between 50 and 100mg of crude extract per litre of medium.

Solvent and Adsorbent Purity

The common solvents (reagent grade) used in these laboratories contain up to 30mg/litre of involatile residue.[6] This would result in an unacceptable level of contamination in samples isolated in milligram quantities. The solvents were therefore distilled prior to use, except diethyl ether which was of sufficient purity to use directly from the container. The various grades of silica gel adsorbent were assumed to be of high purity. They were used for chromatography without further purification.

Column Chromatography

a) Elution Chromatography

Silica gel (Woelm) of particle size less than 230 mesh produced good separation of crude fungal metabolites using elution chromatography. Less resolution was obtained when silica gel of a larger particle size was used. In a typical

separation of one gram of crude extract (see Scheme II, pg. 17) a glass column of 4cm diameter, fitted with a glass wool plug, was filled to a height of 25cm with a slurry of the adsorbent in ethyl acetate. This gave an adsorbent to weight ratio of approximately 100 to 1. The column was kept vertical and vibrated with a mechanical vibrator to remove air bubbles. Ethyl acetate was passed through the column for some time to aid in settling of the adsorbent and to remove non-polar impurities such as plasticizers.

To apply a sample to the column, the solvent level was adjusted to the level of the adsorbent surface. The sample, dissolved in a minimum amount of ethyl acetate, was carefully added to the adsorbent surface. Once all the sample had soaked into the surface, more ethyl acetate was added and the elution was begun (flow rate; ≃10ml/hour).

The initial eluant was discarded until a faint yellow color was observed in the solution, indicating that the least polar components of the crude extract were being eluted. Fractions (10ml) were then collected ('Isco' fraction collector) and monitored by tlc.

The fractions of interest obtained in the above manner were further separated by elution chromatography using a solvent system of methylene chloride/methanol (100:5), as in Scheme II. Typically, a partially purified sample (~50mg) was passed through a silica gel (Woelm) adsorption bed of height 15cm and diameter 2.5cm (adsorbent/sample weight ratio,

100:1). Fractions were collected and monitored as above.

b) Dry Column Chromatography

Dry column chromatography was investigated as a method of separation of C. bulleri metabolites, as mentioned in Section II. The experimental techniques used are essentially those recommended by Loev and Goodman.[20] Typically, a nylon column (~4cm diam; uv transparent) was heat sealed at one end and packed with silica gel (Woelm), activity III - a deactivated silica gel supplied for this technique and containing 1% inorganic phosphor. An adsorbent to sample ratio of at least 100:1 was used.

The crude extract to be separated was dissolved in chloroform and stirred with 2-3gm of the adsorbent. Solvent was evaporated using a rotary evaporator and the sample, now adsorbed on silica gel, was added to the top of the adsorbent bed. The eluting solvent, usually ethyl acetate, was added to the column from a separating funnel, at such a rate as to maintain a 5cm head of solvent above the adsorbent. As mentioned previously, binary solvent systems were unsatisfactory in spite of pre-equilibration of adsorbent, as recommended by Loev and Goodman. Once solvent had completely filled the nylon tube (approximately one hour), the fractions were separated by cutting the column into 1 or 2" sections (no components could be detected by uv light absorption). The adsorbent was extracted with methanol/methylene chloride (10:1), filtered through a sintered glass funnel (2-2.5μ pore

size) and the filtrate evaporated to dryness.

Thin-layer Chromatography

Silica gel G (E. Merck) was the only adsorbent used routinely for tlc. Addition of 1% inorganic phosphor (General Electric, type 118-2.7) to the adsorbent allowed the detection of uv absorbing compounds by irradiating the developed tlc plate with uv light. For both analytical and preparative tlc an aqueous slurry of the adsorbent was spread on glass plates using DESAGA equipment as described in Stahl's handbook.[61] The plates were air dried for two hours, dried at 110°C for a further four hours and then allowed to cool before use. The plates were stored in a dry atmosphere to maintain activity.

For preparative work plates of dimension 20 x 100cm or 20 x 20cm were used with an adsorbent depth of 0.5mm. Samples in solution were applied to the larger plates with a mechanized applicator (designed in this department). A micropipet manufactured from a disposable pipet and fitted with a rubber bulb was used to apply samples to the 20 x 20 cm plates. Microscope slides (7.5 x 2.5cm and 7.5 x 5.0cm; adsorbent depth 0.3mm) were more convenient for analytical work. Samples were applied by touching the adsorbent surface with a capillary tube containing the sample in solution.

Plates were developed in closed tanks containing the required solvent system. A solvent system of methylene

chloride/methanol (10:1) produced the most satisfactory separation of C. bulleri metabolites. Unless otherwise noted, the Rf values quoted in this thesis refer to this solvent system. The following solvent systems have also been used at various times:

methylene chloride/methanol	100:5
methylene chloride/methanol	100:2
benzene/acetone/acetic acid	75:25:1
acetone/Skelly B	30:70

After development of a plate, the components were visualized by uv irradiation of phosphor impregnated plates or by spraying with 30% aqueous H_2SO_4, followed by heating. Occasionally a spray of ceric sulfate in sulfuric acid produced clearer color reactions. In preparative work, the surface area of the plate was protected with a glass plate; only a thin vertical band was treated with spray reagent. The required horizontal bands were then scraped from the plate and washed with a suitable solvent. Filtration and evaporation of the wash solvent gave the purified material.

Gas Chromatography

A Hewlett Packard 5700A gas chromatograph fitted with a flame ionization detector was used for both analytical and preparative work. For analytical separations, a 10' glass column (¼" o.d.) was packed with 3% OV-225 on 100/120 Gas-Chrom Q (Applied Sciences). On a preparative scale a 5'

glass column (¼" o.d.) containing identical packing material was used. The carrier gas flow rate was 60ml (N_2) per minute (anal.); 100ml (N_2) per minute (prep.). Injection and detector ports were kept at 250°C (anal. column); 200°C (prep. column).

A temperature program was selected to produce the best separation of C. bulleri crude extract. The program used routinely was:

Initial temperature; 140°C, held for four minutes

Temperature increased 8°C per minute to

Final temperature; 240°C, held for eight minutes

The relative retention time (Rt) of any peak is defined as the time taken for the peak to appear on the trace after injection of a sample, divided by the total program time. Rt is dependant on the type of column, temperature program and gas flow rate. By keeping these parameters constant, the Rt values were used to identify and compare samples. Rt values quoted in this thesis were obtained using the analytical column and operating conditions mentioned above.

For preparative work a gas flow splitter (10:1) was placed between the column and detector. The collectors[49] consisted of 10cm capillary tubes packed with Amberlite XAD-2 resin (Mallinckrodt). The resin was held in place with glass wool plugs. A teflon disc containing a small hole to accommodate one end of the capillary was clamped over the exhaust port of the chromatograph. As the required compon-

ent appeared at the exhaust port, a capillary tube collector was simply pushed into the Teflon disc. In this manner 1-2mg samples were collected. This method of collection was particularly useful for spectroscopic work. To obtain pmr spectra, the sample was eluted from the collector into an nmr tube with $CDCl_3$ or CCl_4. Similarly, for ir spectra, the sample was eluted into a micro-cell with $CHCl_3$ or CCl_4.

Instrumentation

Mass spectra were recorded on an AEI Model MS-2, MS-9 or MS-12. An interfaced AEI DS50 data system produced a print out of normalized spectra. All mass spectra are recorded in this thesis as a percentage of the base peak. The MS-9 was used for high resolution mass measurement. The small quantity of pure samples available prevented confirmation of molecular formulae by microanalysis.

Ir spectra were recorded on a Unicam SP1000 infra-red spectrophotometer or a Perkin-Elmer Model 421 dual grating spectrophotometer.

Pmr spectra were recorded on a Varian Associates HA-100 with tetramethylsilane as an internal reference. The spectra of small samples ($\simeq$5mg) were recorded on a Varian Associates HA-100 15" instrument equipped with a Digilab Data System and pulser for Pulse Fourier Transform. The ^{13}C magnetic resonance (cmr) spectrum of trans-1,2 dimethylcyclohexan-1-ol(72) was recorded at 22.63 MHz on a Brüker

HFX-90 instrument equipped with a Nicolet 1085 computer. Other cmr spectra were recorded by Prof. J. B. Stothers at the University of Western Ontario.

Uv spectra were recorded using a Cary Recording Spectrophotometer, Model 15.

Optical rotations were measured on a Perkin-Elmer Model 141 Polarimeter.

Cd and ord spectra were recorded on a Durrum Jasco Recording Spectropolarimeter.

Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected.

Isolation of Cybullol, Compound F and Compound B

Cybullol and compound F were both isolated using the procedure outlined in Scheme 1. Experimental details of the chromatographic techniques are given earlier in this section. The chromatographic characteristics and quantities of material isolated are given in sections II-3a(cybullol) and II-4 (compound F).

Cybullol (79)

m.pt; 125-126°C $[\alpha]_D^{25}$ = -16°(C,0.4,methanol)

ms; Figure 3, calc. for $C_{12}H_{22}O_2$,198.1620, meas. 198.1614

ir (KBr disc); Figure 4

pmr ($CDCl_3$); Figure 5, δ0.81(3H,d,J=6Hz,$CHCH_3$), 1.03 (3H,s,CCH_3), ≈3.9(1H,m,CHOH)

Compound F (109):

m.pt; 152°C $[\alpha]_D^{25}$ = -18.5 (C,0.07, methanol)

ms; Figure 21, calc. for $C_{15}H_{28}O_3$ 256.2039, meas. 256.2046

ir (nujol mull); Figure 22

pmr ($CDCl_3$); Figure 23, δ1.04(3H,d,J=7Hz,$CHC\underline{H}_3$), 1.05(3H, s,$CC\underline{H}_3$), 1.26(s,imp.), 1.30(6H,s,$C(C\underline{H}_3)_2$), 3.5(1H,m,C<u>H</u>OH)

Crystalline compound B was isolated on one occasion in the following manner. Crude extract #16 (600mg) was chromatographed on a dry column of deactivated silica gel, as described under the heading 'Dry Column Chromatography.' The column was cut into 13 x 2" segments. Fraction 11 (i.e. the segment between 20 and 22" from the top of column) was eluted with methanol/methylene chloride (10:1). Evaporation of the solvent gave a clear yellow oil (60mg) which, by tlc, consisted of compounds A, B and C (Table 1). Evaporation of an ethereal solution of the oil to a volume of 1 ml resulted in partial crystallization. The solution was allowed to stand at 5°C for 8 hours; the crystals were then separated by centrifugation. Recrystallization from ether/Skelly B solution gave 4mg of colorless crystals, m.pt. 244-248°C. The crystals were classified by their tlc characteristics as compound B (Table 1). Attempts to crystallize more material from this fraction were unsuccessful. It was decided at this time to concentrate on isolation of cybullol, the largest component of the crude

extract; consequently further isolation of compound B was not attempted.

Compound B:

m.pt; 244-248°C

ms; Figure 28, calc. for $C_{13}H_{12}O_4$ 232.0736, meas. 232.0736

ir (nujol mull); Figure 29

pmr ($CDCl_3$); Figure 30, δ1.00(d,J=6Hz), 1.18(s), 1.19(s), 1.24(s), 1.30(s), 7.80(d,J=2Hz) signals not assigned

Dehydrogenation of Cybullol (79)

Cybullol (8.4mg) was heated at 250°C for 4.5 hours in a sealed tube with 5% palladium on charcoal (80mg). The product was extracted with methylene chloride (10ml) and filtered. The remaining solid was extracted with 10% methanol in methylene chloride (10ml). After careful evaporation of solvents, the methylene chloride extract weighed 1.8mg and the more polar extract 1.3mg.

Gc and uv spectral data of the two extracts and of several aromatic compounds is summarized in Table X.

ms:

10% MeOH in CH_2Cl_2 extract: Mono and dimethylnaphthol. Calc. for $C_{12}H_{12}O$ m/e 172.0888, meas. m/e 172.0881 (66%), 171(20), 159(22), calc. for $C_{11}H_{10}O$ 158.0732, meas. 158.0740(100), calc. for $C_{11}H_9O$ 157.0653, meas. 157.0641 (93), 149(45), 129(32), calc. for $C_{10}H_8$ 128.0626, meas. 128.0631(48), 115(30), 111(24), 109(27), 97(30), 95(31),

91(30), 85(24), 83(32), 81(34), 71(42), 71(42), 69(48), 57(66), 55(66), 43(69), 41(78).

CH_2Cl_2 extract: 1-methyl and dimethylnaphthalene Calc. for $C_{12}H_{12}$ m/e 156.0939; meas. 156.0947 (12%), 143(17), calc. for $C_{11}H_{10}$ 142.0783, meas. 142.0779 (100), 141(75), 139(19), 116(14), 115(16), 89(16), 75(15), 74(15), 63(24), 51(18).

Table X

	Gc relative retention time(s) of major peak(s)	Uv spectrum. λmax (methanol) of most intense peaks (nm)
CH_2Cl_2 extract	0.30,0.37	223
10% MeOH/CH_2Cl_2 extract	0.66,0.70	227
Naphthalene	0.24	221
1-Methylnaphthalene	0.30	224
2-Methylnapthalene	0.29	224
Dimethylnaphthalenes	0.34,0.34(sh),0.37	226,229
1-Naphthol	-	210,233[2]
2-Naphthol	0.62	226[2]
2-Methyl-7-naphthol	0.63	229[2]
Azulene	-	275[2]

[1] Column conditions, as pg , except temp. program 80°C→240°C at 8°C p. min.,held at 240°C for 8 min.

[2] Ref. 62.

Attempted Hydrogenation of Cybullol (79)

Cybullol (79; 1.7mg) in 95% ethanol (2ml) containing PtO_2 catalyst was stirred at 25°C under hydrogen (1 atm. pressure) for two hours. The solution was filtered and evaporated giving only recovered starting material (1.7 mg).

tlc: One spot, Rf 0.42

ms: identical with Figure 3 (ms of cybullol).

Acetylation of Cybullol (79)

a) Methylene chloride solvent

Cybullol (79; 8mg) was stirred with acetic anhydride (20mg) and pyridine (2 drops) in methylene chloride (2ml) for 8 hours at 25°C. Removal of solvent under vacuum gave a mixture of starting material and monoacetate (28, 2αOAc, 5αMe, 10αOH) as a yellow oil (5mg).

tlc: Two spots, Rf 0.42 (79), 0.53 (28)

gc: Two peaks, Rt 0.54 (79, 60%), 0.57 (28, 40%).

ms: Peaks from Figures 3 and 7 both present

ir(CCl_4): 3450 (O-H), 1740 (C = O) cm^{-1}

b) Acetic anhydride solvent

Cybullol (79; 4mg) in acetic anhydride (0.5ml) containing pyridine (2 drops) was stirred at 25°C for 4 hours. Removal of the solvent under vacuum gave one product, the monoacetate (28, 2αOAc, 5αMe, 10αOH) as a colorless oil (4.6mg).

tlc: One spot, Rf 0.53

gc: One peak, Rt 0.57

ms: Figure 7,

calc. for $C_{14}H_{24}O_3$ 240.1726, meas. 240.1718

ir (CCl_4): Figure 8

pmr ($GDCl_3$): Figure 9; δ0.80 (3H,d,J=6Hz, $CHC\underline{H}_3$), 1.07 (3H,s,$CC\underline{H}_3$) 2.01 (3H,s,$OCOC\underline{H}_3$) 5.0 (1H,m,C$\underline{H}$OAc).

In another experiment:

Cybullol (2.5mg), acetic anhydride (0.5ml) and pyridine (2 drops) were heated under reflux for one hour. The product (2mg) obtained after removal of the solvent was identical (ms, tlc, gc) with the monoacetate product above.

c) Isopropenyl acetate method

Cybullol (79, 2.2 mg) in benzene was heated under reflux with isopropenyl acetate (5 drops) and p-toluenesulfonic acid (catalytic amount). After one hour the acetone was removed by distillation. The cooled benzene solution was washed with 2% $NaHCO_3$ solution (2 x 3ml), water (3ml), dried (Na_2SO_4) and the solvent evaporated to give an oily residue (2.8mg) consisting of two major products.

tlc: two spots, Rf 0.53, 0.65

gc: two peaks, Rt 0.57 (55%), 0.61 (45%)

The products were separated by ptlc, (silica gel plates, CH_2Cl_2/MeOH (10:1) solvent).

Lower Rf product:

Identical with cybullol monoacetate (ms, gc, tlc).

Higher Rf product:

tlc: Rf 0.65

gc: Rt 0.61

ms: m/e 259(10%), 240(3), 180(M^+, 18), 165(8), 162(33) 147(12), 126(10), 110(14), 109(12), 95(13), 91(13), 89(12), 81(16), 69(14), 67(13), 59(80), 58(15), 57(19), 55(21), 45(75), 43(100), 41(35).

The enhanced m/e 180 peak indicates a dehydration product, possibly 90.

Deuterium Exchange of Cybullol (79)

Cybullol (79; 1mg) was dissolved in methanol-d_1 (CH_3OD; 1 ml). The solution was partially evaporated under nitrogen to give cybullol-d_2

ms: (direct probe dipped into solution), Figure 6.

Jones' Oxidation of Cybullol (79)

Jones' reagent (8N in CrO_3) was added dropwise to a stirred solution of cybullol (8mg) in acetone (2ml) at 25°C until a permanent orange color was produced in solution. The solution was stirred for a further 20 min; isopropyl alcohol was then added to react with excess Jones' reagent (solution orange→green). The pH was adjusted to 7 using 2% $NaHCO_3$ solution. The solution was filtered and the filtrate diluted with saturated NaCl solution (5ml).

Acetone was evaporated and the remaining solution extracted with ether (3 x 5ml). The ethereal solution was dried ($MgSO_4$) and evaporated to give keto-alcohol 80 as a colorless oil (5.6mg).

tlc: one spot Rf 0.60

gc: one peak Rt 0.57

ms: Figure 11; calc. for $C_{12}H_{20}O_2$ 196.2144, meas. 196.2136

ir(CCl_4): Figure 12

pmr ($CDCl_3$): δ0.90 (3H,d,J=7Hz, $CHC\underline{H}_3$), 0.97 (3H,s,$CC\underline{H}_3$), 2.70→2.82 (4H,m,$(C\underline{H}_2)_2$ CO)

ord (C, 0.5, CH_3OH): $[\phi]_{275}$ = -3,000, $[\phi]_{315}$ = +3,400, a =64

cd (C, 0.5, CH_3OH): $\Delta\varepsilon_{296}$ = +1.73

Attempted Base Dehydration of Keto-alcohol 80

The keto-alcohol 80 (1mg) was heated under reflux in 2% ethanolic KOH solution (5ml) for four hours. The cooled solution was diluted with saturated NaCl solution (10ml) and extracted with ether (3 x 5ml). The ethereal solution was washed with water (3 x 10ml), dried ($MgSO_4$) and evaporated to give recovered starting material (≈1mg).

tlc: Rf 0.60

gc: Rt 0.57

ms: identical with Figure 11.

Sodium Borohydride Reduction of Keto-alcohol 80

Keto-alcohol 80 (7.7mg) in 98% ethanol (2ml) was stirred with $NaBH_4$ (5mg) at room temperature. After 15 min. the starting material had been converted to two products (monitoring by gc). Excess $NaBH_4$ was destroyed with water (1ml). The solution was extracted with ether (2 x 5 ml), dried ($MgSO_4$) and evaporated giving a mixture (5mg) of two products; cybullol (79) and its C-2 epimer in a ratio of 1:4 (gc).

tlc: Rf 0.42 (cybullol), 0.50 (epicybullol)

gc: Rt 0.51 (80%; epicybullol), 0.57 (20%; cybullol)

Epicybullol (1mg) was obtained pure by ptlc; (silica gel plate, CH_2Cl_2/MeOH (10:1) solvent).

tlc: Rf 0.50

gc: Rt 0.51

pmr ($CDCl_3$): Figure 10, δ0.79 (3H,d,J=6Hz,$CHC\underline{H}_3$), 1.26 (3H,s,$CC\underline{H}_3$), 4.1 (1H,m,C<u>H</u>OH)

ms: m/e 198(M^+20%), 180(12), 165(10), 162(5), 129(10), 128(100), 127(10), 126(19), 125(12), 123(14), 110(28), 109(22), 99(16), 97(16), 95(20), 86(30), 81(27), 69(22), 57(24), 55(36), 43(38), 41(37).

Ketalization of Keto-alcohol 80

Keto-alcohol 80 (5mg) in benzene (2ml) was heated under reflux for 15 hours with ethylene glycol (500µl) and

p-toluenesulfonic acid (cat. amount). Water was continuously removed by passing the refluxing benzene over 4Å molecular sieves (Fisher). The cooled solution was then washed with 2%$NaHCO_3$ solution (5ml), with water (2 x 5ml) and dried ($MgSO_4$). Evaporation of the solvent gave the ketal (35a, 5αCH_3, 10αOH) plus a small (undetermined) quantity of starting material as a colorless oil (6mg).

tlc: Rf 0.60

gc: Rt 0.57

ms: Figure 13,

calc. for $C_{14}H_{24}O_3$, 240.1726, meas. 240.1718,

calc. for $C_8H_{11}O_2$, 139.0759, meas. 139.0763,

calc. for $C_7H_{10}O_2$, 126.0681, meas. 126.0678,

calc. for $C_6H_9O_2$, 113.0603, meas. 113.0608,

calc. for $C_5H_8O_2$, 100.0524, meas. 100.0525,

calc. for $C_5H_7O_2$, 99.0446, meas. 99.0448

pmr ($CDCl_3$): δ0.79 (3H,d,J=6Hz, CH_3CH), 1.13 (3H,s,CH_3C), 1.25 (s, impurity), 3.90 (4H,m,OCH_2CH_2O) ppm

ir (CCl_4): 3600 (O-H), 1710 (weak; C=O), 1090 (C-O) cm^{-1}.

Thioketalization of Keto-alcohol 80

a) Ethanedithiol as solvent

Keto-alcohol 80 (6.6mg), ethanedithiol (500µl) and boron trifluoride etherate (25µl) were stirred at 25°C for one hour. The solution was diluted with saturated NaCl solution (5ml) and extracted with ether (3 x 5ml). The

ethereal extract was washed with 20% aqueous NaOH solution (3 x 5ml), saturated NaCl solution (2 x 10ml) and dried ($MgSO_4$). Evaporation of solvent gave 3.6 mg of a mixture of four products (gc): 84, 85 and the C-10 epimers of 82.

tlc: (CH_2Cl_2 solvent) Rf 0.50, 0.70

gc: Four peaks, Rt 0.60→0.65 (cf. starting material, Rt 0.57).

The mixture was separated by ptlc (silica gel adsorbent; CH_2Cl_2 solvent).

Rf 0.50 (CH_2Cl_2) product: Epimers of 82

gc: Two peaks Rt 0.63, 0.65

ms: m/e 348 (M^+, 36%), 315(5), 287(6), 256(18), 255(88), 254(22), 195(14), 194(6), 193(8), 162(14), 161(100), 159(11), 131(75), 121(12), 119(25), 113(14), 105(72), 95(15), 93(20), 91(26), 81(32), 79(24), 77(14), 69(15), 67(20), 61(50),59 (18), 55(40), 41(44).

Rf 0.70 (CH_2Cl_2) product: 84 and 85 (tentative assignment)

gc: Four peaks, Rt 0.60→0.65 (Rt 0.63, 0.65 smaller than in original mixture).

ms: All peaks for ms of 82 (above) plus m/e 346, 318, 254, 161, 131 enhanced. Parent peak for 84, 85 = m/e 254.

b) Fieser method

Initially, the keto-alcohol 80 (2.5mg) in glacial acetic acid was stirred with ethanedithiol (4µl) and BF_3Et_2O (4µl) for 2 hours at 25°C. The solution was then diluted with saturated NaCl solution (5ml) and extracted with ether

(3 x 5ml). The ethereal extract was washed with 10% aqueous NaOH solution (3 x 5ml), saturated NaCl solution (2 x 10ml) and dried ($MgSO_4$). Evaporation of solvent gave a mixture of starting material (64%) and the required thioketal 81 (36%) as a colorless oil (2.6mg).

tlc: Rf 0.60, 0.92

gc: Rt 0.57, 0.87

The mixture was separated by ptlc (silica gel G, 2% MeOH in CH_2Cl_2 solvent).

Lower Rf product: 80

Same tlc, gc, ms as 80

Higher Rf product: 81 (<1mg).

tlc: Rf 0.92

gc: Rt 0.87

ms: Figure 14.

In an attempt to optimize the yield of 81, the following reaction conditions were used.

Sample	HOAc	SH SH	BF_3Et_2O	Time	Yield	80	81	82
mg	µl	µl	µl	hr	mg	%	%	%
2.5	50	4	4	2	2.6	64	36	
9*	250	20	20	3	10.7	-	37	35, 27
6.6	500	10	10	2	4.2	45	55	
13	1000	40	10	3.5	5.2	25	75	
7.2	1000	40	10	4.0	4.0	33	67	

* 20% NaOH in work-up.

Raney Nickel Hydrogenolysis of Thioketal 81

a) Initial Reaction Conditions

A mixture of thioketal 81 and keto-alcohol 80 (5.2mg, ratio as above) was stirred with W-2 Raney nickel[47] (excess) in 98% ethanol for 4 hours at 25°C. The solution was filtered and carefully evaporated at room temperature (roto-vac). The volatile residue was taken up in ether (5ml), washed with saturated NaCl solution (5ml), dried and evaporated at room temperature giving a mixture of three products plus unreacted keto-alcohol (total weight 1.2mg).

gc: Rt 0.08 (88 or 89, 27%), 0.15 (90, 15%), 0.17 (76, 58%), 0.57 (80)

gc/ms: (gc conditions, as prep. column below)

76, Figure 15

88, 89, Figure 16

90, Figure 17

Three products were obtained when a purified sample of thioketal 81 (0.7mg) was reacted as above.

b) More Vigorous Reaction Conditions

A mixture of thioketal 81 and keto-alcohol 80 (8mg) was heated under reflux with W-2 RaNi (excess) in 98% ethanol (1ml). The solution was filtered and carefully evaporated at room temperature. The residue was taken up in ether (3ml), washed with saturated NaCl solution (5ml), dried ($MgSO_4$) and partially evaporated to give a solution of 88/89 and 76, plus unreacted keto-alcohol 80 in ether

(≈1 ml).

gc: Rt 0.08 (88/89, 17%), 0.17 (76, 83%), 0.57 (80).

c) Separation of products

The three products 76, 80 and 88/89 were separated by prep. gc using the column and collector described earlier in this section, under 'Gas Chromatography'. The oven temperature was kept constant at 140°C; only the required product (76) was collected.

gc: Rt 0.17

tlc: Rf 0.65 (2%MeOH/CH_2Cl_2 solvent)

ms: Figure 15,

calc. for $C_{12}H_{22}O$ 182.1671, meas. 182.1677

ir (CCl_4): Figure 18

pmr ($CDCl_3$): Figure 20, δ0.75 (3H,d,J=6Hz, $CHCH_3$), 1.01 (3H,s,CCH_3), 1.25 (s,imp.)

trans-1,2-Dimethylcyclohexan-1-ol (72)

MeMgI (12.5gm) in ether (25ml) and 2-methylcyclohexanone (8.4gm) in ether (50ml) were stirred together at 0°C for 90 min. The mixture was poured into 6M H_2SO_4 (30ml) containing ice (30gm). After filtration the mixture was extracted with ether (3 x 50ml). The ethereal extract was washed with 2% Na_2SO_3 solution (2 x 50ml), 2% $NaHCO_3$ solution (2 x 50ml), water (50ml), dried (Na_2SO_4) and evaporated to give 6.8 gm (71%) of two products 72

(70%), 74 (14%) plus starting material (16%).

gc: oven temp. 70°C; Retention times 6.0 (72), 7.0 (73), 8.2 (74) min.

tlc: Rf 0.15, 0.25, 0.30 (CH_2Cl_2 solvent).

The major product (72) was isolated by dry column chromatography using silica gel (Activity III; 600gm) and CH_2Cl_2 solvent.

ms: $C_8H_{16}O$ calc. 128.1202, meas. 128.1206, (M^+, 23%), 113 (16), 112(12), 95(15), 85(40), 71(100), 69(22), 68(46), 58(40), 55(30), 43(50), 41(75).

ir (neat): 3450 cm^{-1} (O-H).

pmr ($CDCl_3$): δ0.87 (3H,d,J=6Hz,$CHC\underline{H}_3$), 1.10 (3H,s,$COHC\underline{H}_3$)

cmr ($CDCl_3$): 15.2 ($CHC\underline{H}_3$), 22.2, 26.1, 28.7 ($COHC\underline{H}_3$), 30.8, 40.1, 40.4 ($\underline{C}HCH_3$) 71.0 ($\underline{C}OHCH_3$) ppm, (relative to TMSi).

Acetylation of Compound F (109)

Compound F (109; 5.4 mg) in acetic anhydride (1 ml) containing pyridine (1 drop) was stirred at 25°C for 5.5 hours. The solvent was removed under vacuum to give a monoacetate derivative (3.5mg) as an oily residue.

tlc: Rf 0.54

ms: Figure 25 calc. for $C_{17}H_{30}O_4$ 298.2144, meas. 298.2136

ir ($CHCl_3$); Figure 26

pmr ($CDCl_3$): Figure 27 δ0.91 (3H,d,J=7Hz, $CHC\underline{H}_3$), 1.08 (3H,s,$CC\underline{H}_3$), 1.26 (s, imp.), 1.31 (6H,s,$C(C\underline{H}_3)_2$), 4.8 (1H, m,$C\underline{H}OAc$).

Attempted Deuterium Exchange of Compound F (109)

Compound F (2mg) was dissolved in methanol-d_1 (CH_3OD; 1 ml). The solvent was partially evaporated under N_2. The sample was introduced into the mass spectrometer by dipping a direct probe into the solution.

ms: (contains E impurity; % ages of ion current therefore not meaningful) m/e 258, 257, 256, 239, 238, 218, 205, 200, 199, 181, 180, 171, 170, 162, 152, 147, 129, 126, 124, 123, 111, 110, 109, 108, 98, 97, 96, 95, 81, 55.

Trichloroacetyl Carbamate Derivatives

The pmr spectra of 67, cybullol (79) and compound F (109) were recorded after the addition of excess trichloroacetylisocyanate to the nmr tube.

Pmr chemical shifts of trichloroacetylcarbamate derivatives ($CDCl_3$): 67 δ0.93 (3H,d,J=6.5Hz,$CHCH_3$), 1.12 (3H,s,CCH_3), 3.72 (s,imp.), 8.10 (1H,s,NHCO)

Cybullol (79) δ1.00 (3H,d,J=6Hz,$CHCH_3$), 1.15 (3H,s,CCH_3), 3.72 (s,imp.), ≈ 5.2 (1H,m,CHOCONH), 8.19 (1H, s,NHCO), 8.28 (1H,s,NHCO)

Compound F (109) (Figure 24) δ1.17 (3H,d,J=6Hz, $CHCH_3$), 1.18 (3H,s,CCH_3), 1.26 (s,imp.), 1.58 (6H,s,$C(CH_3)_2$). 3.72 (s,imp.), 8.08 (1H,s,NHCO), 8.28 (1H,s,NHCO), 8.92 (1H,s,NHCO).

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V APPENDIX

Results of biological testing of C.bulleri crude extract by Smith, Kline and French laboratories, Philadelphia.

February 26, 1975

Method and Material

The serial agar-dilution method used for the assay of fermentation products and for the evaluation of cyathin preparations earlier, was employed. Two-fold dilutions of the crude extract (from 250 to 0.5 µg/ml) were mixed into the Penassay Seed Agar buffered to pH 7. The surface of the agar was inoculated with the relevant dilutions of the suspensions of test micro-organisms using the Steers' multiple inocula replicator. After overnight incubation at 37°C for the bacteria and Candida albicans, and an additional three day incubation at 30°C for Trichophyton mentagrophytes, the median inhibitory concentrations (MIC's) were read and recorded.

The concentrations of the control antibiotics (gentamicin, erythromycin and amphothericin B) used in this evaluation were from 200 to 0.1 µg/ml in the usual two-fold dilutions.

Results

As the data of Table 1 show, SK&F 76543, the crude metabolite of Cyathus bulleri failed to produce any appreciable MIC values against the gram-positive cocci (Nos. 1-3), apathogenic mycobacterium (No. 4), gram-negative bacilli (Nos. 5-13), the yeast-like fungus, Candida albicans and the dermatophyte, Trichophyton mentagrophytes, the microorganisms included in the assay.

Table 1: In-Vitro Activity of SK&F 76543 and Controls

BUFFERED: pH 7.0		PENASSAY SEED AGAR										MIC (µg/ml)				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CODE	SK&F NO.	Staph. aureus HH 127	Staph. aureus SA 910	Strep. faecalis HH 34358	Mycobacterium phlei 1228	Proteus mirabilis PM 444	E. coli SKF 12140	Kleb. pneumoniae SKF 4200	Salmonella gallinarum ATCC 9184	Pseudomonas aeruginosa HH 83	Serratia marcescens ATCC 13880	Proteus morgani P-179	Providencia sp. FR-276	Enterobacter cloacae HH 31254	Candida albicans BC 759	Trichophyton mentagrophytes BC 1253
Dr. Ayer #34	76543	250	> 250	> 250	250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
Gentamicin		1.6	1.6	6.3	1.6	3.1	1.6	0.4	12.5	0.8	1.6	0.8	12.5	1.6	> 200	> 200
Erythromycin		1.6	> 200	> 200	1.6	> 200	100	100	> 200	> 200	200	200	> 200	> 200	> 200	> 200
Amphothericin B		> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	0.4	0.4