CHAPTER—III

Investigation on the root of *Leucas Aspera* spreng

SECTION—A : Extraction:

Dried and powdered root of *Leucas Aspera* (5 kg) was extracted in a Soxhlet apparatus directly with ethyl alcohol for 24 hours.

SECTION—B : Isolation of stigmasterol and a new triterpene dihydroxy lactone:

(i) The alcoholic extract was concentrated under reduced pressure to about 500 cc and it was hydrolysed directly with 7% H_2SO_4 by boiling under reflux for 10 hours. It was extracted with ether and neutral fractions were separated. The neutral fraction of ether layer was concentrated which gave an oily residue (5 gms). This was chromatographed over deactivated alumina and the following fractions were collected:

Table—1

Fraction	Eluent	Residue on evaporation
1	Petroleum ether:benzene (2:3)	White solid m.p.140—50°
2	Benzene:ether (4:1)	White solid m.p. 270—90°

Examination of fraction 1:

Fraction 1 was crystallised from chloroform-methanol mixture and the crystal obtained had the m.p. 164—66°, $[\alpha]_D$ -50°. IR spectrum showed peak at 3430 cm^{-1} for hydroxyl group. The compound was analysed for $C_{29}H_{48}O$. On acetylation with acetic anhydride and pyridine, furnished an acetate, $C_{31}H_{50}O_2$, m.p. 137—9°, $[\alpha]_D$ -34° [M$^+$ 454(Fig.1)], IR spectrum showed peaks at 1720 and 1240 cm^{-1} for acetate groups, which was identified as stigmasteryl acetate, when compared with an authentic specimen (m.m.p. and CO—IR). Thus the fraction 1 contained the compound stigmasterol which was confirmed by comparing with authentic sample (m.m.p. CO—IR, CO—TLC).

Examination of fraction 2:

(ii) Purification and characterisation of the new dihydroxy lactone - leucolactone, $C_{30}H_{48}O_4$:

Fraction 2 was combined and crystallised from chloroform-methanol mixture when shinning crystals (500 mg) of m.p. 310° were obtained. Elemental analysis by Rust's method showed the molecular formula of the compound as $C_{30}H_{48}O_4$ which was supported by the mass spectrum that showed the molecular ion peak at m/e 472. The compound showed no UV absorption in the region 200—350 nm. IR spectrum (Fig.2) of the compound in

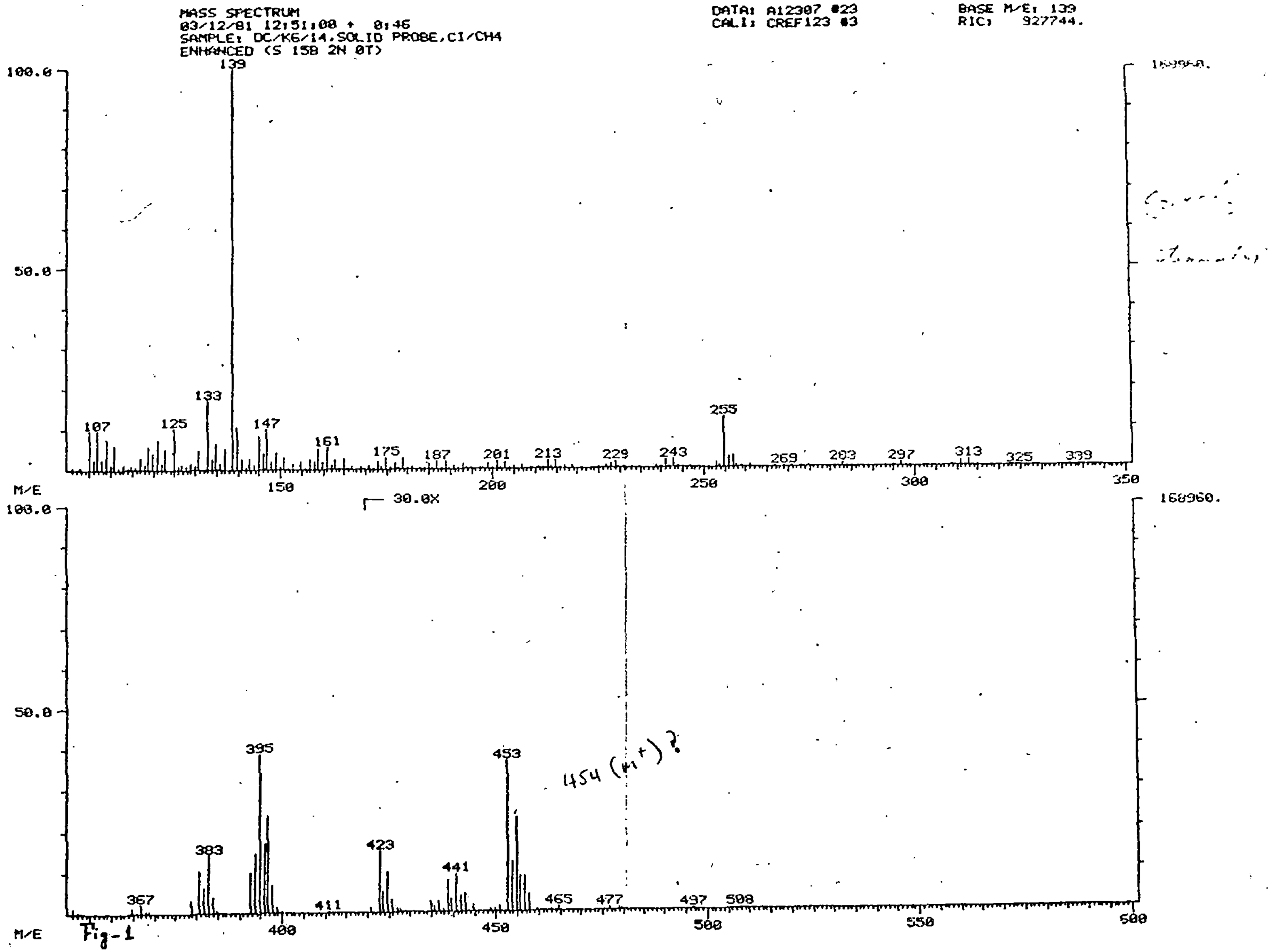


Fig.1: Mass spectrum of stigmasterol.

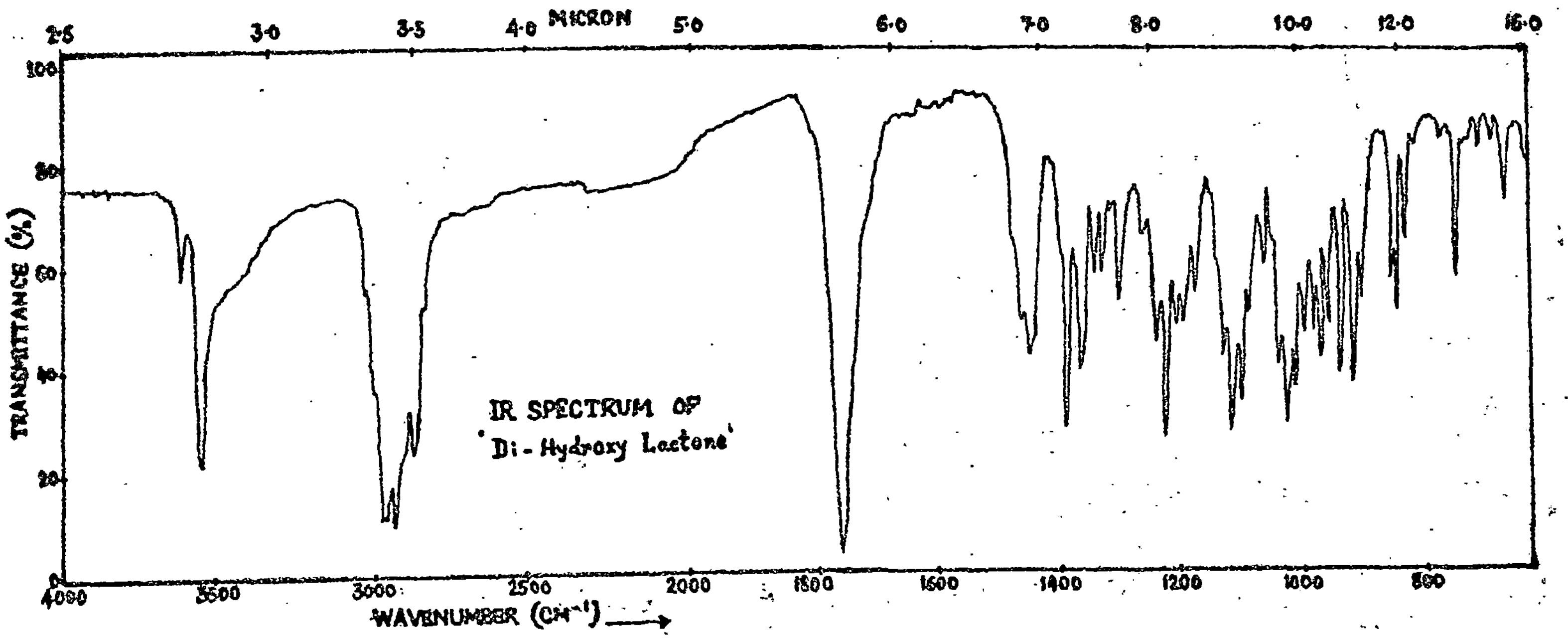


Fig.2: IR spectrum of leucolactone 1.

KBr disc showed the presence of two hydroxyl group at 3607 and 3540 cm^{-1} as two sharp peaks indicating that there is no hydrogen bonding and probably all the three functional groups are situated quite apart from each other. The peak at 1750 cm^{-1} indicated the presence of a γ -lactone ring. Peaks at 1380 and 1355 cm^{-1} indicated the presence of gem dimethyl group. The compound showed positive Libermann - Burtchard test for triterpenoids. It showed the absence of olefinic double bond as indicated by negative TNM test. The compound was designated as leucolactone 1.

(iii) Preparation of acetyl derivative of leucolactone 1—leucolactone diacetate 2, $C_{34}H_{52}O_6$:

200 mg of the hydroxy lactone 1 was acetylated with acetic anhydride and pyridine by keeping over water bath for four hours. Usual work up and chromatography over deactivated alumina furnished a compound of m.p. 270—80°, which on repeated crystallization from chloroform-methanol mixture furnished needle shaped crystalline solid, m.p. 284—85°. Elemental analysis and mass spectrum showed the molecular formula of the acetate as $C_{34}H_{52}O_6$ (M^+ 556). IR spectrum (Fig.3) showed the presence of two acetate carbonyl streaching at ν_{max} 1728 and 1740 cm^{-1}, 1218 and 1255 cm^{-1}. The lactone carbonyl appeared at 1768 cm^{-1}, the gem dimethyl appeared at 1359 and 1379 cm^{-1}.

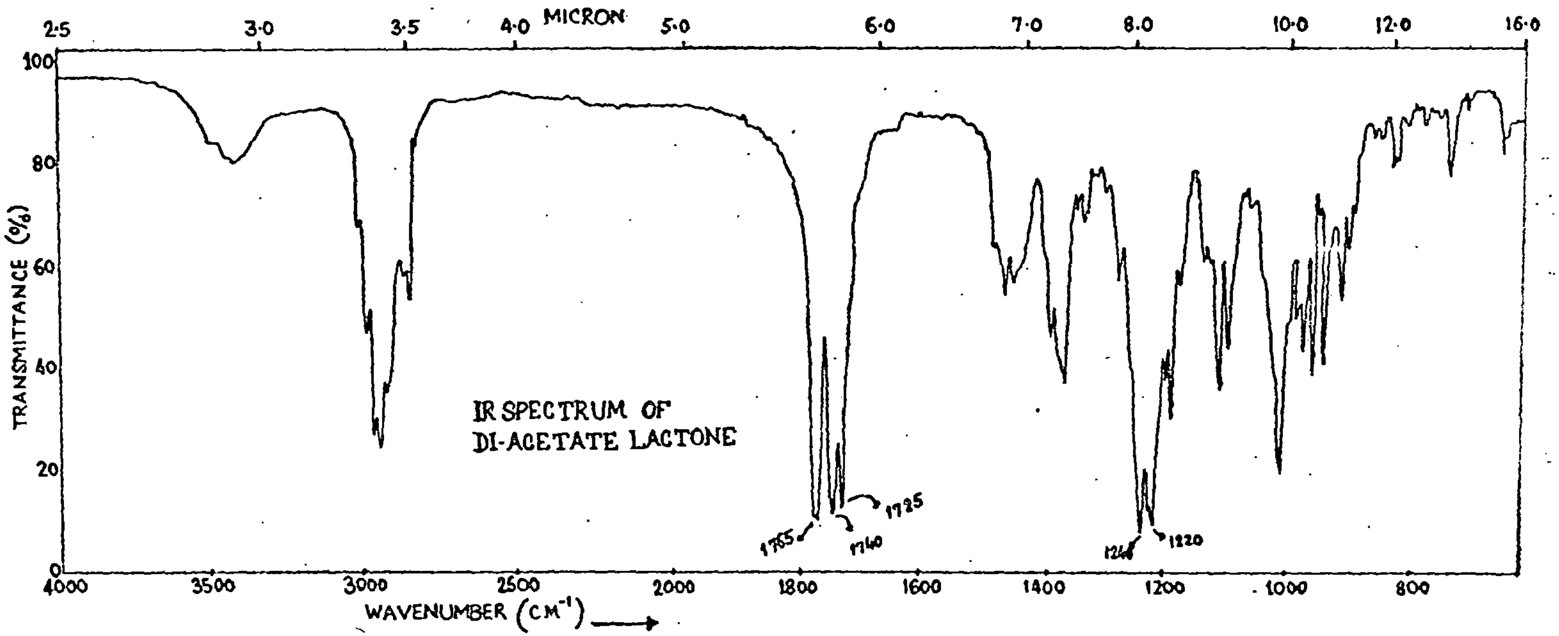


Fig.3: IR spectrum of leucolactone acetate 2.

The compound did not show any peak in the region 700—900 cm^{-1} indicating absence of double bond.

(iv) Oxidation of leucolactone 1 — diketoleucolactone 3:

100 mg of the compound 1 was oxidised by chromium trioxide in presence of pyridine by stirring for about 12 hours. Usual work up and chromatography over deactivated alumina furnished a compound of m.p. 310—20°, which on repeated crystallisation from chloroform-methanol mixture furnished needle shaped crystalline solid m.p. 323—24°. Elemental analysis and mass spectrum showed the molecular formula of the diketolactone 3 as $C_{30}H_{44}O_4$ (M^+ 468). IR spectrum (Fig.4) showed peak at 1760 cm^{-1} (γ-lactone), broad peak in the region 1705—1715 cm^{-1} for the carbonyl functional groups present in the molecule.

From the foregoing data it may be said that the compound 1 contains two hydroxyl groups which are acetylable, one lactone functional group which is probably a γ-lactone, thus accounting for all the four oxygen atoms of the molecule, $C_{30}H_{48}O_4$. The negative TNM test and absence of absorption peaks in the region 700—900 cm^{-1}, 1600—1640 cm^{-1} definitely showed the absence of olefinic double bond. From the molecular formula it may thus be inferred that the compound 1 is probably a saturated pentacyclic triterpenoid. Literature survey of dihydroxy triterpenoid indicated that the compound could be

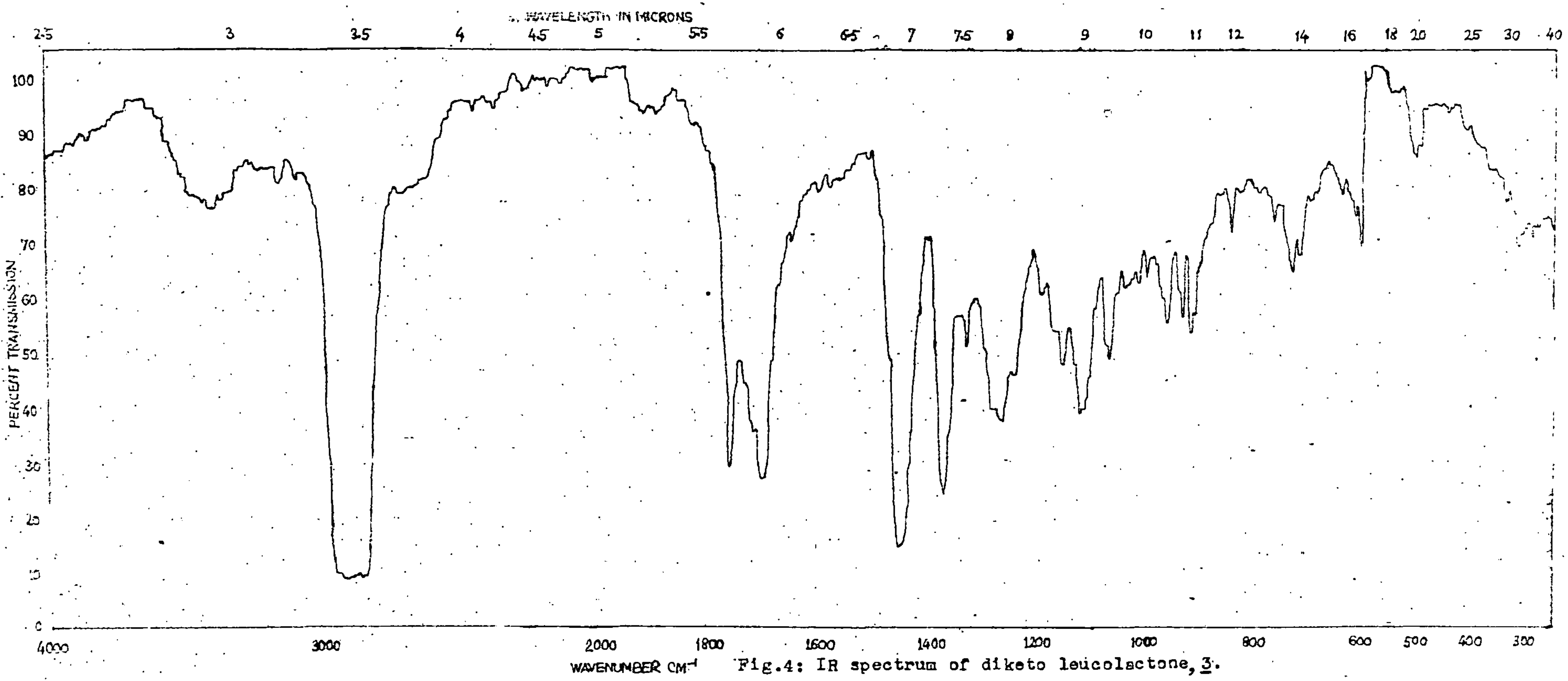


Fig.4: IR spectrum of diketo leucolactone, 3.

a new one. Hence we have carried a thorough physical analysis of the compound 1 and its derivatives which are discussed below:

SECTION—C:

(i) PMR spectral data of leucolactone 1:

^{1}H NMR spectrum of the compound 1 showed peaks at 0.85 ppm for 1-tert-methyl group, at 0.92 ppm for three tertiary methyl protons, at 0.95, 1.18 and 1.28 ppm for one tertiary methyl each as singlet. The multiplets at 2.6 ppm was integrated for one proton. The peak centred at 3.18 ppm with width at half height 8 Hz indicated the presence of axial hydroxyl group and the broad peak centred at 3.85 ppm with width at half height 18 Hz indicated the presence of equatorial hydroxyl group.

(ii) NMR spectral analysis of acetyl derivative of leucolactone 1:

^{1}H NMR spectrum (Fig.5) of the acetate of leulactone 1 showed a singlet at 0.85 ppm which was integrated for protons of four tertiary methyl groups, the peak at 0.92 ppm was integrated for three protons of a tertiary methyl group. The singlets at 1.18 ppm and 1.28 ppm integrated for six protons showing the presence of two tertiary methyl groups.

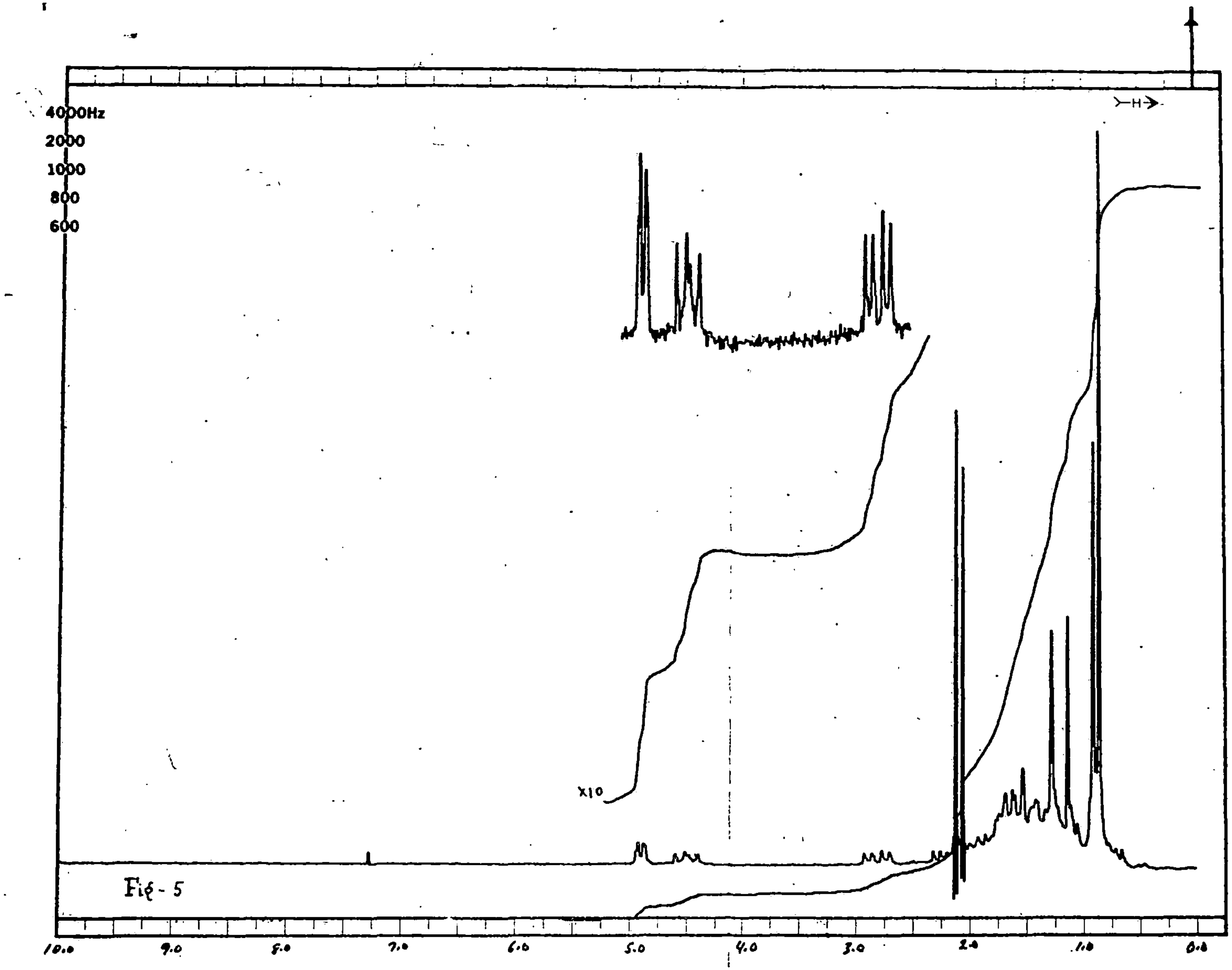


Fig.5: PMR spectrum of leucolactone acetate 2.

The singlets at 2.05 and 2.12 ppm clearly indicated the presence of two acetoxy methyl groups. The presence of two doublets centred at 2.8 ppm with J value about 8 Hz, which was integrable for one proton showed that the lactone carbonyl has an α-proton which had two neighbouring protons that coupled to cause splitting of the peak due to the α-proton. The low coupling constant of 8 Hz indicated that this α-proton must be equatorially oriented and hence the lactone carbonyl should be axially oriented. The peak centred at 4.45 ppm was a triplet that had the J value of 18 Hz. The high coupling constant shows that this proton is axially oriented and is attached to carbon bearing the acetate group which must be equatorially oriented. The last absorption was centred at 4.9 ppm that appeared as a doublet with J value of 8 Hz. This indicated that the proton attached to carbon bearing the second acetoxy group must be equatorially oriented, thus the acetoxy group must be axially oriented. The coupling as a doublet showed that this proton had either only one α-neighbouring proton or it might have two neighbouring protons, the coupling of one of which must be negligible. Decoupling experiments of these multiplets between the range 2.5--5.0 ppm showed that none of these protons were spin coupled with each other showing that carbonyl carbon and the two hydroxyl groups are quite far from each other.

^{13}C NMR spectrum (Fig.6) of the acetate lactone 2 showed the presence of 34 carbon atoms in the range between 15 ppm to 180 ppm. Off resonanse CW-decoupled experiments (Fig.7) showed the presence of 9-CH_3 groups, 10(—CH_2—) groups, 5 (—C—H) groups and 10 (—C—) accounting for the 34 carbon atoms and 52 protons of the acetate, thus confirming the molecular formula as $C_{34}H_{52}O_6$. Again in ^{13}C NMR spectrum (Fig.8) of the acetate lactone 2 revealed that there are three singlets at 176.09, 170.85 and 169.79 ppm for the carbonyl carbon. The one that appeared at 176.09 ppm is probably due to the lactone carbonyl which is either 5 or 7 membered ring. The other two being due to acetate carbonyl groups. The singlet that appeared at 90.2 ppm must be due to the carbon containing the lactyl oxygen atom. The doublet at 80.53 and 71.64 ppm must be due to the carbon bearing the acetyl groups. The two acetyl methyl carbons appeared as quartet at 21.4 ppm.

Before considering the structure of the compound 1 i.e. the leucolactone, we may write the various pentacyclic triterpenoids belonging to different carbon skeletons having 8-methyl groups at various positions.

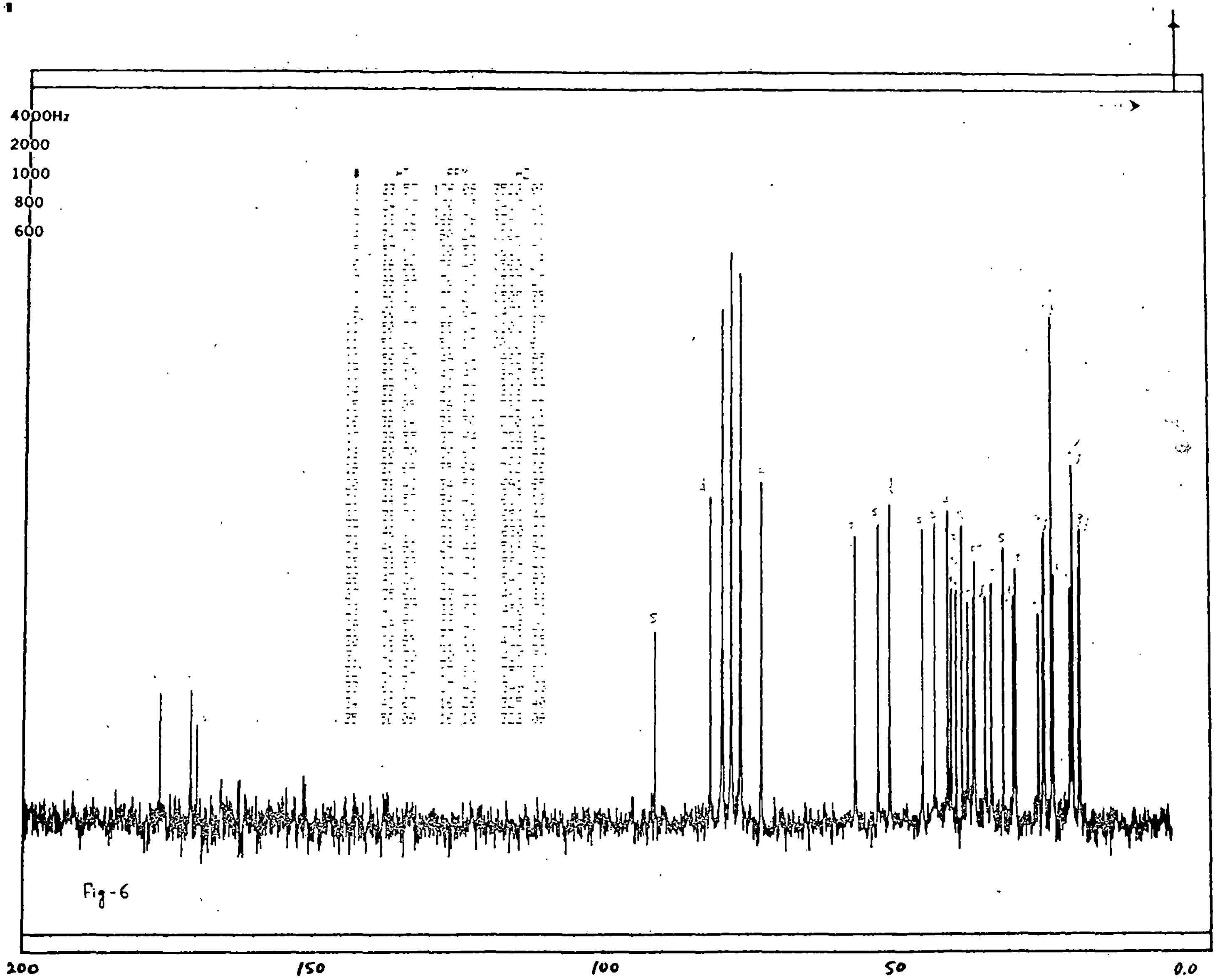


Fig.5: ^{13}C NMR spectrum of leucolactone acetate **2**.

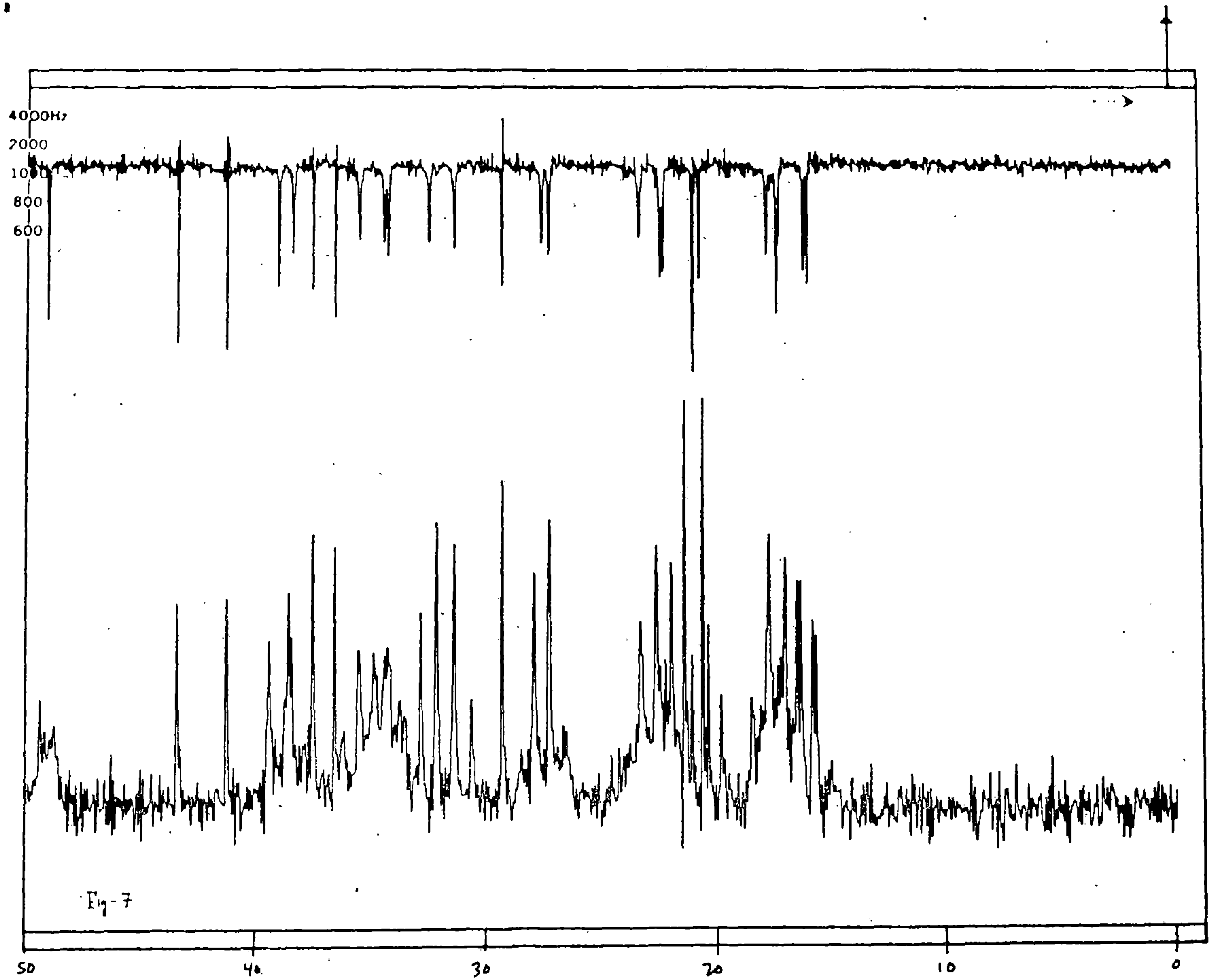


Fig.7: ^{13}C NMR spectrum of leucolactone acetate 2,(off resonance cw decoupled).

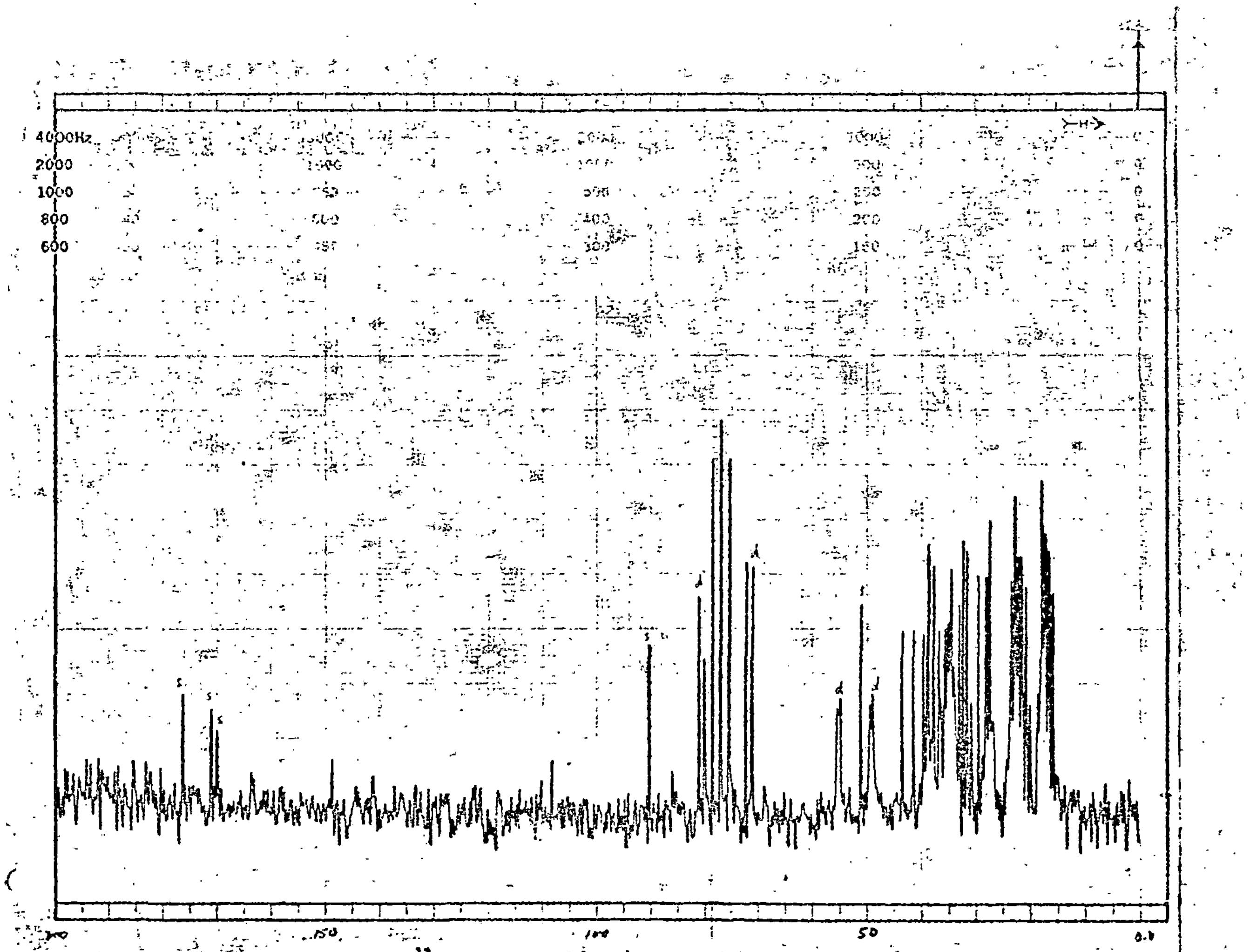
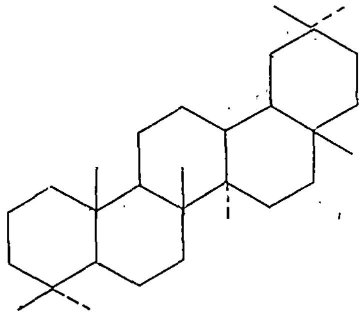
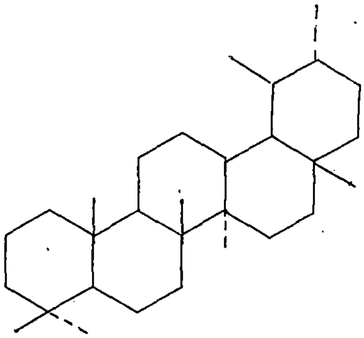


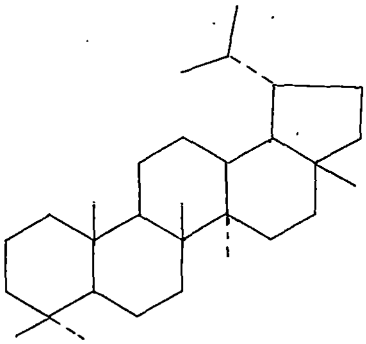
Fig 8 : ^{13}CMR spectrum of leucolactone acetate, **2** (off resonence CW decoupled)



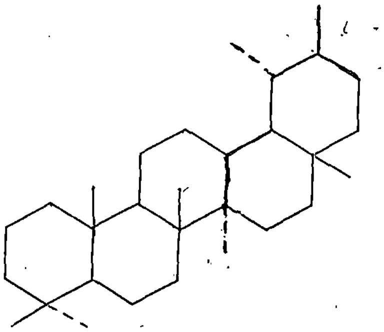
Oleanane



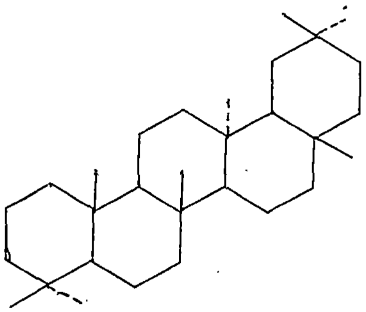
Ursane



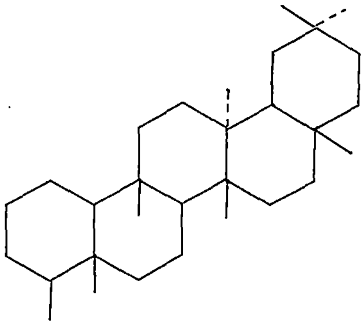
Lupane



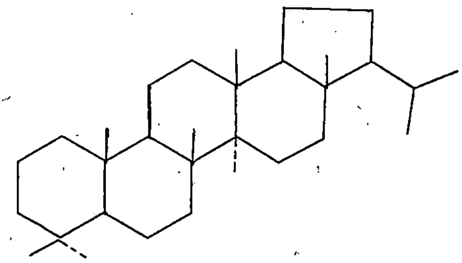
Taraxastane



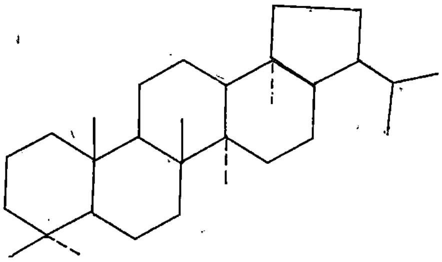
Taraxarene



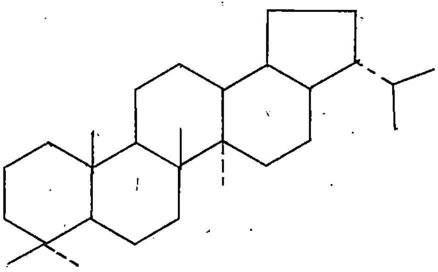
Friedelane



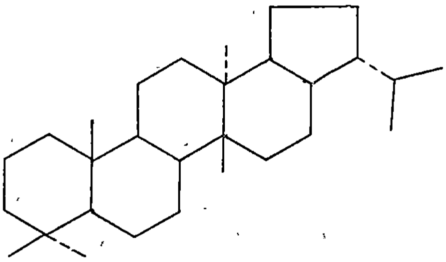
Arborane



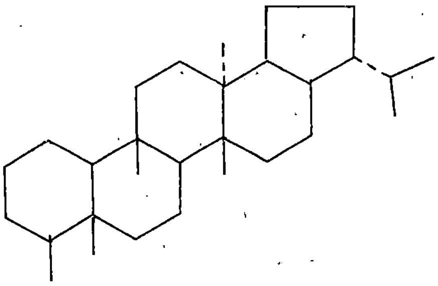
Hopane



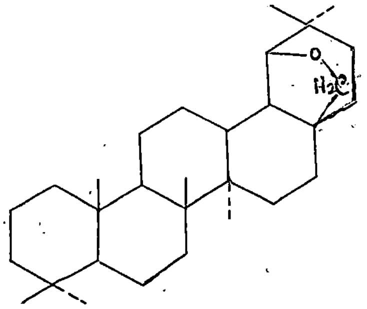
Isohopane



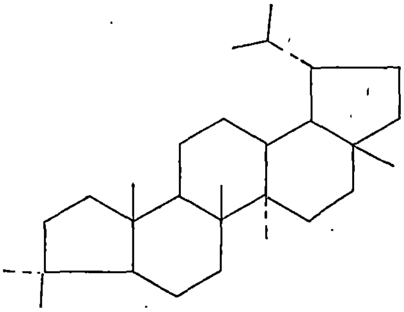
Fernane



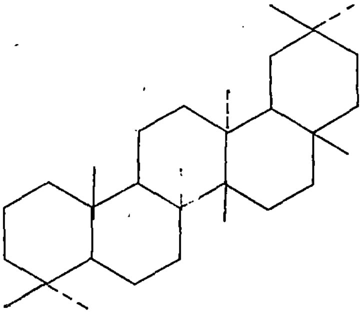
Filicene



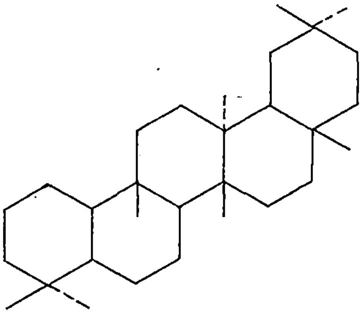
Allobetulin



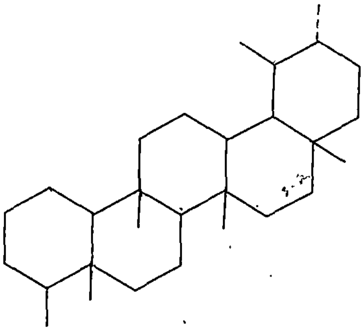
Ceanothane



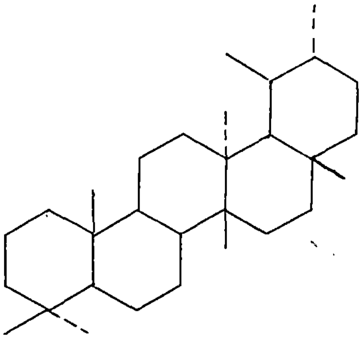
Multiflorane



Glutane

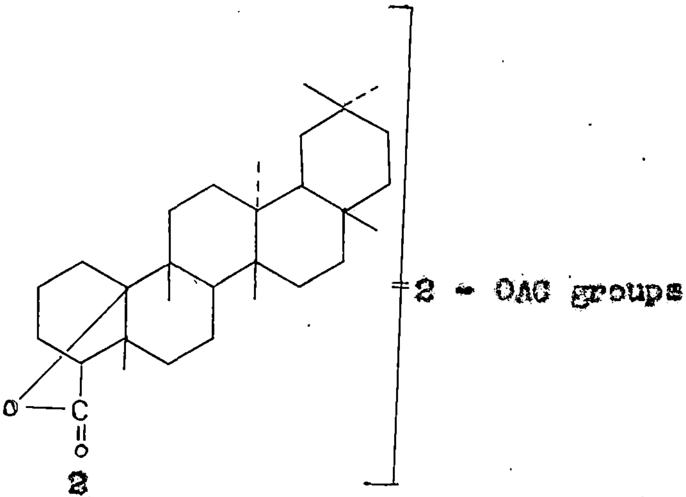


Eupacane



Baurane

Of all the above skeletons of the pentacyclic triterpenoids if we assume that the lactone carbonyl is the C—23 carbon of friedelaneskeleton with the lactyl oxygen being attached to the C—10 carbon atom then we would be able to explain partially the ^{1}H NMR and ^{13}C NMR spectral data mentioned above. So the probable partial structure of leucolactone acetate can be written as 2.



2

Since the proton α-to the lactone carbonyl group is having two α-protons that coupled with each other it indicates that there is no oxygen function at the usual C—3 position. Hence the two hydroxyl groups may be present at any other secondary carbon atoms except the position C—3.

(iii) NMR spectral analysis of diketoleucolactone 3:

XL—300 PMR spectroscopy of the ketolactone 3 (Fig.9) showed the presence of seven tertiary methyl groups

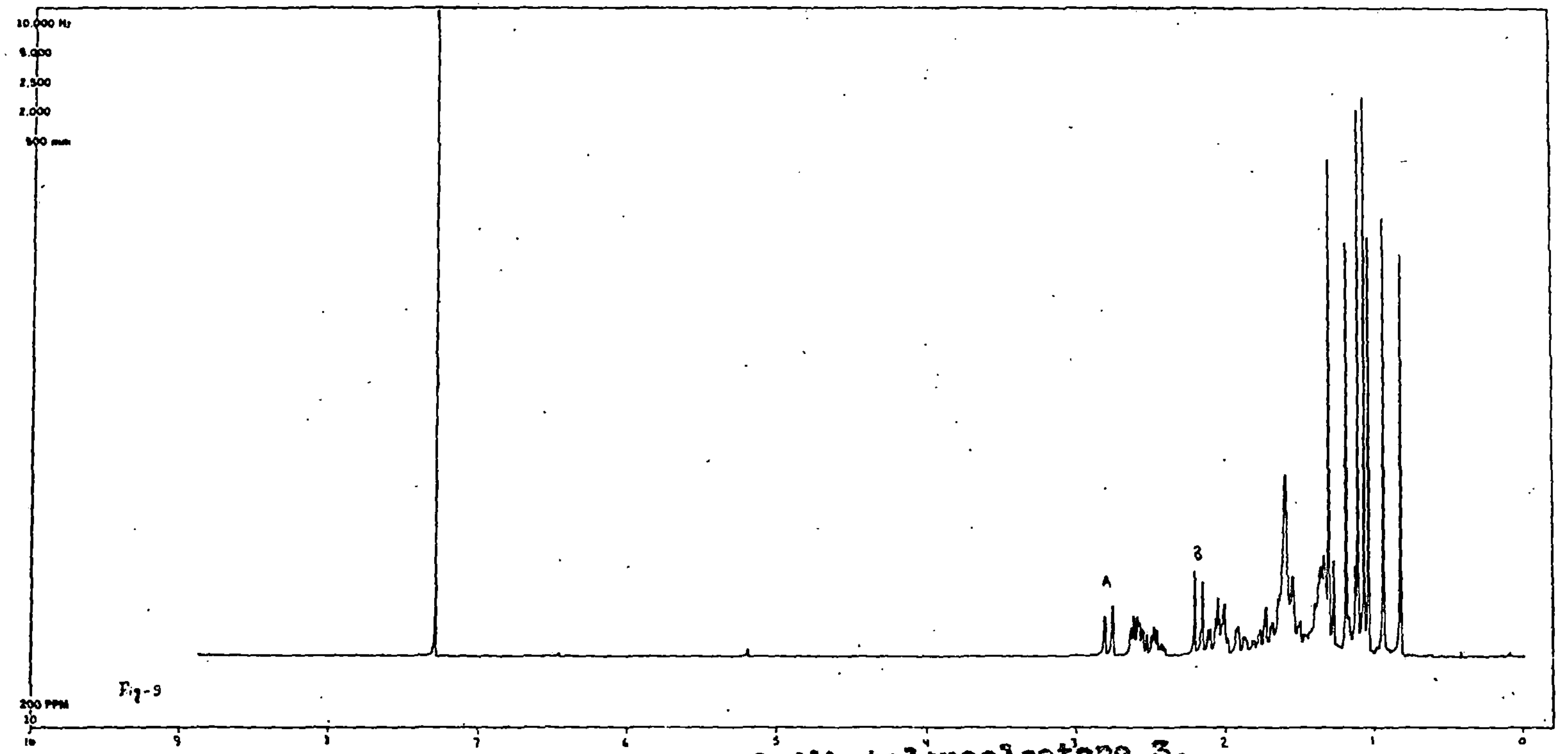


Fig.9: PMR spectrum of diketoleucolactone 3.

at 0.82, 0.93, 1.02, 1.06, 1.10, 1.18 and 1.30 ppm, it showed a series of multiplets between 2.0 to 3.0 ppm, accounting for five protons situated at the α-carbon atoms of the carbonyl carbons. It was already shown that the lactone carbonyl has only one α-proton so the two ketonic carbonyl groups are far apart from each other. The protons that appeared as doublets at 2.18 and 2.78 ppm are coupled with a gem coupling that are separated by 0.6 ppm. One appeared to be shifted upfield and the other downfield presumably by the anisotropic effect of the adjacent carbonyl. These protons have no other proton neighbour showing that the carbonyl functional group can not be present at C—7 position. Otherwise if the oxygen function would have occupied C—7 position it would have 3 α-protons which is not possible in this case. Again it cannot be at C—6 position, as the proton at C—8 would couple with α-protons at C—7 position. The proton at 2.78 ppm shows a coupling to the methyl at 1.18 ppm with a very small J-value (Fig.11) of 0.5 Hz which has been confirmed by decoupling experiments to the methyl at 1.18 ppm as shown in the Fig.10 where the irradiation at 1.18 ppm gave singlets at 2.18 and 2.78 ppm. Therefore, this —CH_2— group can not be at C—1 or C—21 positions because a gem-dimethyl peak would be broadened by CH_3—CH_3 coupling and would not show the coupling observed and 1.18 ppm. This eliminates the presence of oxygen function at C—2 or C—22 positions.

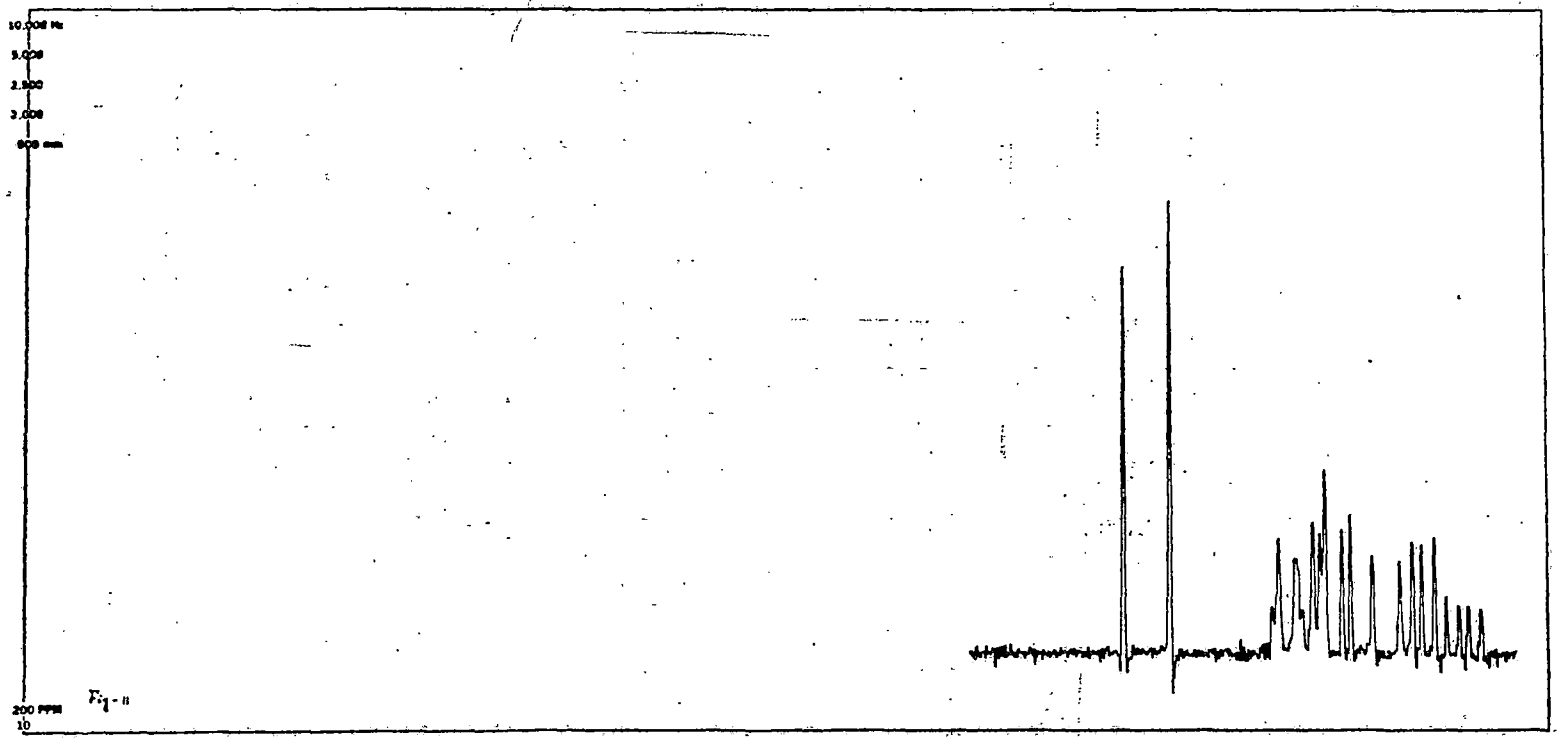


Fig.10: PMR spectrum of diketo leucolactone 5 (decoupled methyl at 1.18 ppm.

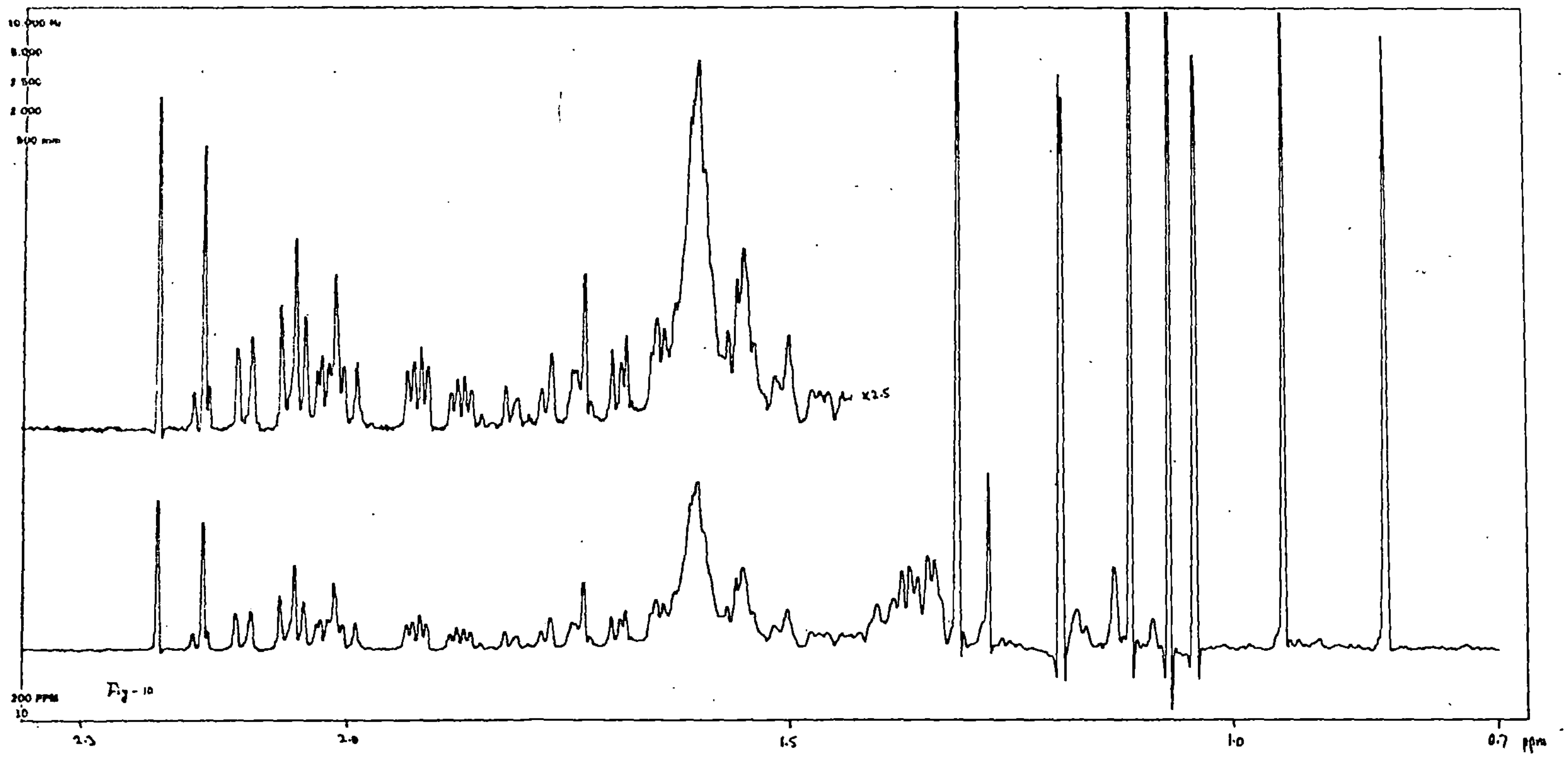


Fig.11: PMR spectrum of diketo - lactone 5 (plot expansion)

We have mentioned that the doublet that appeared at 4.9 ppm of the acetate lactone (Fig.5), would be due to the presence of one neighbouring proton α- to the methine carbon containing the acetate group. Had therebeen only one α-proton, then we could very easily place one of the two hydroxyl groups at C—19 position of the friedelaneskeleton but the presence of two α-protons in each of the carbonyl groups of the ketone established that the oxygen function is not present at C—19 position. Had the carbonyl group been present in C—19 position we would observe a singlet for one proton due to the C—18 proton which is not observed in the NMR spectrum of the diketolactone. From the above derivations the ketone functions or the hydroxy function must occupy any two of the carbon sides at C-1, C-11, C-12, C-15, C-16 and C-21 positions. The multiplets of eight lines between 2.4 and 2.5 ppm (Fig.12) showed that these protons i.e. one of the —CH_2— protons next to one of the ketones has two vicinal neighbours, such a situation is possible only if one of the ketone functions is located at C—1 positions, the α-protons at C—2 would then couple with protons at C—3 positions. Thus we may fix one of the oxygen functions at C—1 position. Hence the second oxygen function may occupy any one of the positions at C-11, C-12, C-15, C-16 or C-21.

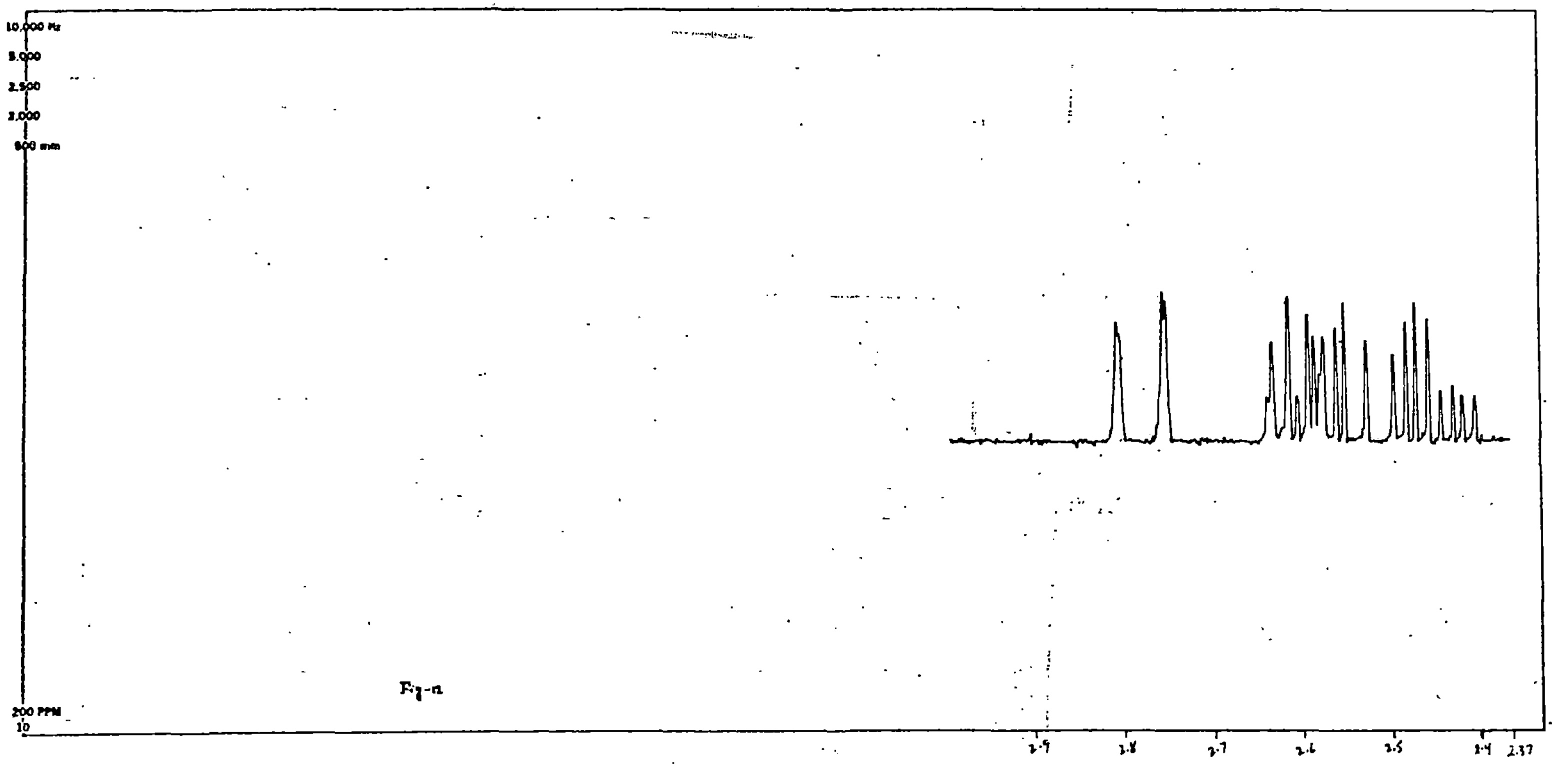
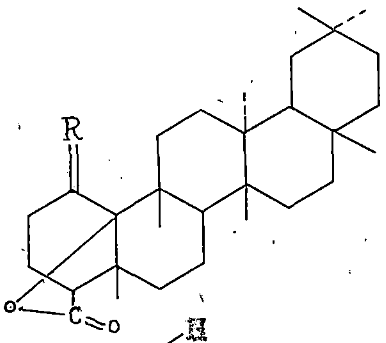


Fig.12: PMR spectrum of diketoleucolactone 5 (plot expansion).

Thus we may write the partial structure of leucolactone 1, leucolactone acetate 2 and diketoleucolactone 3 as:



1, R = $<^{H}_{OH}$

2, R = $<^{H}_{OCOCH_3}$

3, R = O

Another R group may be at any of the positions C—11, C—12, C—15, C—16 and C—21 positions.

Further discussion on the methyl signals of diketolactone 3:

The individual chemical shifts of the various methyl groups in the ^{1}H NMR spectra of friedelin have been assigned by Crowford *et al*[5]. It was suggested that the most downfield signal at 1.16 ppm to the C—28 methyl. Anjaneyulu *et al*[6] have assigned signals for different methyl groups of various D:A friedooleanane derivatives. A comparison of the methyl signals of the various derivatives of the compound 1 showed that the most downfield methyl signal appear at 1.28 ppm which must be the C—24 methyl group deshielded by the anisotropic effect of the lactone carbonyl group. The signal at

1.18 ppm as confirmed earlier[6], can be assigned to C—28 methyl group. Now these methyl protons split as a doublet (J = 0.5 Hz) as mentioned earlier which must be due to protons at either C—16 or C—22 position. Thus we may assume now that the second oxygen function is attached to either C—15 or C—21 position.

SECTION—D:

Mass spectral analysis of the compound leucolactone 1 and its derivatives:

Mass spectrum (Fig.13) of the compound 1 showed fragments at m/e 187, 189, 202, 203, 207, 220 (base), 221, 233, 234, 251, 384, 411, 421, 439, 454, 472 (M^+). The peak at m/e 472 being molecular ion peak, the other peak at m/e 454 must be due to loss of a molecule of water from the molecular ion. The peak at 439 is probably due to loss of a methyl group from the fragment at m/e 454, the one at m/e 421 may be due to loss of another water molecule from the fragment at m/e 439. Peak at m/e 411 may be considered due to the loss of —CO group from the fragment at m/e 439. The peak at m/e 202 may be due to loss of water from the fragment at m/e 220. The fragment at m/e 189 may be due to loss of water from fragment at m/e 207.

Mass spectrum (Fig.14) of the acetate of compound 1 viz. leucolactone diacetate 2 showed peaks at m/e 556, 496, 481, 453, 436, 421, 414, 393, 327, 301, 263, 262, 249, 233, 216, 212, 203, 189 (base), 187. The fragment at m/e 496 was due to the loss of CH_3COOH from M^+ ion peak. The peak at m/e 481 was due to loss of one methyl group from the fragment at m/e 496. The peak at m/e 453 was due to loss of CO from the fragment at m/e 481. The peak at m/e 436 was due to loss of CH_3COOH from the fragment at m/e 496. The peak at m/e 421 was due to loss of acetic acid from the fragment at m/e 481. The peak at m/e 202 was due to loss of CH_3COOH from the fragment at m/e 262 and the peak at m/e 189 might be due to loss of CH_3COOH from the fragment at m/e 249.

Mass spectrum of the ketolactone 3 showed molecular ion peak at m/e 468 and the other fragments at m/e 453, 425, 230, 250, 240, 218, 212, 210, 203, 196, 189, 182 etc.

The presence of fragments at m/e 220 and 202 in the hydroxylactone 1, 262 and 202 in the acetate lactone 2 and the fragments at m/e 218 in the diketolactone 3 shows that one of the oxygen must be present either in ring D or E, so as to form the fragment a.

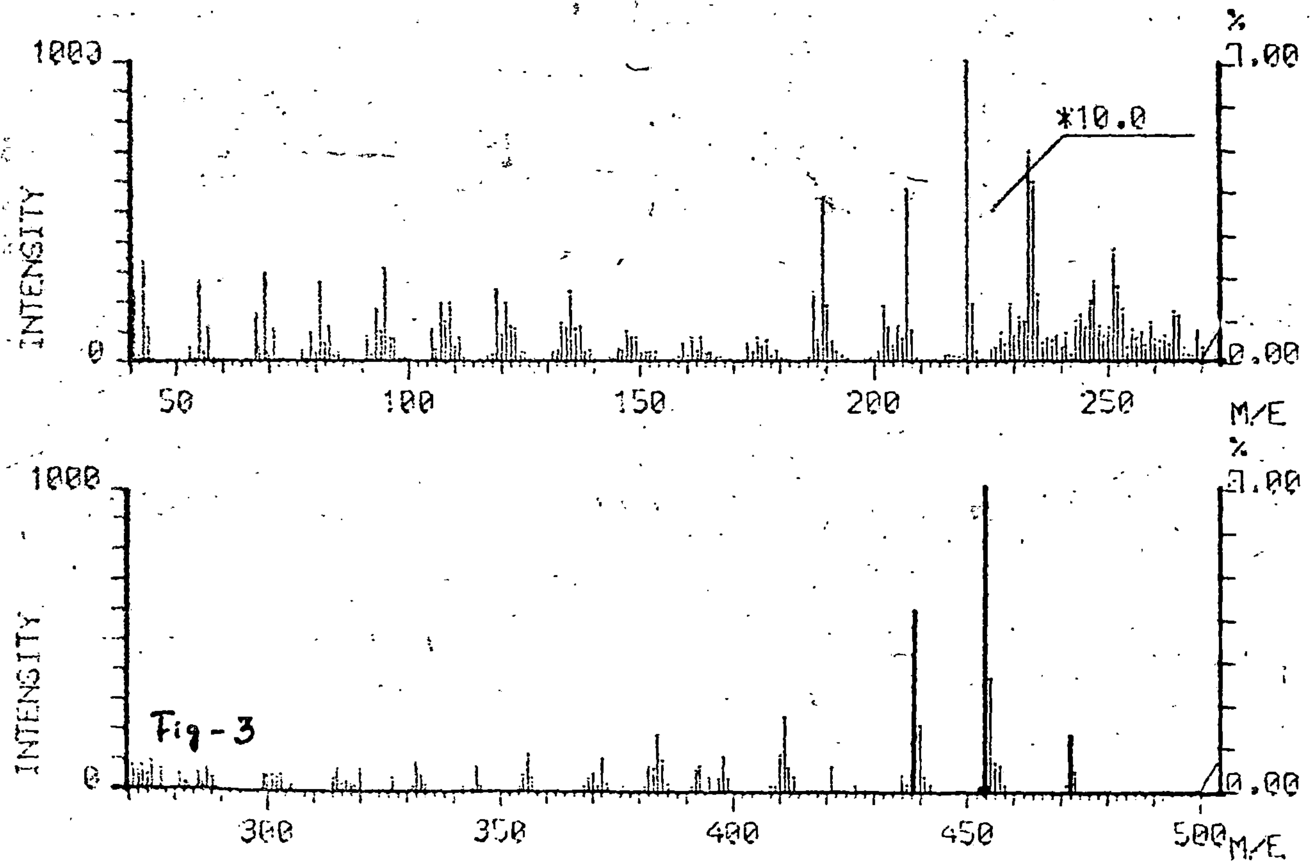


Fig-3

Fig.13: Mass spectrum of leucolactone **1**.

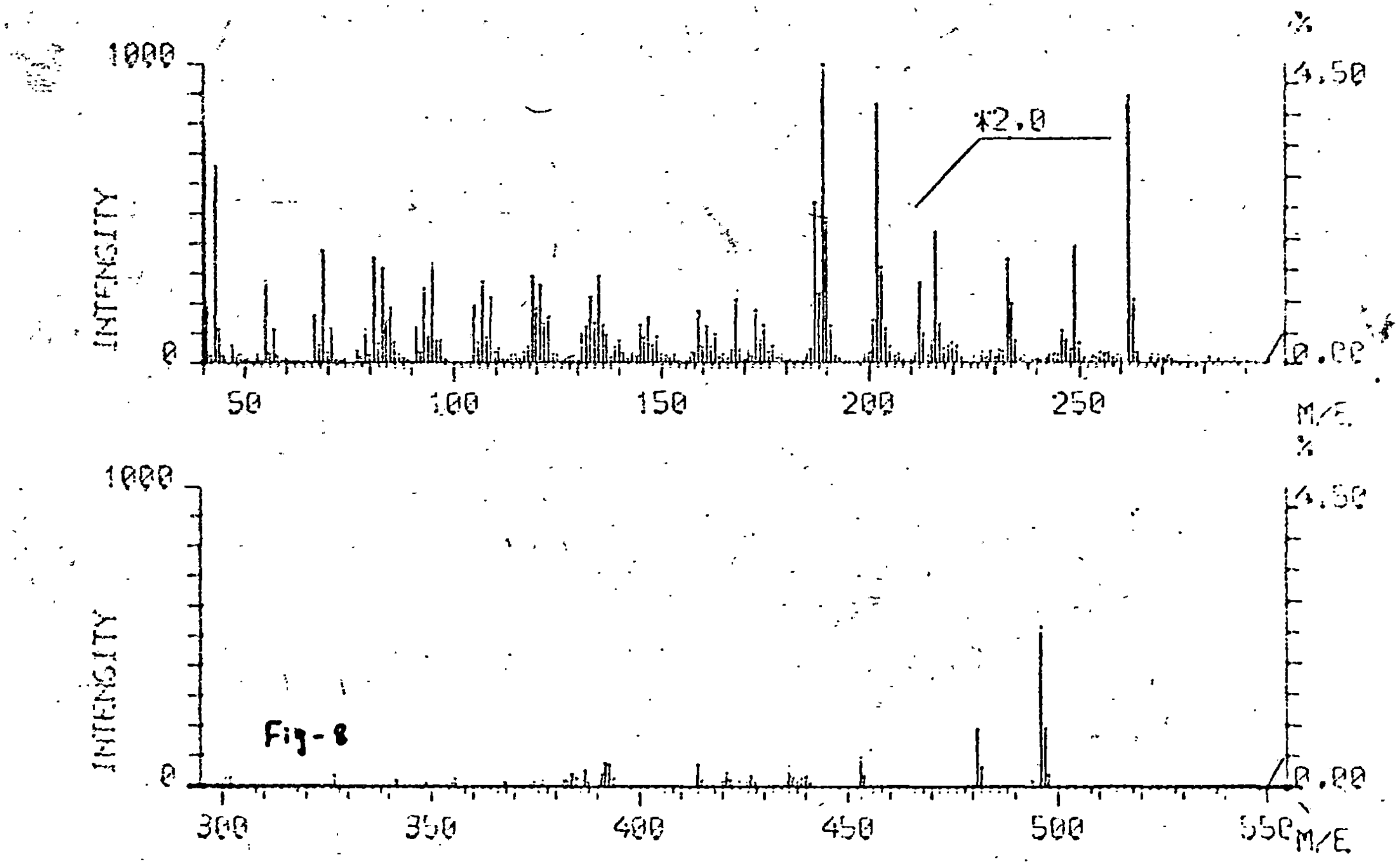
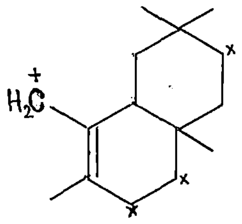


Fig-8

Fig.14: Mass spectrum of leuctolactone acetate 2.

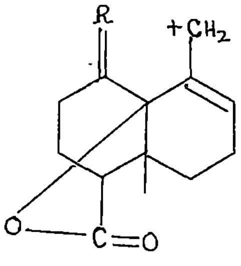


a

+ Any one of the positions containing group R.

$$\begin{tikzcd}
\text{When R = OH, } \underline{a} = m/e\ 220 \arrow[r, "-H_2O"] & m/e\ 202 \\
\text{R=}CH_3COO\text{, } \underline{a} = m/e\ 262 \arrow[r, "-CH_3COOH"] & m/e\ 202 \arrow[dd, "-CH_3"] \\
\text{R=O, } \underline{a} = m/e\ 218 \arrow[d, "-CH_3"] & \\
m/e\ 203 & m/e\ 187
\end{tikzcd}$$

Whereas the fragments at m/e 221, 203 in the hydroxy lactone 1 and fragments at m/e 263 and 203 in the spectra of the acetate lactone 2 and fragments at m/e 219 in the case of diketolactone 3 showes that the fragment b is formed which may be depicted as follows:



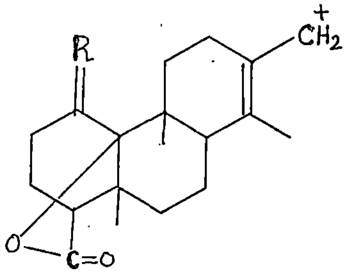
b

When, $R = \left< \begin{matrix} OH \\ H \end{matrix} \right.$, $\underline{b} = m/e\ 221 \xrightarrow{-H_2O} m/e\ 203$

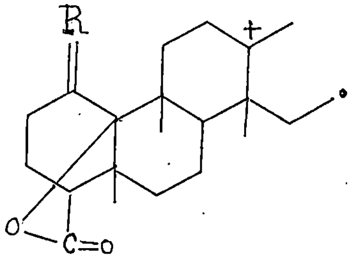
$R = \left< \begin{matrix} OCOCH_3 \\ H \end{matrix} \right.$, $\underline{b} = m/e\ 263 \xrightarrow{-CH_3COOH} m/e\ 203$

R= O, $\underline{b}$ = m/e 219.

The presence of peaks at m/e 303 and 332 for the hydroxy lactone 1, fragments at m/e 301 and 330 for the diketolactone 3, indicated the formation of fragments c and d.



c

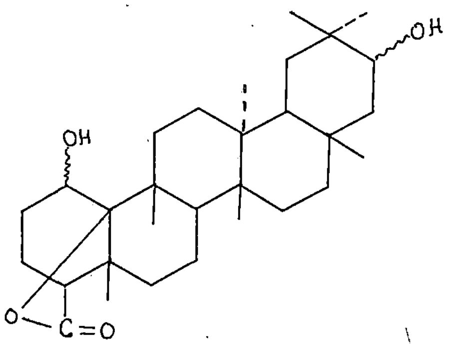


d

R = $\langle^{OH}_{H}$, c = m/e 303 R = $\langle^{OH}_{H}$, d = m/e 332

R=O , c = m/e 301 R=O , d = m/e 330

The fragments a, b, c and d are typical fragments which have been found in other compounds belonging to the friedelane[7] skeleton. Thus from the above mass fragmentation analysis it may be inferred that the second oxygen function is probably not present in C—11, 12, 15 or 16 positions, thereby leaving only one probable position i.e. C—21 for the second hydroxyl group. Thus we may write the probable structural formula of the leucolactone as 1.



1

Though we have isolated the above compound once, attempts to reisolate the same, collected during the next following seasons failed to afford the above leucolactone thereby causing delay in further chemical investigation for the structural establishment of the compound. We are trying to reisolate the compound in a larger quantity currently, collection of which have already been started. It is hoped that we may be able to complete the establishment of the structure of this compound by further chemical and physical studies within next one year.