CHAPTER II

Oldenlandia diffusa

Discussion and Result

Criteria for Activity

As stated previously in the introduction, the goal of the research is the isolation, study of the chemical constituents and structure elucidation of tumour inhibitors from Oldenlandia diffusa and Acanthus (ilicifolius and ebracteatus)

There are two systems of tumour activity test evaluation. (7, 8) These are survival tumour systems and tumour weight inhibition systems. Survival tumour systems are evaluated on the basis of survival time and are reported in the form of percent T/C. T is mean or median survival time of the test animal and C is the median survival time of control animal. Percent T/C is, therefore, the ratio of survival time of treated animals to control animal; expressed as percent. In general, a minimal increase in survival of treated animal over controls resulting in a T/C of 125 or more is a necessary condition for further experimental work. Tumour weight inhibition systems are evaluated on the basis of tumour inhibition. There are two ways of reporting the activity, percent T/C and ED_{50}. Percent T/C is the ratio of the tumour weights of treated animals to control animals expressed as percent. In general a minimal reproducible tumour weight

inhibition of test over control animals resulting in a T/C of 42 percent or less is necessary for further experimental testing. ED_{50} is the dose that inhibits tumour growth to 50 % of the control growth. If a compound is found to inhibit tumour growth to 50 % of the control growth at a dose of 4 $\mu g/cm^3$ or less ($ED_{50} \leq 4\mu g/cm^3$) it is deemed significantly cytotoxic. A compound is considered to be devoid of activity if the ED_{50} is greater than 100 $\mu g/cm^3$. For crude natural products of demonstrated minimal in vivo activity, in vivo testing is used, when possible, for biological assay during fractionation leading to isolation of the antitumour material.

In vitro testing is used primarily for bioassay related to fractionation of in vivo active natural products. Normally, materials are not developed further on the basis of in vitro activity alone. Currently, however, new natural products with on ED_{50} of 4 $\mu g/cm^3$, are evaluated for further testing.

In subsequent testing, activities substantially greater than the minimal end points described above are required (eg., a T/C % of 150 in 3LE testing). Criteria for acceptance as a candidate for development toward clinical trial are dependent on a number of factors, including the predictive value and overall sensitivity of the tumour, as well as on the nature and physical-chemical properties of the test material. These criteria are reviewed periodically by the chemotherapy staff, NCI and are flexible

Examination of Oldenlandia diffusa

Preliminary studies involving ethanol and chloroform crude extracts were assayed for inhibitory activity in vitro against cells derived from P_{388} lymphocytic leukemia (PS) carried in cell culture. According to the Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 20014 U.S.A., the results of screening data were summerized as followed

Table I

Activity of the Constituents of Oldenlandia diffusa*

Crude Extraction	Dose (mg/kg.)	% T/C
EtOH fraction	400	117
	200	111
	100	108
$CHCl_3$ fraction	400	110
	200	108
	100	102

The data clearly indicated that both ethanol and chloroform fractions were not the active constituent of the plant against P_{388} lymphocytic leukemia (PS). This is because of

* All tests were on P_{388} lymphocytic leukemia in CDF_1 strains of mice. Injections were intraperitoneal using alcohol as a vehicle.

a minimal increase in survival of treated animal (as compared to control animal) resulting in percent T/C less than 125.

The powder of Oldenlandia diffusa was extracted with light petroleum ether and ethanol. The ethanol crude extraction, after evaporating the solvent in vacuo, was equilibrated between chloroform and water. The light petroleum ether extract, after chromatography on a standardized alumina column, yielded two compounds which from now on will be designated as compound I and compound II.

The structure elucidation of compound I was done by using and comparing the spectroscopic data, melting point, and mixed melting point of the parent as well as its derivatives. The information suggested that compound I was

$$CH_3 - (CH_2)_n - CH_2 - OH$$

$$n = 28$$

Compound I

Compound I; which was eluted from the column with 10 % ether petroleum ether in the fraction No. 10 to No. 20, crystallized as amorphous solids from petroleum ether-benzene, m.p. 87-88°C, analyzed as $C_{30}H_{62}O$. It was soluble in chloroform, ether, benzene, methanol and insoluble in water. It gave a negative test with Liebermann-Burchard reagent and could not change the colour of bromine in carbon tetrachloride, suggesting the absence of unsaturation.

The Infra-red Spectrum of Compound I[9]

The IR spectrum of compound I shows a broad absorption band at 3350 cm^{-1}, which corresponds with polymeric association of OH stretching vibration. A medium absorption at 1060 cm^{-1} is C-OH stretching vibration of primary alcohol. The strong bands at 2915 and 2845 cm^{-1} are assigned to the asymmetrical and symmetrical C-H stretching vibration of methyl and methylene group repectively. Two absorption bands at 1475 and 1460 cm^{-1} are assigned to the scissoring vibration of the methylene C-H bonds and the out-of-plane bending of the methyl C-H bonds, respectively. The doublet medium bands at 735 and 720 cm^{-1} are indicative of the methylene rocking, in phase, vibration for straight chain. There are no absorption peaks of olefinic linkage, branched chain skeleton or carbonyl group. These information are suspected to be a saturated long chain primary alcohol.

Compound I Acetate

Acetylation of compound I with acetic anhydride in pyridine yielded the acetylated product which crystallized as white amorphous solids from light petroleum ether, m.p 74-75°C.

The Infra-red Spectrum of Compound I Acetate[9]

The IR spectrum shows a sharp absorption band at 1740 cm^{-1} which corresponds with the carbonyl stretching vibrations of the acetoxy group[10] and an absorption at 1230 and 1030 cm^{-1} are assigned to the C-O stretching vibration of saturated esters.

The rest of the spectrum is similar to that of compound I, except for the absence of peak due to the alcoholic hydroxy group in the region 3400-3200 cm^{-1}.

Compound I Benzoate

Benzoylation of compound I with benzoyl chloride in pyridine yielded the benzoate product which crystallized as white amorphous solids from light petroleum ether, m.p 67-68°C.

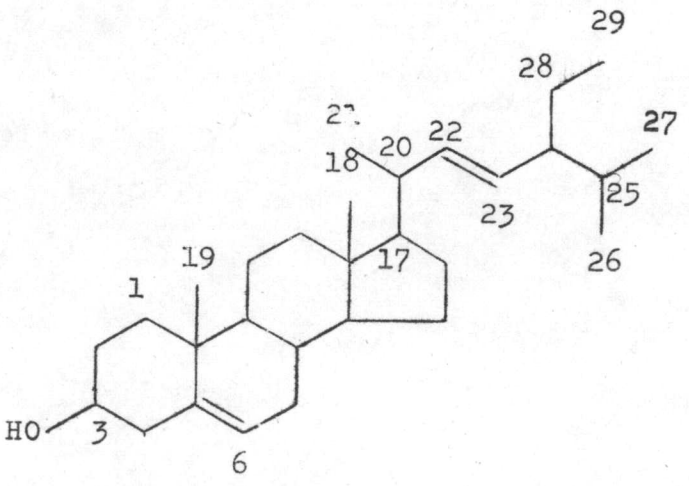
The Infra-red Spectrum of Compound I Benzoate[9]

The IR spectrum shows a sharp and strong absorption band at 1725 cm^{-1} which corresponds with the carbonyl stretching vibration. This is at lower frequency than that of a normal ester C=O stretching (ca. 1750-1735 cm^{-1}), due to the presence of carbonyl gr. in conjugation with phenyl group. Two broad and strong absorption bands at 1290 and 1130 cm^{-1} are assigned to the C-O stretching vibration of ester. The absorption bands at 1608, 1585 and 1465 cm^{-1} are characteristic of the out-of-plane bending vibration, involving carbon to carbon stretching within the ring, together with peaks at 1190, 1085, 1035 cm^{-1} and a weak peak in the region between 3100-3000 cm^{-1} are indicative of the in-plane bending vibration and C-H stretching vibration of benzene, respectively. The strong absorption bands at 2950, 2870 cm^{-1} and doublet bands at 700, 690 cm^{-1} are assigned to the asymmetrical and symmetrical C-H stretching vibration of methyl and methylene group rocking in phase for straight chain, respectively.

Conclusion

From the comparison of their IR spectra (including the derivatives), together with the negative test for unsaturated hydrocarbon by using bromine in carbon tetrachloride, compound I is suspected to be a saturated long chain primary alcohol. The carbon-hydrogen analysis of compound I shows C 82.34 %, H14.48 % and corresponds to the myricyl alcohol, $C_{30}H_{62}O$ (Cal. C82.11 %, H14.24 %). With the supporting evidence of mixed melting point with the authentic sample, it is concluded that compound I is myricyl alcohol.

The structure elucidation of compound II was done by using and comparing the spectroscopic data, melting point, and mixed melting point of the parent compound as well as its derivatives. The information suggested that compound II was



Compound II

Compound II, which was eluted from the column with 40 % ether- petroleum ether in the fraction No. 32 to No. 42, crystallized as white needle-shaped crystals from petroleum ether or 95 % ethanol, m.p. 168-169°C, $[\alpha]_D^{CHCl_3}$ -41.24 degree at room temperature (reported; specific rotation, $[\alpha]_D^{22}$ -52 degree in chlorofrom). It gave a deep green colour with Liebermann-Berchard reagent, which suggested the possibility of a steriod or triterpene[11, 12] and gave a positive reaction for unsaturated hydrocarbon by using bromine in carbon tetrachloride. It was precipitated by the addition of 0.5 % digitonin in 95 % ethanol. The formation of digitonide indicated the presence of a 3 β-hydroxyl group in the ring A.[13, 14]

Carbon-hydrogen analysis of compound II contained C 83.98 % and H 11.92%. The mass spectrum gave a molecular ion M^+ = 412, therefore its molecular formula should be $C_{29}H_{48}O$ (Cal, C 84.4 % and H 11.72 %).

The Infra-red Spectrum of Compound II[19-26]

The IR spectrum of compound II shows an absorption band at 3400-3200 cm^{-1}, a hydroxy stretching vibration. The absence of the band at 1680-1580 cm^{-1} eliminates the possibility of conjagated ethylenic double bonds stretching vibration; this suggests that compound II has non-conjugated ethylenic double bonds.[15] Moreover, compound II gives no ultra-violet absorption bands, this observation indicates that there is no conjugation in the compound II, which agrees well with the IR spectrum. At

frequency 3100-3000 cm^{-1} is the absorption band of a C-H bond stretching vibration in the system -C = C-H while in a saturated alkyl system the corresponding bands lie between 3000-2800 $cm.^{-1}$ (15, 16) The two weak bands near 830, and 790 cm^{-1} are assigned to the out-of-plane bending vibration of a hydrogen at C-6 attached to a trisubstituted olefin, it seems probable that at least one of these bands is brought forth by the presence of C 5-6 double bond and these bands are not seen in their reduction products or in the steriod of other olefin.(17-19) Furthermore, a frequency at 1060 cm^{-1} for Δ^5 sterol rather than 1033 cm^{-1} for Δ^7 suggests that the compound II is Δ^5 sterol.(13) The two significant absorption bands in the 970 and 960 cm^{-1} region are characteristic of trans configuration for the C 22-23 double bond. (13, 18) The C-H stretching vibration at 3000-2800 cm^{-1} and the out-of-plane C-H bending vibration at 1450-1350 cm^{-1}, both are the absorption of methylene and methyl skeleton in side chain and ring system.(10, 19)

The Nuclear Magnetic Resonance of Compound II

The NMR spectrum of compound II shows a complex multiplet at δ 5.14 to δ5.4 with a relative intensity of three protons, characteristic of olefinic hydrogens, whose signal at δ5.4 (1H, broad triplet) is thought to be due to the vinylic proton attached to a methylene group at C-6 ($>$ C = CH-CH_2); at δ5.14 (2H, broad multiplet like structure) there appears a resonance

signal which corresponds to olefinic protons attached to two methylidene group at C 22-23 (>CH - CH = CH - CH<).[13, 22-25] A broad multiplet at δ3.3 - 3.8 with an integration for 1-proton is typical for a hydrogen geminal to a hydroxyl group at C-3 (>CH-OH); a singlet δ1.57 with a relative intensity of 1-proton, disappears upon shaking with D_2O indicating the presence of hydroxylic proton at C-3.[13-22] The singlets at δ0.73 (3H) and δ1 (3H) are considered to be due to the angular methyl groups at C-18 and C-19, respectively. The doublet at δ1.2; with a relative intensity of three protons, is the characteristic of the methyl group adjacent to a single proton (CH_3 - CH< , probably at C-21[13, 22, 32] and the doublet at δ0.82; with an integration for 6-protons, is attributed to the isopropyl group at C 25-26-27 (CH_3 CH_3>CH-). The triplet at δ0.87 (J = 6Hz); with a relative intensity of 3-protons, indicates the methyl group adjacent to methylene proton (CH_3-CH_2-, probably at C-29).[13, 22, 28, 32] The signals arising from the remaining protons in the molecule are broad and ill-defined (δ 0.55-2.4), the broadness is due to extensive spin-spin coupling between protons of similar chemical shift, which manifests itself in a very large number of unresolved multiplets.[28]

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The Mass Spectrum of Compound II[13, 24]

The molecular ion (M^+) at m/e 412 shows that the molecular weight of compound II is 412. The other peaks are interpreted as follows: m/e 397 ($M^+ - CH_3$), m/e 394 ($M^+ - H_2O$), m/e 273 (M^+ - R,

R = side chain), m/e 271 $\left[M^+ - (R + 2H)\right]$, m/e 255 $\left[M^+ -(R+H_2O)\right]$, m/e 253 $M^+ -\left[(R + 2H + H_2O)\right]$, and m/e 213 $M^+ -\left[(R + H_2O + 42)\right]$. The peak at m/e 300 could conceivably have been formed by cleavage within the side-chain at the 20-22 carbon to carbon bonds together with one hydrogen atom derived from the remaining fragment.(13, 33, 34) The peaks at m/e 273 and 271 establish that side-chain of this sterol is composed of $C_{10}H_{19}$ (mol. wt. 139). The presence of intense peak at m/e 271 $\left[M^+ - (R + 2H)\right]$ and 253 $\left[M^+ - (R + 2H + H_2O)\right]$ suggests that this sterol involves the double bond in the side-chain.(33) The presence of double bond in the side-chain is also confirmed by the fact that the peak at m/e 271 is more intense that at m/e 273 ($M^+ - R$).(13)

Compound II Acetate

Acetylation of compound II with acetic anhydride in pyridine yielded the acetylated product which crystallized as white glossy crystal plates, m.p. 143-144°C and its specific rotation, $\left[\alpha\right]_D^{CHCl_3}$ -43.3 at room temp. (reported; specific rotation, $\left[\alpha\right]_D^{22}$ -55.5 degree in $CHCl_3$)

The Infra-red Spectrum of Compound II Acetate

The IR. spectrum shows the absorption band at 1742 cm^{-1}, a carbonyl stretching vibration for acetoxy group(10) and the strong single absorption band at 1270-1250 cm^{-1}, C-O stretching vibration, suggesting an acetate group of secondary alcohol in ring A is equatorial.(18) It agrees well with compound II by the

addition of 0.5 % digitonin in 95 % ethanol which indicates the presence of a 3β hydroxyl group in the ring A. The rest of the spectrum is similar to that of compound II, except for the alcoholic group disappears in the region near 3500-3200 cm^{-1} which indicates that compound II is completely acetylated.

The Nuclear Magnetic Resonance of Compound II Acetate[13, 22]

The NMR spectrum of compound II acetate shows a broad multiplet band at δ4.9-4.3, with a relative intensity of 1-proton, is a typical of a hydrogen geminal to an acetoxy group at C-3. The presence of singlet at δ2.01, with an integration for 3-protons, is assigned to a proton of acetoxy group at C-3. The signals arising from the remaining protons are the same as that of compound II, except for the disappearance of alcoholic group at δ1.57. This information confirms the IR spectrum that compound II acetate is completely, acetylated.

The Mass Spectrum of Compound II Acetate

In the case of compound II acetate, the mass spectrum gives the prominent peaks at m/e 394, 379, 255. The peak at $[M^+]$ m/e 454 corresponding to the molecular ion peak of compound II acetate is not observed. However, a relatively high peak is seen at m/e 394 corresponing to that for the loss of acetic acid from the molecular ion (M^+ - 60) of compound II acetate. The other peaks are interpreted as follows: m/e 379 $[M^+ - (AcOH + Me)]$, m/e 255 $[M^+ - (R + AcOH)$, R = side-chain$]$[13], m/e 253 $[M^+ - (R + AcOH + 2H)]$ and m/e 213 $[M^+ - (R + AcOH + 42)]$.

Hydrogenation of Compound II

Hydrogenation of compound II was done under normal and under pressure (26 psi), hydrogenated products under both condition are the same and crystallized as white crystal plate from petroleum-ether or ethanol, m.p. 135-136°C. It gave a negative test for unsaturated hydrocarbon by using bromine in carbon tetrachloride.

The Infra-red Spectrum of Hydrogenated Compound II[9]

The IR spectrum shows an absorption band at 3400-3200 $cm.^{-1}$ a hydroxyl stretching vibration, and absorption at 1060 $cm.^{-1}$, a C-O stretching O-H deformation vibration of secondary alcohol. The absorption bands at 2915 and 2880 cm^{-1} are assigned to the asymmetrical and symmetrical C-H stretching vibration, respectively.[9] Two absorption bands at 1460 and 1385 cm^{-1} are characteristic of asymmetrical and symmetrical methyl bending vibration and absorption band at 1475 cm^{-1} is assigned to the scissoring vibration of methylene group ($-CH_2-$).[9] There are no absorption bands in the region near 840-800 cm^{-1} and 970-960 cm^{-1} suggesting that the double bonds are completely hydrogenated, and agree well with the negative test for unsaturated hydrocarbon by using bromine in carbon tetrachloride.

The Nuclear Magnetic Resonance of Hydrogenation of Compound II

The NMR spectrum shows a broad multiplet band at $\delta 3.55$; with a relative intensity of 1-proton, is a typical of a hydrogen geminal to hydroxyl group at C-3. The presence of singlet at $\delta 1.63$ with an

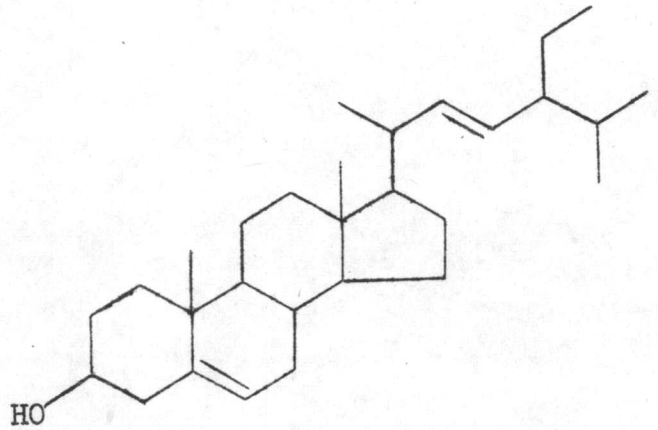
integration for 1-proton, is a hydroxylic proton at C-3. The doublet at δ0.84 (9H, J = 6 Hz) is the characteristic of a methyl group adjacent to one-proton (CH_3- CH< , probably C-21, C-26 and C-27) and the triplet band at δ0.83 (3H, J = 6Hz) is indicative of the methyl group attached to a methylene proton ($\underline{CH_3}$ - CH_2 -, probably at C-29). The disappearance of NMR spectrum at δ5.4 - 5.1 confirms that compound II is completely hydrogenated, this observation agrees well with the I R spectrum and with the negative test for unsaturated hydrocarbon by using bromine in carbon tetrachloride.

The Mass Spectrum of Hydrogenation of Compound II

The molecular ion (M^+) at m/e 416 shows that the molecular weight of the hydrogenated compound is 416. The other peaks are interpreted as follows: m/e 401 (M^+ - Me), m/e 383 $\left[M^+ - (H_2O + Me)\right]$, m/e 275 ($M^+$ - R, R = side-chain), m/e 257 $\left[M^+ - (R + H_2O)\right]$, m/e 255 $\left[M^+ - (R + H_2O + 2H)\right]$, and m/e 215 $\left[M^+ - (R + H_2O + 42)\right]$. From the mass spectrum of hydrogenated compound M^+ 416 is compared with compound II, the loss of 4 mass unit indicates that compound II contains two double bond in the molecule and is supported by chemical test, NMR and I R spectrum.

Conclusion

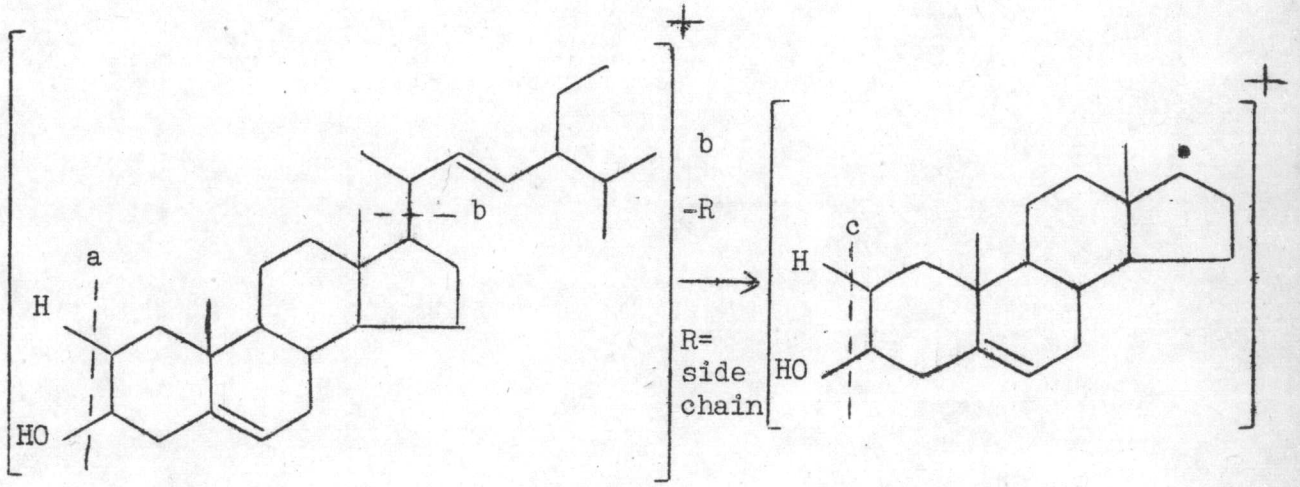
Thus the above spectral evidence of compound II, compound II acetate, and hydrogenated compound II supported each other, so that compound II was assigned as stigmasterol and its structure is as followed.



From the literature; the fragmentation patterns of stigmasterol have not been reported, so that the fragmentation of stigmasterol and its derivative are proposed as follows:

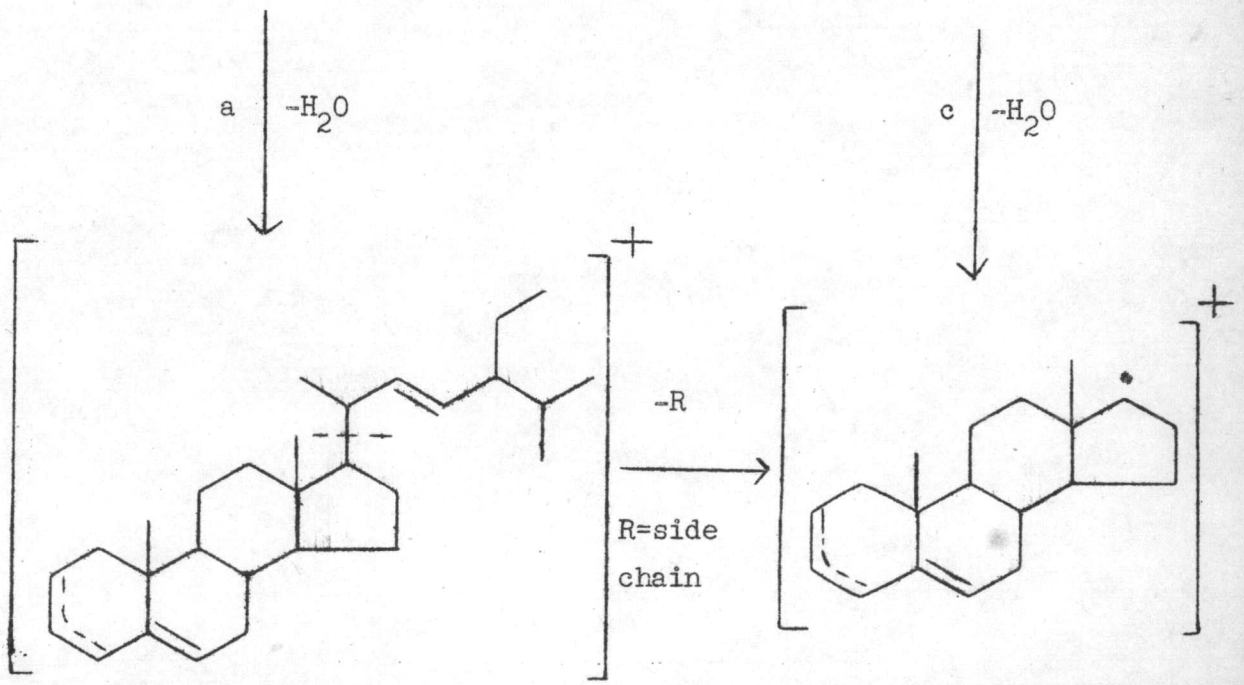
Scheme I

The mass spectrum pattern seems to fragment via the following mechanism:



M^+ 412

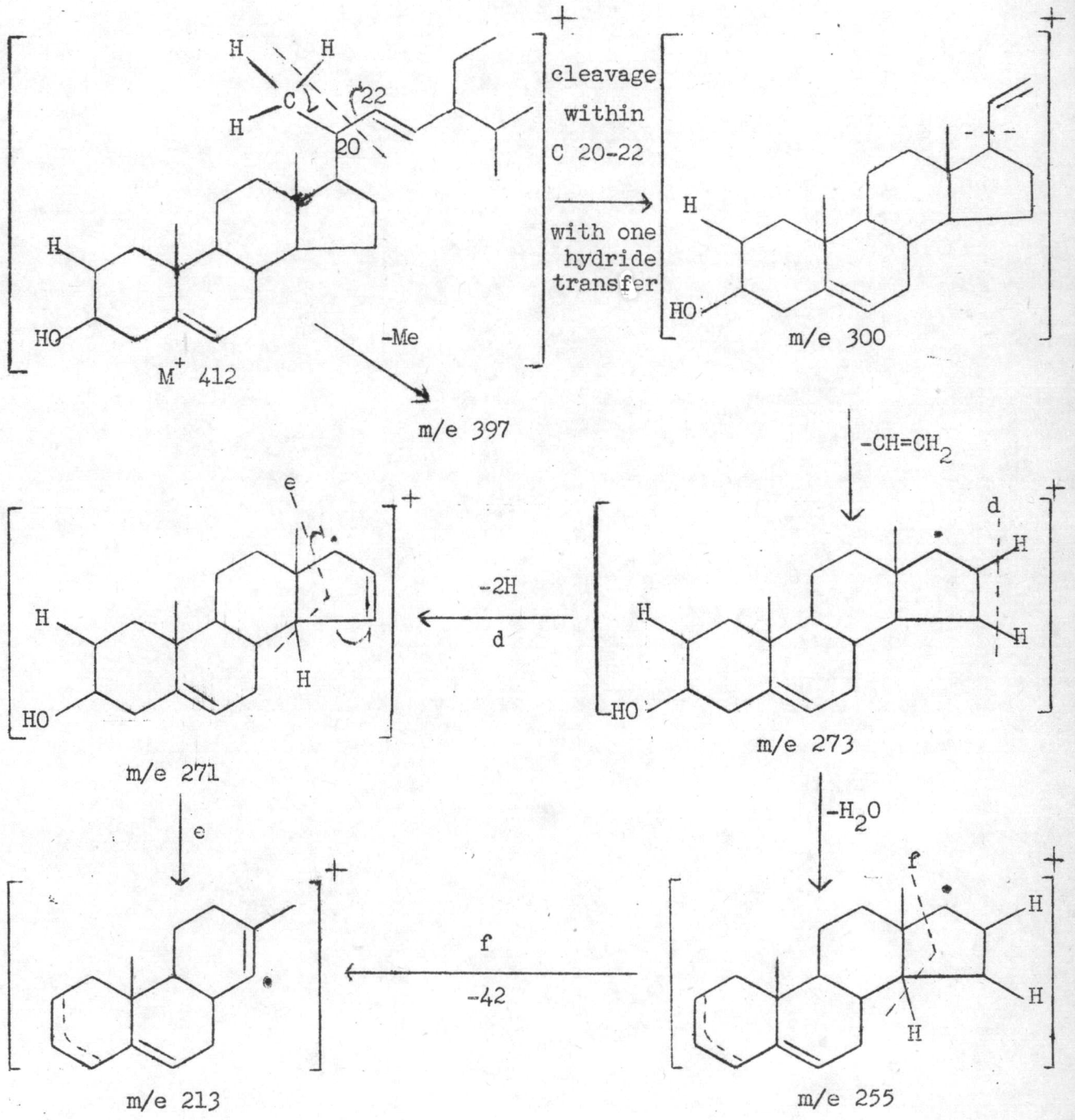
m/e 273



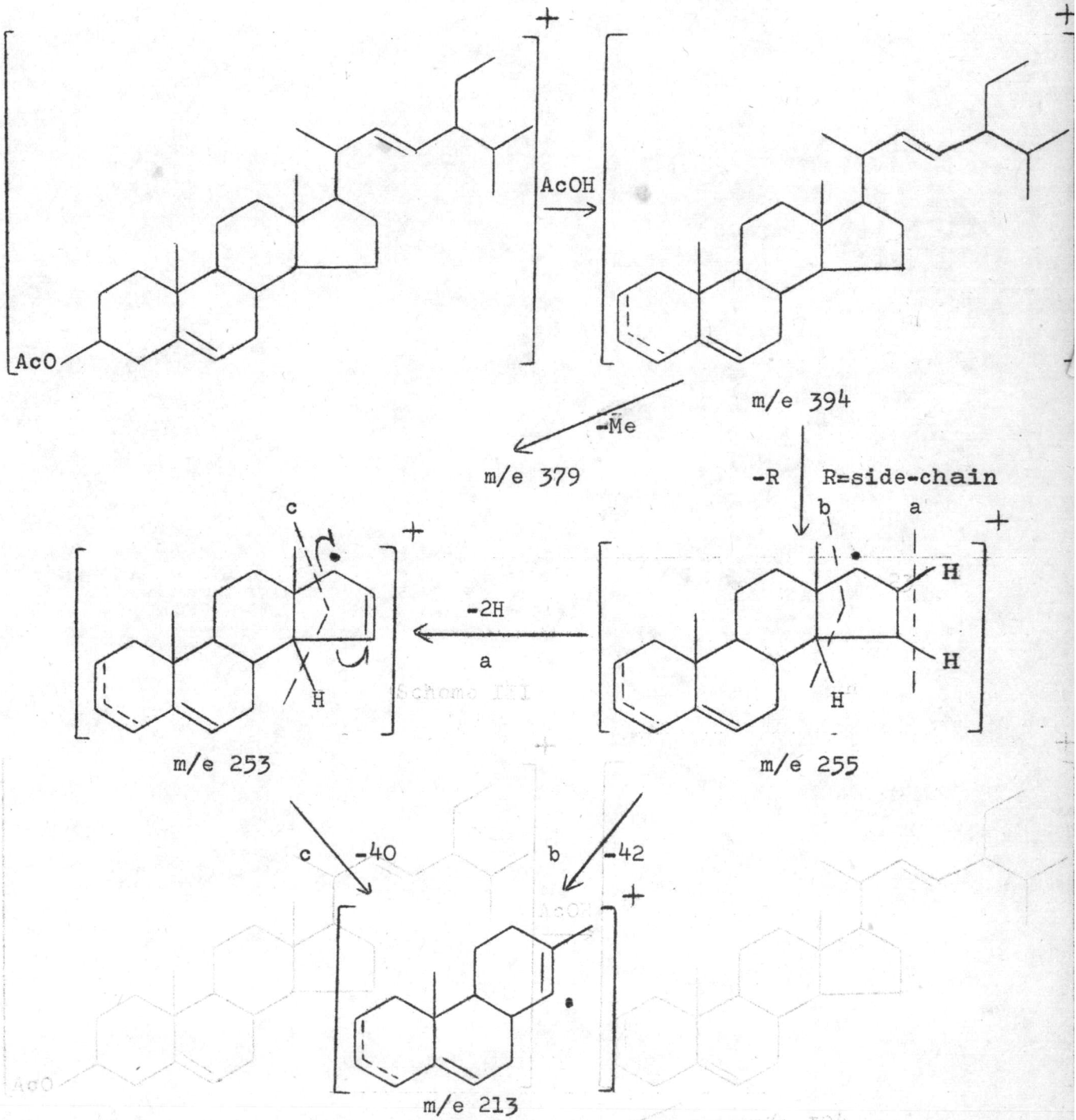
m/e 394

m/e 255

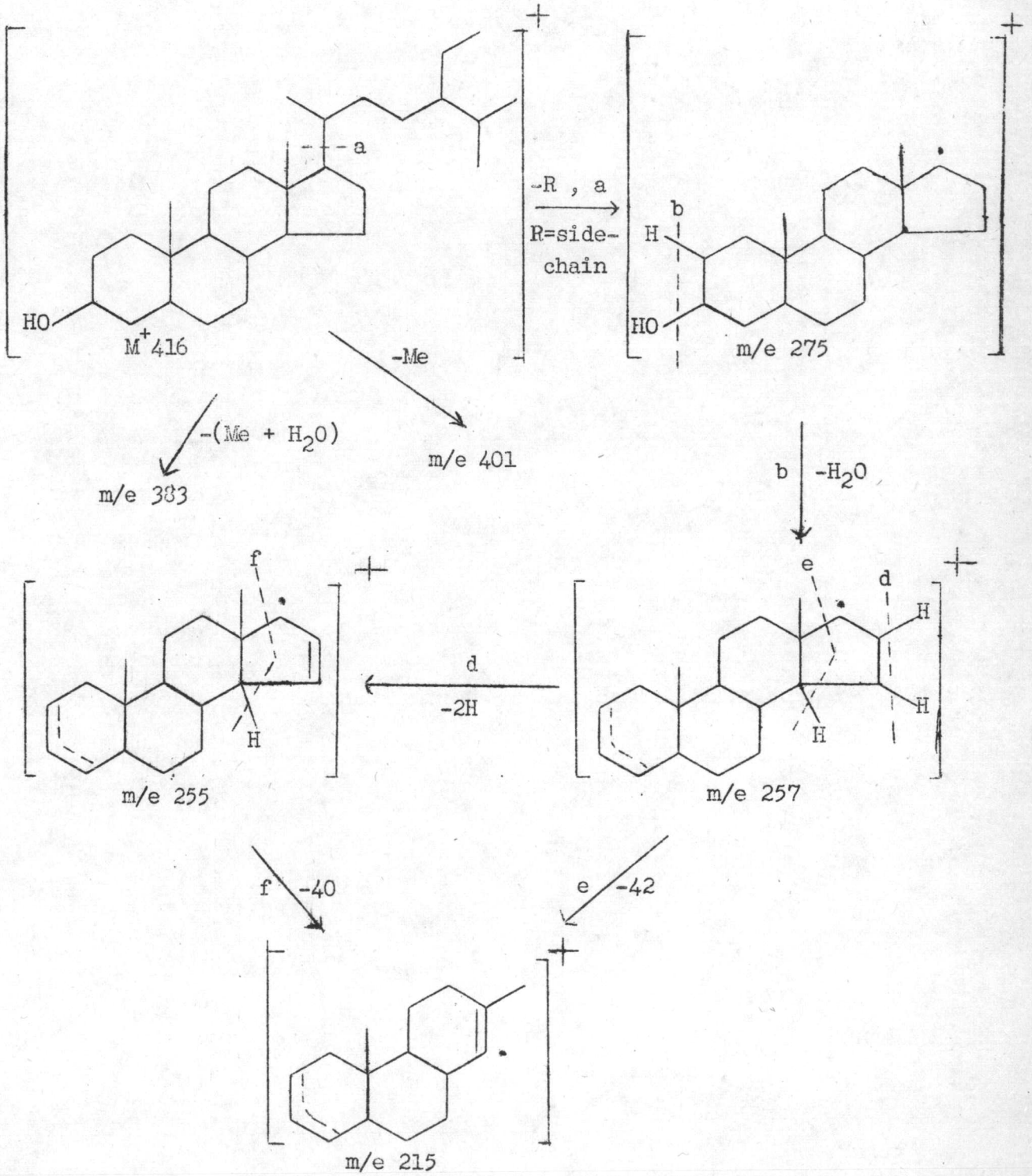
Scheme II


cleavage within C 20-22
with one hydride transfer
-Me
M+ 412
m/e 397
m/e 300
-CH=CH2
m/e 273
-2H
d
m/e 271
e
-H2O
m/e 255
f
-42
m/e 213

Scheme III



Scheme IV


a
HO
M+ 416
-R , a
R=side-chain
b
H
HO
m/e 275
-Me
m/e 401
-(Me + H2O)
m/e 383
b -H2O
f
H
m/e 255
d
-2H
e
d
H
H
H
m/e 257
f -40
e -42
m/e 215

The 95 % ethanolic extract of O. diffusa, after evaporating the solvent in vacuo, was equilibrated between chloroform and water. The chloroform soluble fraction was preliminary tested with the reagents as follows. It gave a red to deep violet colour with Shinoda's reagent, a yellow colour with aqueous sodium hydroxide solution, and a reddish orange colour with concentrated sulphuric acid. These information suggested a flavonoid compound.(35) And then the crude chloroform soluble fraction was chromatographed by using both alumina 90 (standardized and neutral alumina and silica gel (70-325 mesh ASTM). A little flavonoid was separated and due to paucity of sample, its structure could not be investigated. Attempts to separate further methods via the formation of its basic salts(36) gave only traces amount of flavonoids. In addition, the chloroform soluble fraction had no significant antitumour activity against P_{388} lymphocytic leukemia, so that the chloroform soluble fraction was not further studied.

The aqueous fraction, after evaporating the solvent in vacuo, was dissolved in a small volume of methanol. The insoluble precipitate mainly consisted of chloride salts of sodium 28.9 %, potassium 15 %, magnesium 0.00425 %, manganese 0.0009 % and traces amount of copper cobalt, zinc, calcium, and lead.