

STEROIDAL SAPONINS AND SAPOGENINS

FROM

AGAPANTHUS praecox Willd.

A thesis submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy in the Department
of Chemistry, University of Cape Town.

by

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S U M M A R Y

Agapanthin, $C_{51}H_{84}O_{24}$, a steroidal saponin, isolated from rhizomes of Agapanthus praecox Willd., yielded, on acid hydrolysis, the steroidal sapogenin agapanthagenin and galactose and rhamnose in the molecular ratio of 3:1. The structure assigned to agapanthagenin has been confirmed on the basis of further synthetic and spectroscopic evidence. The structure of a closely associated steroidal sapogenin, praecoxigenin, $C_{27}H_{40}O_4$, has been partially elucidated.

ABBREVIATIONS

p.p.m.	parts per million
i.r.	infrared
m.u.	mass units
n.m.r.	nuclear magnetic resonance
s.	singlet
d.	doublet
t.	triplet
q.	quartet
m.	multiplet
TLC	thin layer chromatography
Me	methyl CH_3 .
Ac	acetyl CH_3CO .
Ts	tosyl
Ms	mesyl
Tosylate	p-toluenesulphonate
Mesylate	methanesulphonate

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INTRODUCTION

Steroidal sapogenins occur in nature as plant glycosides which have the properties of forming a soapy lather in water and the ability of haemolysing blood. Studies by Marker (1) and more recently by Wall (2) have shown that they occur in monocotyledons found in a narrow segment of the plant kingdom. The more important genera of the plant families (Table 1) where steroidal sapogenins are known to occur are the Dioscorea, Agave and Yucca.

Table 1. Plant families containing steroidal sapogenins

<u>Liliaceae</u>	<u>Amaryllidaceae</u>	<u>Dioscoreaceae</u>
<u>Agapanthus</u>	<u>Agave</u>	<u>Dioscorea</u>
<u>Chlorogalum</u>	<u>Manfreda</u>	
<u>Nolina</u>		
<u>Smilax</u>		
<u>Yucca</u>		
<u>Trillium</u>		

These particular plant glycosides on enzymatic

or acid hydrolysis yield a sugar moiety and an aglycone which is structurally related to the steroidal hormones. The aglycones are thus called steroidal saponinins and can be converted into steroidal hormones by well tried degradative procedures.

Although the Liliales have proved to be the main source of these steroidal glycosides, their presence along with cardiac glycosides in commercial preparations of digitalis from one of the Scrophulariaceae, D. purpurea, was reported as early as 1875 by Schmiedeberg who named the principal saponin digitonin. Similar glycosides were subsequently found in the leaves D. purpurea and D. lanata.

In work conducted during the period 1890 to 1918 Kiliani (3) identified the sugars of the principal digitalis saponins and devised procedures for oxidation of the corresponding saponinins to a succession of acidic degradation products. It was only in 1935 as a result of the study of the products of selenium dehydrogenation that Simpson and Jacobs (4) established that the saponinins possess the steroid ring system. This coupled with the evidence for a C₈ side-chain strengthened the case for the formulation of

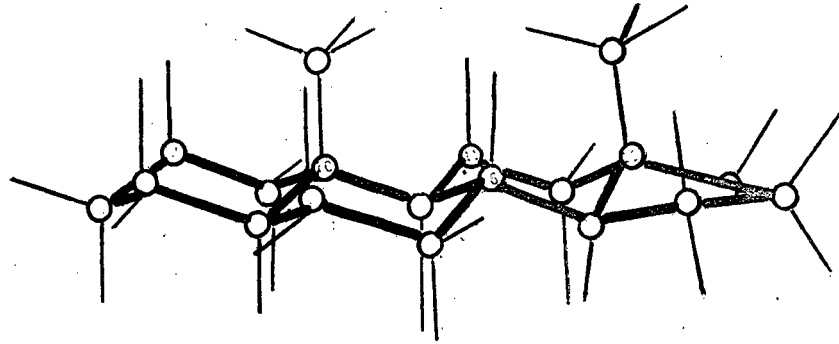
these sapogenins as C_{27} compounds.

The classic researches of Marker and co-workers (5, 6, 7), resulted in the spiroketal formulation for the terminal part of the sapogenin side chain which consists of a five and six-membered ring joined by a spiro carbon atom at C_{22} .

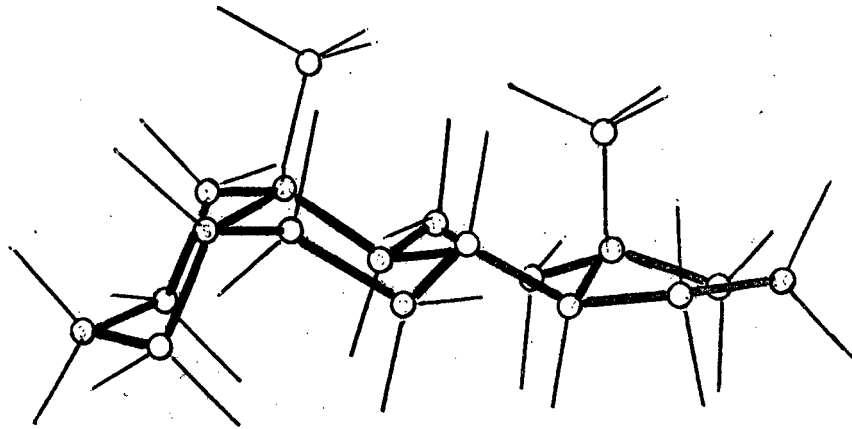
Conformation of steroid portion of the molecule

In the steroid portion of the molecule rings B and C are locked in the chair conformation by trans-fusion. The five membered D ring is also transfused to ring C. Rings A and B, however, can either be transfused or cis fused giving rise to the androstane ($5-\alpha$) or $5-\beta$ androstane series respectively (Fig. 1).

In the androstane series the 5α -hydrogen is axial to both ring A and B and the 10-methyl and 13-methyl groups are both axial and lie above the plane of the ring backbone. In the 5β -androstane series, however, the 5β -hydrogen is axial with respect to ring A and equatorial with respect to ring B. The 10-methyl group is equatorial to ring A and axial to ring B and the 13-methyl group is axial to both.



Androstane

 5β -AndrostaneFig. 1 Ring conformations of the androstane and 5- β androstane series

The stereochemistry of the spiroketal side chain

The natural steroid sapogenins occur in two isomeric series, the "normal" and "iso" which differ in steric orientation in the side chain. Marker and Rohrman (5) found that sarsasapogenin on being refluxed with alcoholic hydrochloric acid is converted into isosarsasapogenin which is identical with smilagenin.

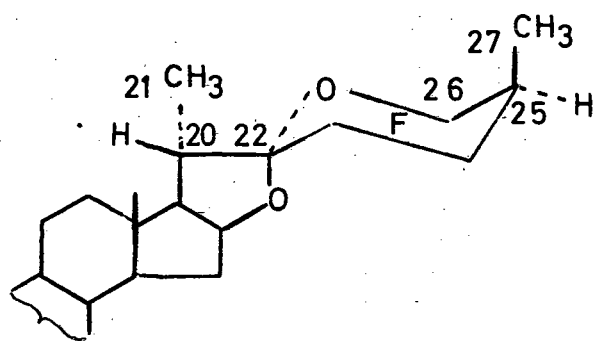
They suggested that this isomerisation consists of a change of configuration at C₍₂₂₎ involving the opening and reclosure of ring F. Scheer, Kostic and Mosettig (8) showed that the two series differ in configuration at C₍₂₅₎ and James (9) related the configuration at this asymmetric centre to glyceraldehyde so that the normal and iso series may be referred to as 25L and 25D respectively. Pettit (10) suggested the R and S system in order to provide a consistent system of nomenclature, naming the normal sapogenins 25S and the iso sapogenins 25R.

There remained the possibility that the two series differ in configuration at C₍₂₂₎ as well as at C₍₂₅₎. Scheer et al. (11) showed that sarsasapoge-

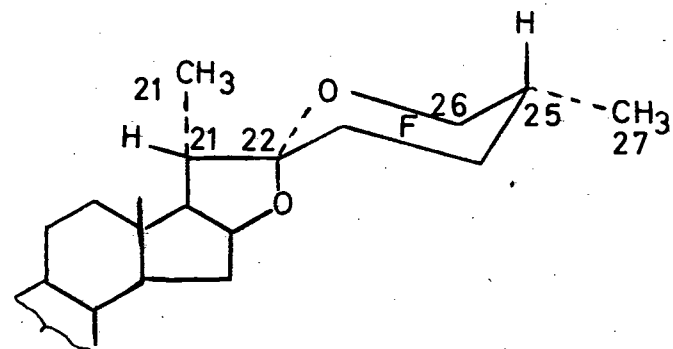
nin and smilagenin on treatment with acetic anhydride gave dihydro compounds which differ only at C₂₅.

However, the possibility that inversion at C₂₂ had occurred during the opening of ring F could not be excluded. Callow (12) prepared the unsaturated derivations of neotigogenin (25L) and tigogenin (25D), which had a double bond between C₂₅ and an adjacent carbon atom, by a method unlikely to cause isomerisation at C₂₂, and found they were identical. As the asymmetry at C₂₅ had been destroyed by the introduction of the double bond the identity of the unsaturated compounds obtained from neotigogenin (25L) and tigogenin (25D) shows that there is only a single difference between the two series namely the configuration at C₂₅.

It has been shown by Djerassi and Klyne (13) from optical rotatory dispersion measurements that the conformation of ring F with the methyl group axial is assigned to the normal sapogenins and the conformation, with the methyl group equatorial, to the iso sapogenins. Both these conformations (Fig. 2) have the oxygen atom in the α -position which is consistent with the known relative stabilities of the two series.



NORMAL
25L
25S



ISO
25D
25R

FIG.2 Conformations of ring F in steroidal sapogenins

The introduction of the prefix "neo" has caused much confusion in the naming of steroidal sapogenins. Fieser (14) has taken the prefix to indicate the 25L series of the natural steroidal sapogenins in which the methyl group at C-20 is behind ring E and known as 20α . Rosen (15) and other workers have retained the prefix "normal" for the natural steroidal sapogenins of the 20α series and have called the sapogenins in which the methyl group at C-20 is in front of ring E the neo or 20β -sapogenins. These latter sapogenins are more correctly known as the cyclopseudo-sapogenins.

When the corresponding spirostans are heated with acetic anhydride at 200° opening of ring F takes place with the formation of compounds known as furostans or pseudosapogenins. These can be converted back to the corresponding sapogenins by refluxing with alcoholic hydrochloric acid. Very mild treatment with acetic acid in ethanol, however, produces the cyclopseudosapogenins. These latter compounds under more vigorous acid catalysis are isomerised to the sapogenins formed, under the same conditions, from the pseudo compound. The use of the term cyclopseudosapogenins rather than "neo" has now been

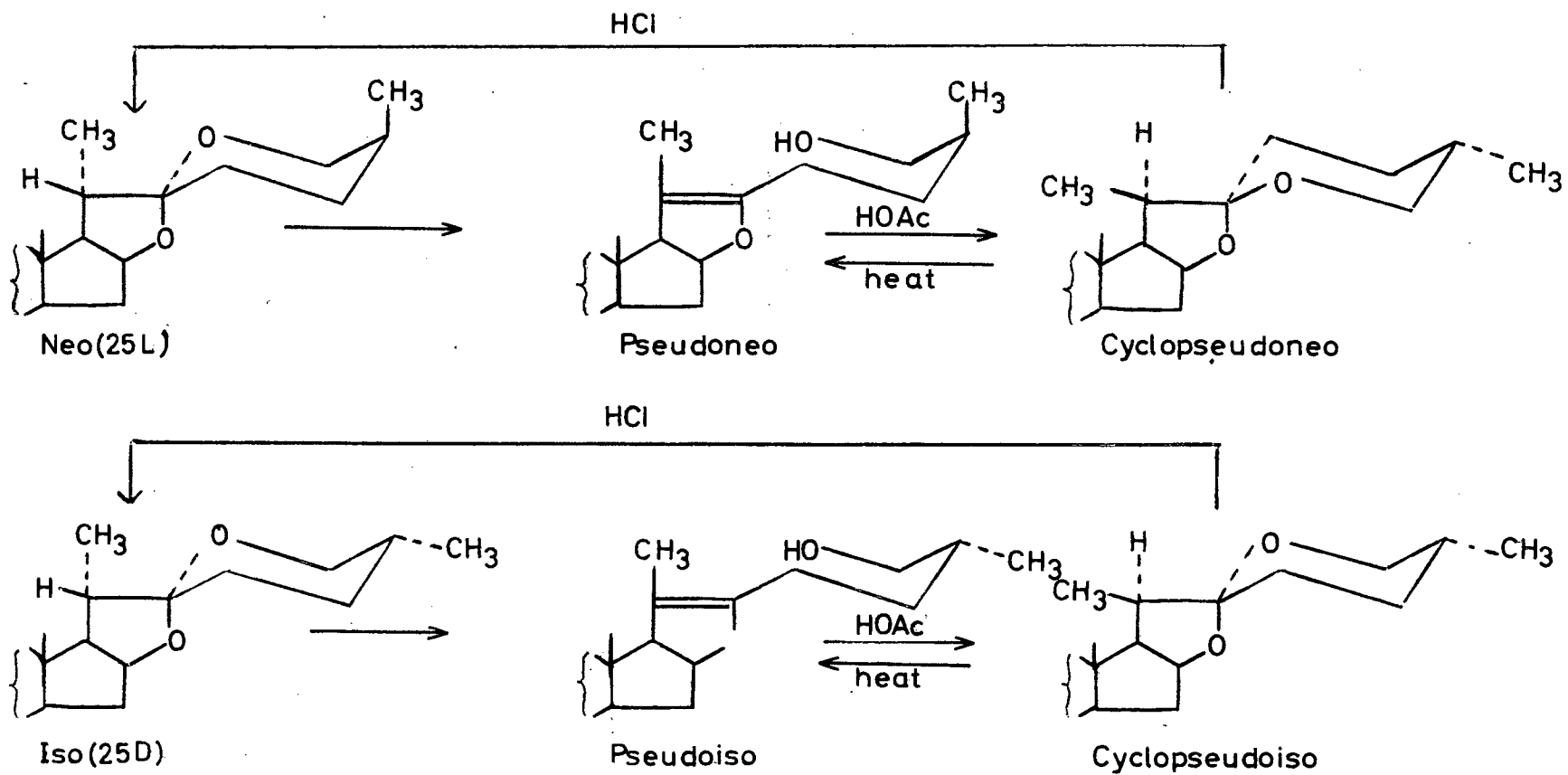
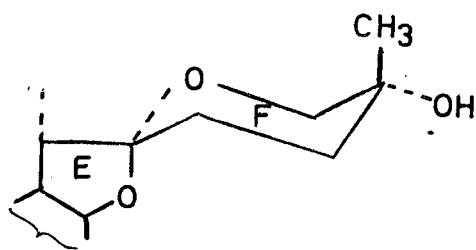


CHART 1 The isomerism of the cyclopseudosapogenins

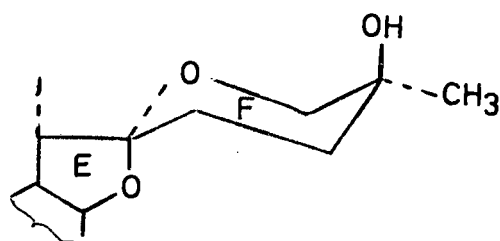
generally accepted and therefore for the sake of clarity the use of the term "neo" should fall away. The structural relationship between these compounds is shown in Chart 1.

Whereas most steroidal sapogenins conform to the normal or iso pattern certain steroidal sapogenins have been described which have unusual spiroketal side chains. These variations are as follows:

- I. Sapogenins in which the hydrogen attached to C_{25} has been replaced by a hydroxyl group. Examples of these are reineckiagenin, asperagenin, and iso-reineckiagenin of partial structure XIV, XV and XVI.

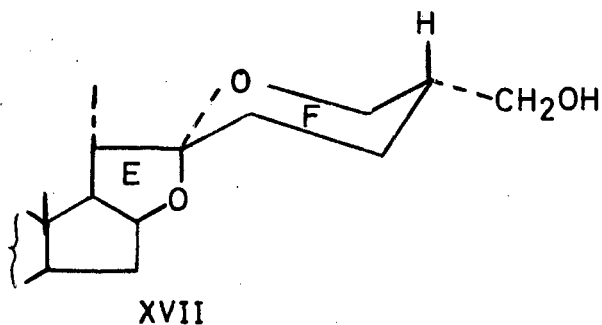


XIV and XV

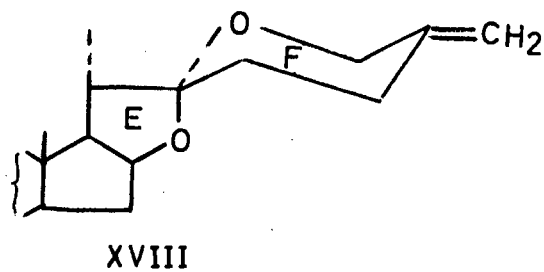


XVI

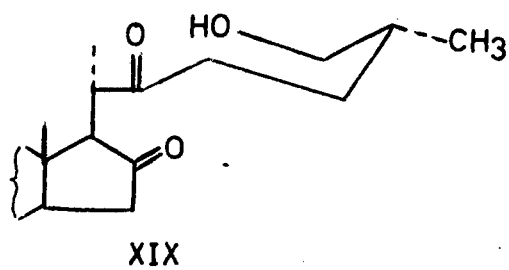
II. Sapogenins in which the methyl group attached to C_{25} has been replaced by a $-CH_2OH$ group (hydroxy methylene group) examples of these are isonarthogenin, igagenin, isocarneagenin, of partial structure XVII.



III. Sapogenins in which there is a double bond attached to C_{25} . Examples of these are neoruscogenin, convallamarogenin, $\Delta^{25(27)}$ -gitogenin, $\Delta^{25(27)}$ -manogenin, $\Delta^{25(27)}$ -dehydromanogenin of partial structure XVIII.



IV. Sapogenins with a non spiroketal side chain. An example is kryptogenin of partial structure XIX.



Structural types

The principal known sapogenins of established structure have been classified according to the number of hydroxyl groups in the molecule (Table 2). Sapogenins of unusual spiroketal structure are shown in Table 3.

Table 2. Steroidal sapogenins of known structure

Monohydroxy

Name	C ₂₅	C ₅	Substituents	m.p.	[α] _D
Sarsasapogenin	normal	β	3 β OH	200°	-75°
Smilagenin	iso	β	"	183°	-66°
Laxogenin	iso	α	3 β OH,6-CO	210-212°	-83.5°
Willagenin	normal	β	3 β OH,12-CO	168°	+5°
Neotigogenin	normal	α	3 β OH,	203°	-65°
Tigogenin	iso	α	"	204°	-67°
9-Dehydrohecogenin	iso	α	Δ^9 ,3 β -OH,12-CO	235°	-11°
Yamogenin	normal	Δ^5	3 β -OH	201°	-123°
Diosgenin	iso	"	"	208°	-121°
Correlogenin	normal	Δ^5	3 β -OH,12-CO	211°	-60°
Gentrogenin	iso	"	"	216°	-57°
Pamusgenin	iso	Δ^5	3 β -OH,11-CO	180-2°	-75°

Dihydroxy

Name	C ₂₅	C ₅	Substituents	m.p.	[α] D
Markogenin	normal	β	2 β , 3 β -(OH) ₂	257°	-70°
Samogenin	iso	"	"	202°	-74°
Rhodeasapogenin	normal	β	1 β , 3 β -(OH) ₂	295°	-72°
Isorhodeasapogenin	iso	"	"	243°	-71°
Yanogenin	iso	β	2 β , 3 α -(OH) ₂	243°	-53°
Gitogenin	iso	α	2 α , 3 β -(OH) ₂	272°	-73°
Neochlorogenin	normal	α	3 β , 6 α -(OH) ₂	270°	-
Chlorogenin	iso	"	"	276°	-45°
Rockogenin	iso	"	3 β , 12 β -(OH) ₂	220°	-64°
Chiapagenin	normal	Δ^5	3 β , 12 β (OH) ₂	257-259°	-130°
Iso chiapagenin	iso	Δ^5	3 β , 12 β (OH) ₂	236-237°	-121°
Yonogenin	iso	β	2 β , 3 α -(OH) ₂	-	-
Mexogenin	iso	β	2 β , 3 β -(OH) ₂ , 12-CO	238°	-6°
Manogenin	iso	α	2 α , 3 β -(OH) ₂ , 12-CO	246°	-5°
Kammogenin	iso	Δ^5	2 α , 3 β -(OH) ₂ , 12-CO	245°	-53°
Dehydromanogenin	iso	α	Δ^9 , 2 α , 3 β (OH) ₂ , 12-CO	240°	-16°
Neoruscogenin	normal	Δ^5	1 β , 3 β -(OH) ₂	-	-
Ruscogenin	iso	Δ^5	"	210°	-127°
Lilagenin	normal	Δ^5	2 α , 3 β -(OH) ₂	246°	-
Yuccagenin	iso	"	"	243°	-120°

Trihydroxy

Name	C ₂₅	C ₅	Substituents	m.p.	[α] _D
Tokorogenin	iso	β	1 β ,2 β ,3 α -(OH) ₃	268°	-50°
Metagenin	iso	β	2 β ,3 β ,11 α -(OH) ₃	274°	-82°
Neodigitogenin	normal	α	2 α ,3 β ,15 β -(OH) ₃	279°	-82°
Digitogenin	iso	α	"	296°	-61°
Agapanthagenin	iso	α	2 α ,3 β ,5 α -(OH) ₃	285°	-
Agavogenin	iso	α	2 α ,3 β ,12-(OH) ₃	242°	-62°
Diotigenin	normal	β	2 β ,3 α ,4 β -(OH) ₃	221-3°	-
Convallagenin A	normal	β	1 β ,3 β ,5 β -(OH) ₃	268-9°	-28°
Cacogenin	iso	α	2-,3 β ,6-(OH) ₃ , 12-CO	278°	-

Tetrahydroxy

Kogagenin	iso	β	1 β ,2 β ,3 α , 5 β -(OH) ₄	322°	-26°
Convallagenin B	normal	β	1 β ,3 β ,4 β , 5 β -(OH) ₄	-	-

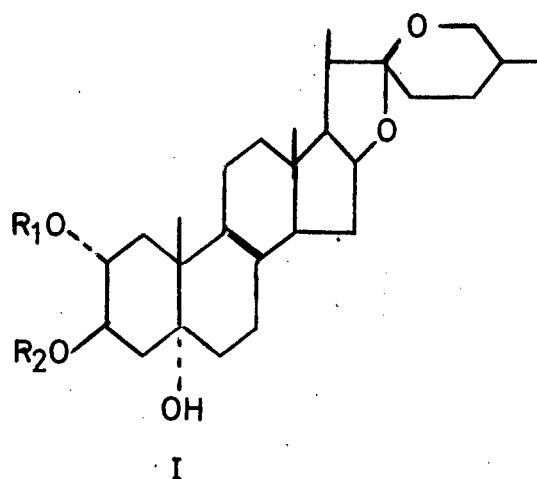
Table 3. Unusual spiroketal structures

Name	C ₂₅	C ₅	Substituents	m.p.	[α] _D
Kryptogenin			No ring F		
Reineckia- genin	normal	β	1 β , 3 β , 25-(OH) ₃	-	-
Isoreineck- iagenin	normal	β	1 β , 3 β , 25-(OH) ₃	-	-
Asperagenin	normal	β	3 β , 2 α , 25-(OH) ₃	264-8°	-135.9°
Isonartho- genin	iso	Δ^5	3 β , 27-(OH) ₂	236-8°	-110°
Igagenin	iso	β	2 β , 3 α , 27-(OH) ₃	253-256°	-
Isocarnea- genin	iso	β	1 β , 3 β , 27-(OH) ₃	-	-
Neorusco- genin	-	Δ^5	$\Delta^{25(27)}_{1\beta}$, 3 β -(OH) ₂	-	-
Convallama- rogenin	-		$\Delta^{25(27)}_{1\beta}$, 3 β -(OH) ₂	259-61°	-79.1°
$\Delta^{25(27)}$ gito- genin	-	α	$\Delta^{25(27)}_{2\alpha}$, 3 β -(OH) ₂	242-243°	80.1°
$\Delta^{25(27)}$ mano- genin	-	α	$\Delta^{25(27)}_{2\alpha}$, 3 β -(OH) ₂ , 12-CO	238-240°	-
$\Delta^{25(27)}$ dehy- dromano- genin	-	α	$\Delta^{25(27)}$, $\Delta^9_{2\alpha}$, 3 β -(OH) ₂ , 12-CO	230-32°	-36°

Discussion of the present investigation

Although most members of the Liliaceae have been extensively investigated as a source of steroidal sapogenins, little work has been done on Agapanthus. Watt and Breyer-Brandwyk (16) have reported the isolation of yuccagenin from A.africanus Hofmngs. and A.pendulus. Agapanthus africanus has now been reclassified by Leighton (17, 18) as A.praecox a species which is commonly found in and around Durban. Stephen (19) reported the isolation, from several unknown species growing in the Transvaal, traces of yuccagenin together with two other sapogenins. The main component was identified as a new sapogenin named agapanthagenin and the other was a complex.

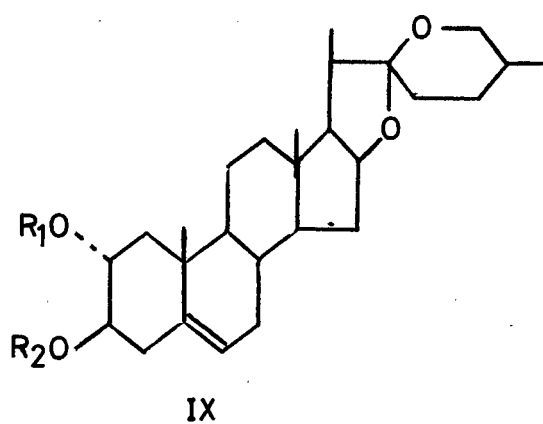
Agapanthagenin, $C_{27}H_{44}O_5$, was proved to be a new sapogenin and formulated as 22a-spirostan-2 α :3 β :5 α -triol (Ia). Evidence for the presence of the three hydroxyl groups was based on the fact that it formed a diacetate, the infrared spectrum of which showed hydroxyl absorption. The one hydroxyl group was thus considered to be tertiary a claim supported



(a) $R_1 = R_2 = H$

(b) $R_1 = R_2 = Ac$

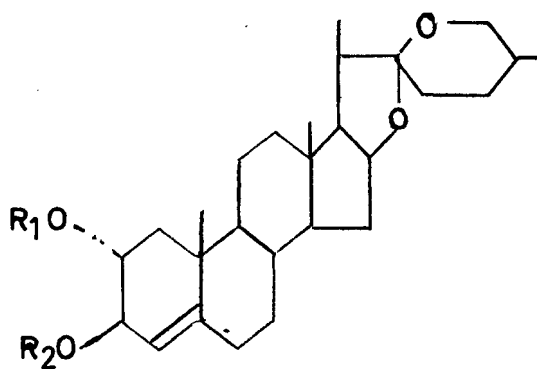
by the fact that dehydration of its diacetate with thionyl chloride in pyridine gave yuccagenin diacetate (IXb) a dihydroxy sapogenin of known structure.



(a) $R_1 = R_2 = H$

(b) $R_1 = R_2 = Ac$

The third saponin, $C_{54}H_{86}O_9$, was reported by Stephen, on the basis of later work, to be a complex consisting of an equimolecular mixture of agapanthagenin and yuccagenin. This complex which was derived directly from the plant could also be produced by refluxing agapanthagenin with alcoholic hydrochloric acid. Presumably the complex was formed as dehydration of agapanthagenin occurred. Acetylation of the complex followed by chromatography was reported to yield agapanthagenin diacetate (Ib) and an isomer of yuccagenin diacetate (VIIIb). The latter was presumed to have been formed by isomerisation of yuccagenin diacetate during chromatography.

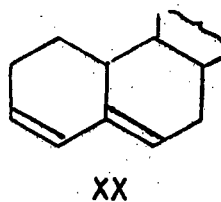
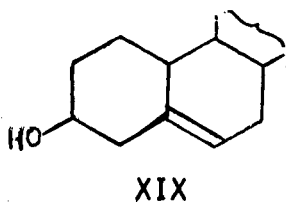


VIII

(a) $R_1 = R_2 = H$ (b) $R_1 = R_2 = Ac$

These findings raised a number of interesting queries as to the nature of the saponins in Agapanthus and the sapogenins obtained from them.

Due to the ease with which agapanthagenin undergoes dehydration it could be claimed that yuccagenin is merely an artefact, produced during the extraction process, and not a naturally occurring sapogenin. The formation of artefacts during the extraction of sapogenins from plant material is well known. Peal (20) reported the formation of 25D-spirosta-3:5-diene (XX) as an artefact produced during the isolation of diosgenin (XIX) from tubers of *Dioscorea* spp.



The fact that the species worked on by Stephen had not been identified left some doubt as to whether

it was A. praecox, the species from which Watt et al. reported the isolation of yuccagenin, or some entirely different species.

The present investigation was undertaken with a view to establishing the nature of the saponins in A. praecox and their hydrolysis products. During the course of the investigation agapanthagenin was isolated as one of the main components of this species together with a new sapogenin named praecoxigenin. Further evidence is presented to confirm the structure of agapanthagenin beyond doubt.

The suggested formulation of praecoxigenin (IIa) as a $2\alpha:3\beta$ -spirostadiene has been partially confirmed.

1. Steroidal saponins from Agapanthus praecox

Whereas the steroidal sapogenins have been extensively investigated, their precursors in the plant, the saponins, have received little attention. Stoll and Jucker (21) in a review of the steroidal sapogenins list fifteen saponins which have been isolated and characterised. More recently Kawasaki and Miyahara (60) have added five further saponins to the list of those which have definitely been isolated in a pure form (Table 4).

Although there is a wide variation in the nature of the aglycone portion of the molecule (Tables 2 and 3), the nature of the sugar moiety associated with these sapogenins is comparatively simple. Only five different sugars have been reported as found in the steroidal saponins (Chart 2) as compared with the cardiac glycosides where no fewer than sixteen different sugars have been identified.

Table 4. Steroidal saponins and their hydrolysis products

Saponin	Sapogenin	Sugar moiety
amolonin	tigogenin	1 galactose 3 glucose 2 rhamnose
chloronin	chlorogenin	6 sugar molecules
digitonin	digitogenin	4 galactose 1 xylose
dioscin	diosgenin	1 galactose 2 rhamnose
dioscorea-sapotoxin	diosgenin	glucose rhamnose
gitonin	gitogenin	4 galactose 1 xylose
F-gitonin	gitogenin	2 galactose 1 glucose 1 xylose
gracillin	diosgenin	2 galactose 1 rhamnose
kammonin	kammogenin	6 sugar molecules
kibubasaponin	diosgenin	3 galactose 1 rhamnose

nolonin	nologenin	not investigated
sarsasaponin	sarsasapogenin	2 glucose 1 rhamnose
smilonin	smilagenin	5 sugar molecules
tigonin	tigogenin	2 glucose 2 galactose 1 rhamnose
timosaponin A-I	sarsasapogenin	1 galactose
timosaponin A-III	sarsasapogenin	1 galactose 1 glucose
trillarin	diosgenin	2 glucose
trillin	diosgenin	1 glucose
yononin	yonogenin	1 arabinose
yucconin	yuccagenin	4 sugar molecules

The nature and site of the sugar linkage to the aglycone portion was the subject of earlier investigations. Marker and Lopez (23) reported

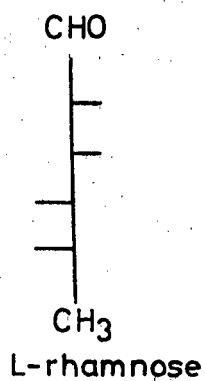
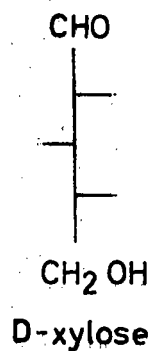
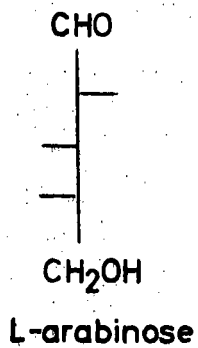
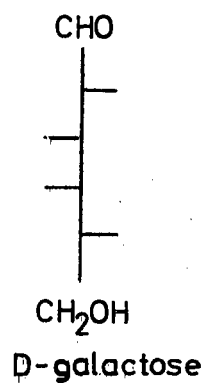
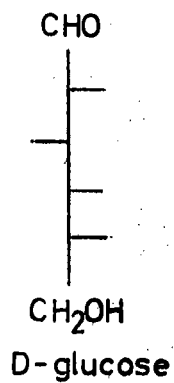
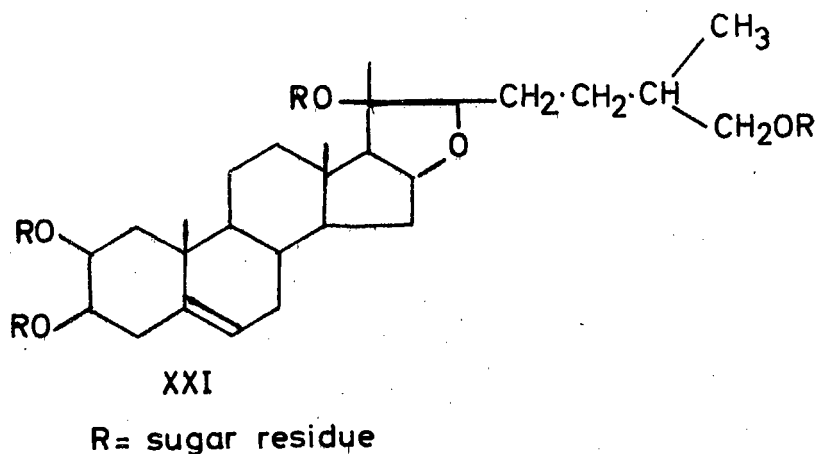


Chart 2 Sugar components of steroidal saponins

the isolation of yucconin from Yucca schottii which they claimed as having structure, XXI, a hydroxylated open chain



in place of the spiroketal ring, to which sugar molecules were attached. They claimed that acid hydrolysis of this glycoside with ring closure gave the usual usual spiroketal ring structure of the isosapogenins. Krider and Wall (24) disproved this theory by showing that the infrared spectrum of dioscin gave absorption bands in the $850-1000\text{ cm}^{-1}$ region typical of the spiroketal ring structure proving that the sugars must be linked to hydroxyl substituents in the steroidal nucleus.

A survey of the literature disclosed that all the

work carried out on the nature of the sugar moiety and the site of its linkage with the aglycone was done on saponins which had an aglycone with a single hydroxyl group at C-3. The nature and location of the sugar linkages in sapogenins with more than one hydroxyl function, as is the case with agapanthagenin, could form the subject of an separate investigation.

1.1 Evidence of the presence of saponins in A.praecox

Sliced sections of leaves, lower stems and rhizomes of freshly cut plant material exuded a sticky mucilage which formed a soapy lather in water, and when placed on a blood gelatine plate at 19° showed haemolysis indicative of the presence of saponins.

1.2 Extraction of saponins from the plant material

Saponins were extracted from the plant material by the method of Wall et al. (22) which was modified in certain respects. Freshly collected rhizomes were sliced, minced and dried at 60°. The finely

ground residue was exhaustively extracted with ethanol (95%) and the alcoholic extract evaporated to low bulk. Water was added and the watery extract was defatted by extraction with benzene in a liquid-liquid extractor. After the addition of salt and adjustment of the pH to between 3 - 4 the solution was extracted several times with butanol saturated with water. Tests indicated that the saponins showed preferential solubility in the butanol layer. In this way the saponins were separated from other plant products such as sugars, gums and proteins. Butanol was distilled off under reduced pressure and the residue taken up in the minimum amount of methanol and after filtration of the precipitated sodium chloride the methanol extract was added dropwise to acetone from which a dark brown residue separated out. This was dried in vacuo over calcium chloride to provide a crude saponin extract.

1.3 Chromatographic examination of the crude saponin extract

The extract examined by thin layer chromatography, using solvent I and spraying with sulphuric acid (20%),

after drying, revealed seven distinct spots. Similar chromatograms sprayed with a suspension of guinea pig blood cells showed faint white spots on a light brown background at Rf values 0.17, 0.37, 0.70.

It was therefore assumed that these three spots were indicative of three saponins in the crude saponin extract and that the other spots shown in chromatograms sprayed with sulphuric acid were due to impurities responsible for the dark brown colour of the crude extract.

1.4 Isolation of saponins from the crude extract

All attempts at crystallisation of the saponins from the crude extract were unsuccessful due to the presence of resinous impurities from which the saponins were unseparable.

The saponin, found in highest concentration in the extract, was ultimately isolated chromatographically by the procedure outlined in Chart 3. This separation showed limited success as the saponin mixture formed a jelly-like mass on the column which inhibi-

CRUDE SAPONIN EXTRACT

Acetylated and chromatographed
on neutral alumina

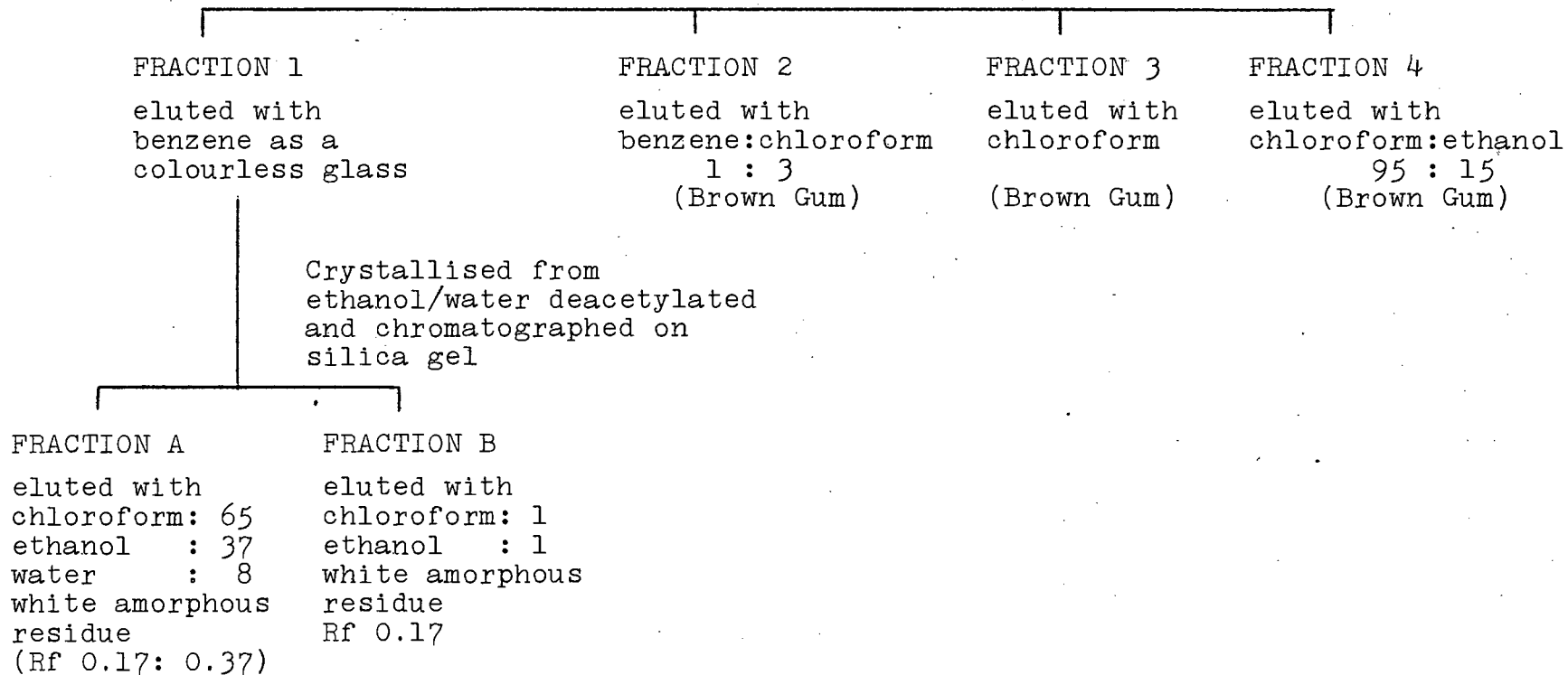


CHART 3. Separation of the crude saponin mixture from A.praecox

ted elution. Continuous elution under mild suction yielded small amounts of chromatographically pure saponin named agapanthin (Rf 0.17, Solvent I). All attempts at isolating the remaining two saponins were unsuccessful.

1.5 Chromatographic examination of saponins and derived sapogenins

Three purified saponin extracts from the plant, one containing a mixture of all three saponins, the other the two predominant saponins and the third a solution of agapanthin, were tested by thin layer chromatography. Each extract was then hydrolysed and the precipitated sapogenins compared chromatographically with samples of known sapogenins (Table 14).

These experiments showed that the three saponins originally present in the plant were yuconin which yielded yuccagenin on hydrolysis, agapanthin which on acid hydrolysis gave agapanthagenin and a third saponin named praecoxin which yielded praecoxigen. This evidence proved, beyond doubt,

that yuccagenin was not an artefact produced during the extraction of the sapogenins from the plant but was, indeed, derived from yuconin one of the saponins in the rhizomes.

2. The characterisation of agapanthin

2.1 The nature of the aglycone obtained on acid hydrolysis

During the hydrolysis of agapanthin with hydrochloric acid in 50% ethanol a white flocculent precipitate separated out which was crystallised from chloroform to give rhombic plates (m.p. 281-283°) showing no depression in melting point when mixed with pure agapanthagenin. The infrared spectrum of this hydrolysis product was identical with that of agapanthagenin.

2.2 Composition of the sugar moiety

The filtrate obtained from the hydrolysis was passed through a ion exchange column and evaporated to dryness. Paper chromatography of the residue compared with known sugars showed the presence of two sugars, galactose and rhamnose.

2.3 Qualitative estimation of the ratio of galactose to rhamnose in the hydrolysate

A visual comparison of the intensity of the spots on the above chromatogram showed that galactose was the predominant sugar present. In order to obtain an empirical idea of the ratio of the two sugars present, mixtures of galactose and rhamnose were made up in various molar ratios and after suitable dilution were compared chromatographically with the acid hydrolysate of agapanthin. From the intensity of the spots it appeared that galactose and rhamnose were present in the approximate molar ratio of 3 : 1.

2.4 Determination of the molecular formula of agapanthin and its peracetate

The formula of agapanthin was determined by the following method outlined in brief. A weighed sample of agapanthin was quantitatively hydrolysed. From the weight of agapanthagenin formed the molecular weight was calculated and, from elemental data, possible formulae were calculated which agreed as closely as possible with the molecular weight. The

molar ratio of the sugars was then determined in the acid hydrolysate and by reference to Table 5 the molecular formula was established. The formula of the peracetate was also established on the basis of elemental analysis and its % acetyl.

The success of this method depended upon the careful determination of the molecular weight and also on the molar ratio of the sugars obtained as a result of hydrolysis. The method was carefully standardised at every stage.

2.4.1 Hydrolysis of agapanthin

For the purposes of this determination it was important to establish the completeness of the acid hydrolysis step. Incomplete hydrolysis would result in the formation of prosapogenins. Kawasaki and Yamauchi (26) recorded that timosaponin on refluxing with 0.5N sulphuric acid for two hours yielded a prosapogenin and in order to obtain complete hydrolysis to sarsapogenin it was necessary to increase the hydrolysis time to one hour using 2N hydrochloric acid. Too high an acid concentration or too long a hydrolysis

Table 5. Possible molecular formulae for agapanthin and its peracetate

	(1)		(2)		(3)		(4)		(5)	
	Genin + 1 Galactose + 2 Rhamnose		Genin + 2 Galactose + 1 Rhamnose		Genin + 1 Galactose + 3 Rhamnose		Genin + 3 Galactose + 1 Rhamnose		Genin + 2 Galactose + 2 Rhamnose	
	glycoside + 3H ₂ O	nonacetate + 2H ₂ O	glycoside + 2.5H ₂ O	deca- acetate + 2H ₂ O	glycoside + 2H ₂ O	undeca- acetate + 2H ₂ O	glycoside	tredeca- acetate	glycoside	dodeca- acetate
	C ₄₅ H ₇₇ O ₁₈	C ₆₂ H ₉₂ O ₂₇	C ₄₅ H ₇₄ O ₁₉	C ₆₅ H ₉₄ O ₂₉	C ₅₁ H ₈₄ O ₂₂	C ₇₃ H ₁₀₆ O ₃₃	C ₅₁ H ₈₄ O ₂₄	C ₇₇ H ₁₁₀ O ₃₇	C ₅₀ H ₈₄ O ₁₃	C ₇₄ H ₁₀₈ O ₃₆
	·3H ₂ O	·2H ₂ O	2·5H ₂ O	·2H ₂ O	·2H ₂ O	·2H ₂ O				
C%	56.48	57.07	56.08	56.76	56.45	56.66	56.67	56.83	56.39	56.39
H%	8.36	7.36	8.2	7.13	8.1	7.1	7.78	6.77	7.89	6.89
Ac%		29.7		31.3		30.6		34.38		32.9
Mol. Wt.	956		963		1084		1080		1064	

time, on the other hand, could result in decomposition of the sugars and also cause structural changes in the aglycones themselves. The conditions for the quantitative hydrolysis of saponins were carefully investigated and reported on by Rothman et al. (27). On the basis of a large number of experiments he arrived at the following conclusions:

- (i) Hydrolysis of saponins with 2N acid resulted in low sapogenin yields.
- (ii) Destruction of sapogenins with excess heating time occurred only when 6N hydrochloric acid was used.
- (iii) Hydrolysis with 4N hydrochloric acid for two hours gave quantitative yields of sapogenins.

On the basis of these findings and those of Wall et al. (28) agapanthin was hydrolysed under reflux with 4N hydrochloric acid in ethanol (50%) at 80° for two hours. After hydrolysis the solution was diluted with water and the precipitated aglycone quantitatively collected on a tared sintered glass crucible which was then dried to constant weight at 105°.

The filtrate containing the sugar moiety was placed in a deep freeze refrigerator overnight and then freeze dried for 12 hours. By this process all the water and hydrochloric acid was removed. This modification appeared to offer less danger of loss of sugars than the recommended procedures used for the removal of hydrochloric acid such as passage through ion exchange columns or treatment with silver carbonate followed by precipitation of the excess silver ion with hydrogen sulphide.

The freeze dried sugars were made up to a known volume with water. The sugars in aliquot portions were separated by paper chromatography, eluted out of the paper by a method similar to that recommended by Borel et al.(29), and the sugar concentration of the eluate determined colorimetrically by a method recommended by Leopold (54).

2.4.2 Acetylation of agapanthin and the determination of the percentage acetyl of the product

Agapanthin was repeatedly acetylated with equal volumes of acetic anhydride and pyridine at room tem-

perature until the acetylated product showed only one spot on examination by thin layer chromatography. The percentage acetyl of the crystalline acetyl, dried in vacuo over P_2O_5 , and determined by the Kuhn-Roth method, was, on average 33.4%.

The analytical data obtained with agapanthin and its peracetate agreed closely with calculated molecular formulae shown in Table 5. Due to their non volatile nature it was not possible to obtain an accurate mass of either agapanthin or its peracetate. The molecular weight of the agapanthin calculated from the weight of agapanthagenin obtained on acid hydrolysis suggested molecular formulae (3) or (4). The quantitative sugar determination showed that galactose and rhamnose were present in the molar ratio of 3 : 1 which established the formula of agapanthin as (4). It thus has a molecular formula of $C_{51}H_{84}O_{24}$ and consists of 1 mole of agapanthagenin linked with 3 moles of galactose and 1 mole of rhamnose. Its peracetate has thirteen acetyl groups and is formulated as $C_{77}H_{110}O_{37}$.

The infrared spectrum of agapanthin (Figure 3) showed absorption bands at 866, 900, 920, and 982 cm^{-1} .

which are typical of a spiroketal ring structure of "iso" configuration and indicated that the sugars could only be linked to the hydroxyl groups of the steroidal nucleus. The infrared spectrum of its peracetate (Figure 4) gave an absorption peak at 3500 cm^{-1} which is indicative of a hydroxyl group. This is possibly the hydroxyl group at C-5 which being sterically hindered resisted acetylation. Insufficient material was available to investigate the nature of the sugar linkages by permethylation and hydrolysis.

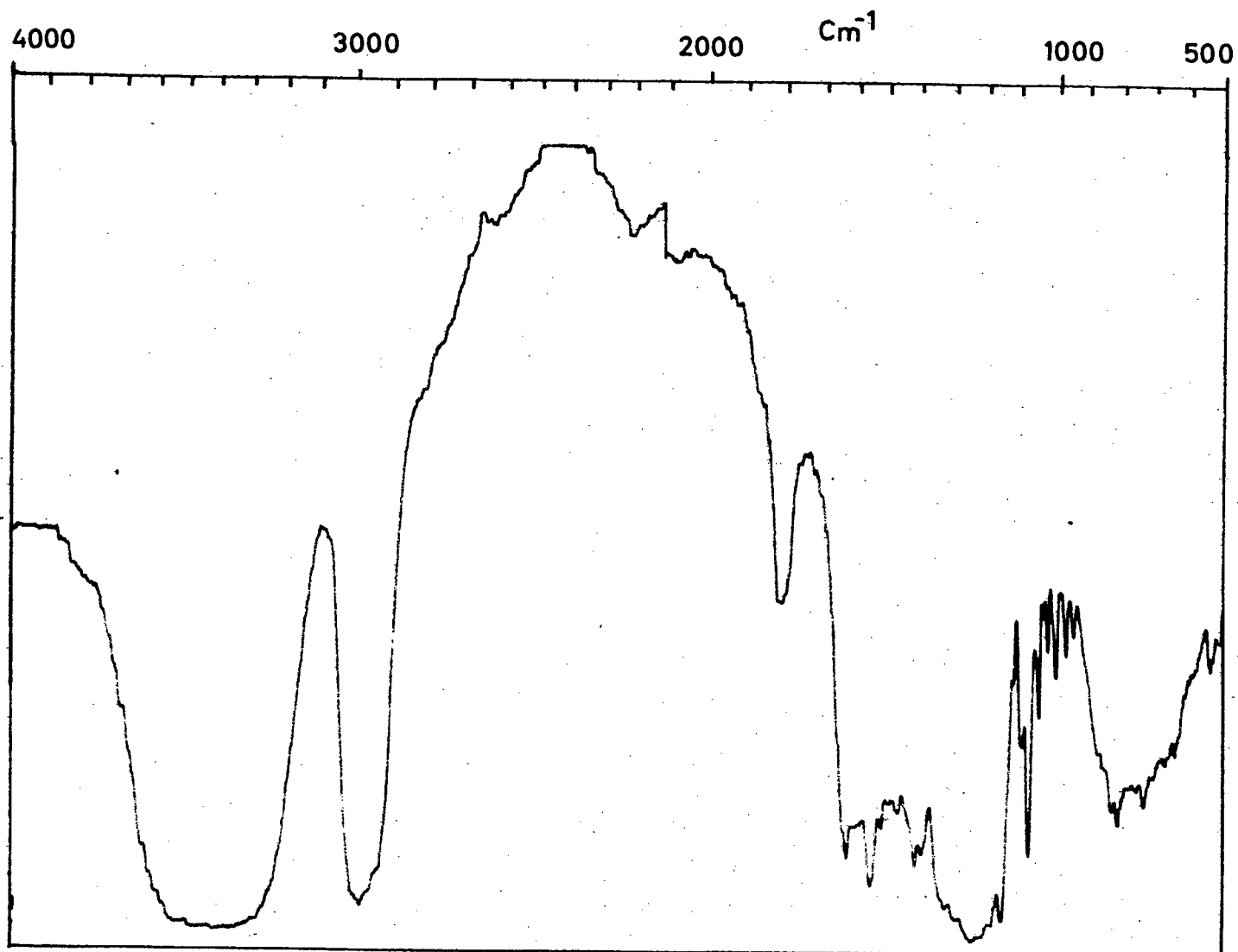


Fig.3 IR spectrum of agapanthin

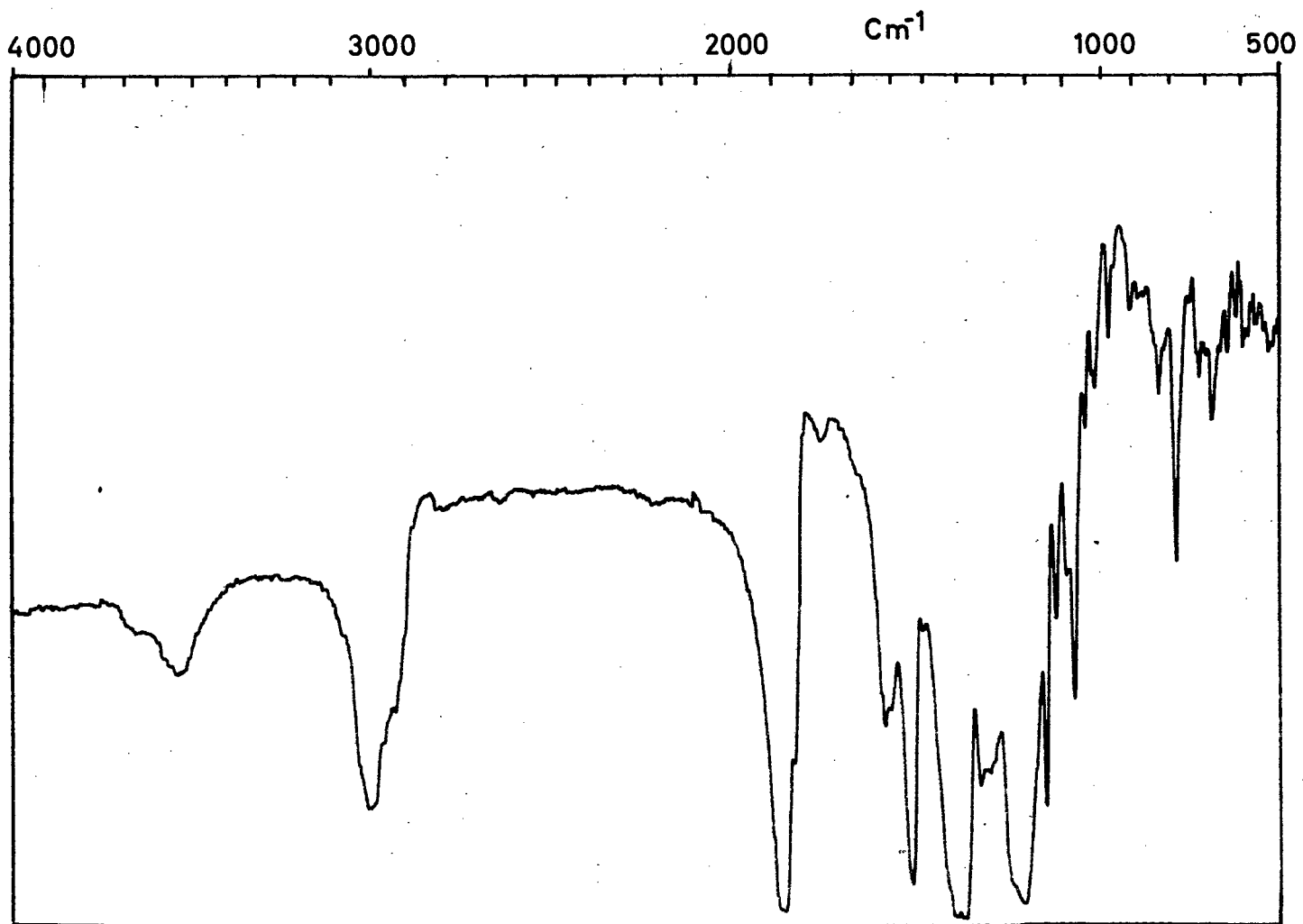


Fig. 4 I.R. spectrum of agapanthin peracetate

3. Steroidal saponinins from Agapanthus praecox

3.1 Procedures used for the extraction, evaluation and isolation of the saponinins

Two general methods have been recommended for the extraction of saponinins from plant material. By one method, developed by Wall et al. (28), the saponinins were first isolated in a crude form by alcoholic extraction of the plant material. To a solution of this crude saponin extract in ethanol (25%) sufficient concentrated hydrochloric acid was added to make it 4N. The solution was refluxed for 3 to 4 hours, cooled and filtered and the crude tarry saponin precipitate was washed with aqueous ethanol (50%) and refluxed with a mixture of benzene and methanol containing potassium hydroxide. After washing with water the solvent was removed to provide an extract consisting of a mixture of crude saponinins containing resinous material (approximately 50%). This method proved unsatisfactory when applied to the hydrolysis of the crude saponin extract of agapanthus rhizomes. The crude saponinins obtained as a result of the hydrolysis were insoluble in the benzene layer and thus caused emulsions which were

difficult to break. In a second method, fresh minced rhizomes were hydrolysed with hydrochloric acid and after drying the hydrolysed residue was extracted with carbon tetrachloride, using a modified type of soxhlet process. The sapogenins were only slightly soluble in cold carbon tetrachloride so that on filtering the cold carbon tetrachloride extract they were obtained in a reasonable state of purity and separated from fats, waxes and the other plant steroids. A better yield of sapogenins was obtained with this process and thus it was adopted as the standard procedure for extracting the sapogenins from the rhizomes.

Thin layer chromatography of the residue from the carbon tetrachloride extract of the hydrolysed rhizomes using Solvent III showed the presence of three sapogenins. The sapogenin showing as a faint spot at Rf 0.83 because of its low concentration could not be isolated in a state of purity. The two sapogenins at Rf 0.57 and 0.48 were isolated as a mixture which proved to be extremely difficult to separate. They were tenaciously held on alumina columns and were only separated from each other by repeated chromatography on silica gel. The sapoge-

nin at Rf 0.48 was identified as agapanthagenin and only when these investigations were near completion was it found that it could be obtained pure by repeated fractional crystallisation from chloroform. The second sapogenin (Rf 0.57) was isolated in small quantities in a state of purity. An investigation of the literature showed it to be a new sapogenin and has been named praecoxigenin. Some investigations into its structure have been carried out and are reported on later.

3.2 Seasonal variations in the concentration of sapogenins from rhizomes

As insignificant yields of sapogenins were obtained from rhizomes extracted during the winter months, it was considered appropriate to investigate possible assay procedures for the determination of sapogenins in extracts of plant material. Two general methods have been proposed for the assay of sapogenins in plants. One described by Blunden et al.(30) consists of isolating the sapogenin by thin layer chromatography and quantitatively evaluating the absorbance of the spot by using a photoelectric den-

sitometer coupled to an integrating logarithmic recorder. A second method developed by Wall et al. (28) is based on the comparison of the absorbance of the acetylated crude extract, in carbon disulphide, with that of a standard sapogenin acetate solution, at one of the characteristic sapogenin absorption peaks of the infrared spectrum.

A modification of the procedure recommended by Wall was finally adopted.

The dried, minced rhizomes (10 g) were hydrolysed with hydrochloric acid, the residue after filtration was dried and extracted with carbon tetrachloride. After removal of the solvent the residue was acetylated and the absorbance measured at 980 cm^{-1} in carbon disulphide at a concentration of 0.7 to 2% in a cell of path length 1.0 mm. The absorbance of a crystalline sample of mixed sapogenin acetates obtained from agapanthus rhizomes was taken as a standard.

A detailed study showed that there was a distinct seasonal variation in the sapogenin content of rhizomes (Table 6). During the winter dormant period the sapogenin content fell off to an insignificant value

and at the flowering period and for two months thereafter it was at a maximum.

Although this method gave a comparative estimation of the percentage of sapogenins in plant material many factors could affect its accuracy and it was only considered to be semi quantitative, especially, as the extracts examined contained considerable amounts of non steroidal matter. For the purpose of a comparative examination, however, it proved to be satisfactory.

Table 6. Seasonal variations in sapogenins from agapanthus rhizomes

Period of year	Sapogenin content % w/w
Summer season	0.37
Late Autumn	0.24
Winter period	0.07
Early Spring	0.12

3.3 Chromatographic examination of sapogenins from

A. praecox

3.3.1 Preliminary investigations

Paper chromatographic separations of the sapogenins in crude extracts were tried according to methods described by Heftmann and Hayden (31), McAleer and Kozlowski (32) and Wall et al. (33). It was found that either the spots remained fixed to the starting line or when more polar solvents were used, lack of resolution due to tailing took place. As a tool for the separation and identification of the more polar dihydroxy saturated and unsaturated sapogenins it proved most unsatisfactory.

Thin layer chromatography has been employed by many workers for the separation of steroidal sapogenins. Sander (34) used this technique most successfully for the separation of the dihydroxy sapogenin gitogenin from several monohydroxy sapogenins. Smith and Foell (35) also reported success with the separation of the C₂₇ sapogenins with starch bound silica gel using phosphomolybdic acid as a detecting agent. Bennett and Heftmann (36) reported on the use of

zaffaroni-type partition systems with silica gel containing only gypsum as a binding agent to permit the use of sulphuric acid as a detecting agent. Later workers have reported that the partition solvent system, as suggested by Bennett, gave erratic results.

It was found that thin layer chromatography with kieselgel G as an adsorbent with solvent systems which depended on adsorption rather than partition principles effected satisfactory separations of both sapogenins and their acetates.

3.4 Comparative thin layer chromatography of sapogenins, sapogenin extracts and their acetylation products

In order to establish whether the species from the Transvaal reported on by Stephen (19) was in fact A.praecox, thin layer chromatography of the extract from this species together with the complex isolated from it were compared with extracts of A.praecox. Pure sapogenins were used as standards and solvent system III used for development which was carried out in a controlled temperature room at 20°. Similar

chromatograms were carried out on their acetylated derivatives using solvent system V. The results are given in Table 7.

Discussion of results

The chromatogram showed quite clearly that the species worked on by Stephen was not A. praecox. The Transvaal species appeared to contain two sapogenin, agapanthagenin and yuccagenin, and the complex appeared to be pure yuccagenin as it gave one spot at the same Rf value. The acetylated extract of the Transvaal species showed the presence of three sapogenin acetates, yuccagenin diacetate, agapanthagenin diacetate and an unidentified acetate (Rf 0.18). The complex was also resolved into two acetates one being yuccagenin diacetate and the other the acetate (Rf 0.18). It, therefore, appeared that the complex was a mixture of yuccagenin and an unidentified sapogenin of similar Rf value. These results for the composition of the complex were at variance with those reported by Stephen who recorded the complex as a mixture of agapanthagenin and yuccagenin. At the time when work on this species was carried out by Stephen, thin layer

Table 7. TLC of sapogenins and acetylated derivatives

Sample	Sapogenin		Acetylated derivative	
	Rf value	Colour after development	Rf value	Colour after development
Extract from Transvaal Agapanthus	0.83 0.48	purple purple	0.67 0.27 0.18	purple purple purple
Extract from Agapanthus praecox	0.83 (faint) 0.57 0.48	purple olive green purple	0.67 (faint) 0.27 0.22	purple purple olive green
Complex from Transvaal Agapanthus	0.83	purple	0.67 0.18	purple purple
Agapanthagenin	0.48	purple	0.27	purple
Yuccagenin	0.83	purple	0.67	purple
Δ^4 isomer of yuccagenin	0.93	pink	0.90	tan
Praecoxigenin	0.57	olive green	0.22	olive green

chromatography had not been developed as an analytical tool.

These results showed the presence of three saponin-
genins in A. praecox, yuccagenin which was present in
low concentration, agapanthagenin and praecoxigenin.

The small difference in the Rf values of agapan-
thagenin and praecoxigenin suggested similarity in
structure with possibly only differences in configu-
ration of substituent groups as a result of epimeri-
sation. It has been shown by Heftmann (31) that con-
figurational changes give rise to small differences in
Rf values of saponin-
genins (Table 8).

Table 8. Paper chromatography of normal and iso saponin-
genins

Saponin	Rf value of spot	Difference
sarsasapogenin (25L)	0.68	0.04
smilagenin (25D)	0.72	
yamogenin (25L)	0.48	0.04
diosgenin (25D)	0.44	

Subsequent investigations showed large structural differences between these two sapogenins. Agapanthagenin is a 2:3:5-triol and praecoxigenin is considered to be a diene diol. The additional hydroxyl group in agapanthagenin should make it more polar and give rise to a lower R_f value. The fact that the third hydroxyl group is tertiary and sterically hindered could possibly explain why these two sapogenins have R_f values which are so similar.

4. Confirmation of the structure of agapanthagenin

The structure assigned to agapanthagenin by Stephen (19) was based mainly on analytical data and its dehydration to yield yuccagenin as one of the products. Although it is a fair assumption that the tertiary hydroxyl group was attached to C-5, its position and configuration had not been established beyond doubt. The configuration of the 2:3 diol was not unequivocally established. It was therefore deemed necessary for more synthetic and instrumental evidence to be presented to establish the structure of agapanthagenin beyond doubt. The results of further investigations for this purpose are reported in this chapter.

Further support for the assignment of agapanthagenin as a sapogenin of the 'iso' series was obtained from a study of its infrared, mass and nuclear magnetic resonance spectra and those of its derivatives. The glycollic nature of the secondary hydroxyl groups attached to C-2 and C-3 was confirmed by oxidative fission with lead tetraacetate and chromic anhydride and its configuration as a diequatorial $2\alpha:3\beta$ -diol was established on evidence obtained from the kinetics of glycol fission with lead tetraacetate, the nature

of the proton shift in the low field region of the n.m.r. spectrum of its diacetate, the extent of hydrogen bonding in the 3 micron region of the infrared spectrum of its 5α -monoacetate, and the solvolysis of its dimesylate of an olefine.

Support for the location of the tertiary hydroxyl group at C-5 and its 5α -configuration was obtained from its synthesis by a series of proved stereospecific reactions.

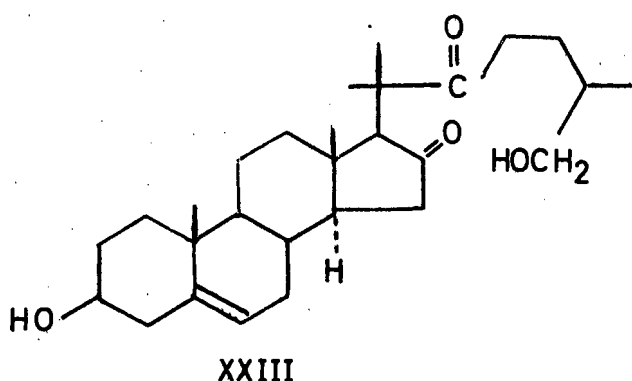
On the basis of this evidence the structure of agapanthagenin has been confirmed as 22a-spirostanstan- $2\alpha:3\beta:5\alpha$ -triol.

4.1 Nature and configuration of ring F of agapanthagenin

Infrared spectra

It has been shown by Wall et al.(28) that the spiroketal ring system of steroidal sapogenins gives rise to four well defined bands in the $800-1000\text{ cm}^{-1}$

region of the infrared spectrum, sapogenins of the 'iso' series give bands at 865,900,920,981 cm^{-1} and the 'normal' sapogenins, bands at 860,900,920,981 cm^{-1} . These four bands appear to be specific to ring F as the infrared spectrum of kryptogenin XXIII, in which the F ring is absent shows none of these bands.



The relative intensities of the bands at 900 and 920 cm^{-1} have also been used to distinguish between the iso and normal sapogenins. The 900 cm^{-1} band is twice as strong as the 920 cm^{-1} in the iso series and in the normal series the 920 cm^{-1} band is the more intense. The infrared spectra of agpanthagenin and its diacetate (Fig. 5 and 6) show absorption bands at 866, 900,921,982 cm^{-1} and the band at 900 has almost twice the intensity of the band at 921 cm^{-1} indicating that

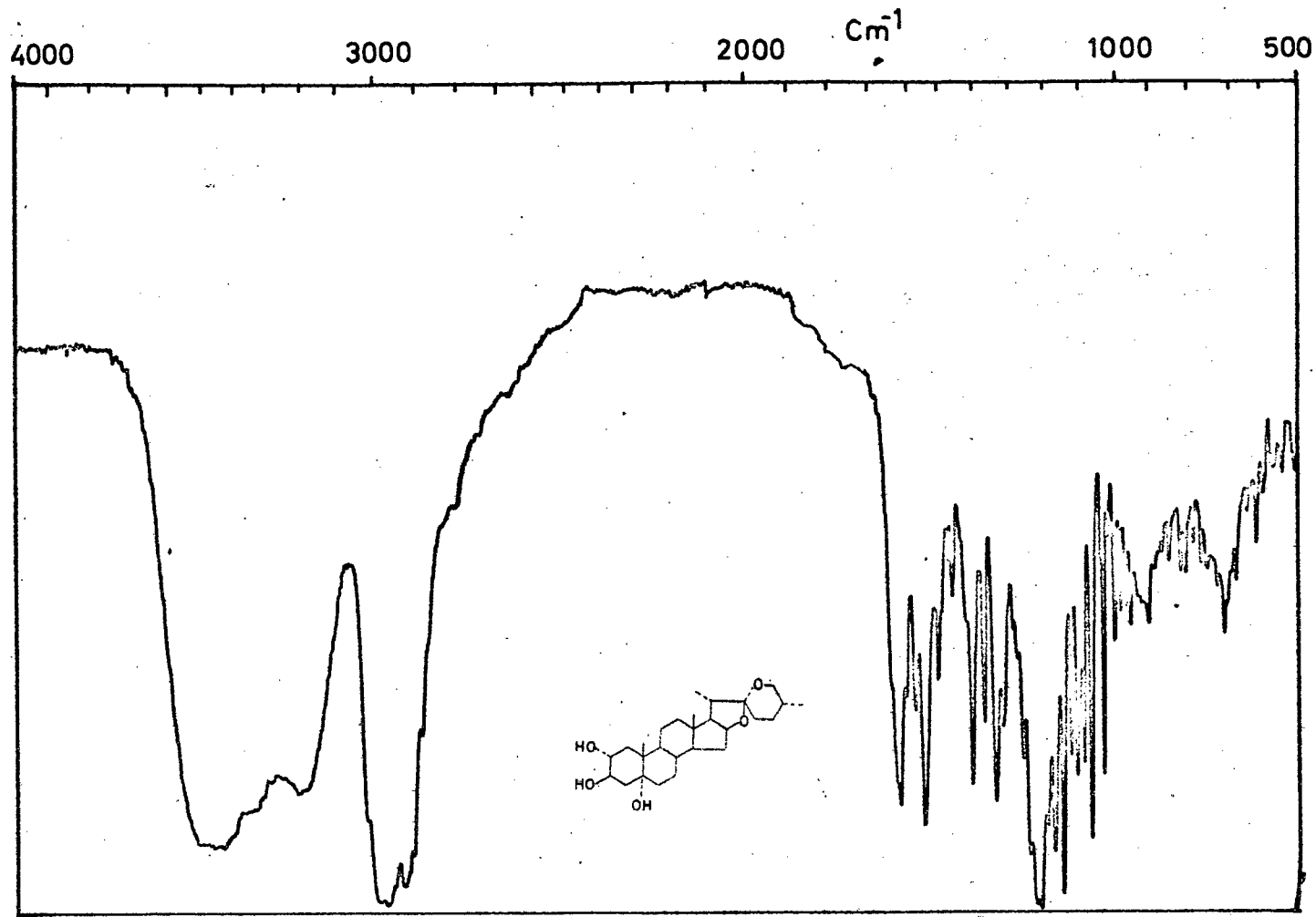


Fig. 5 I.R.spectrum of agapanthagenin

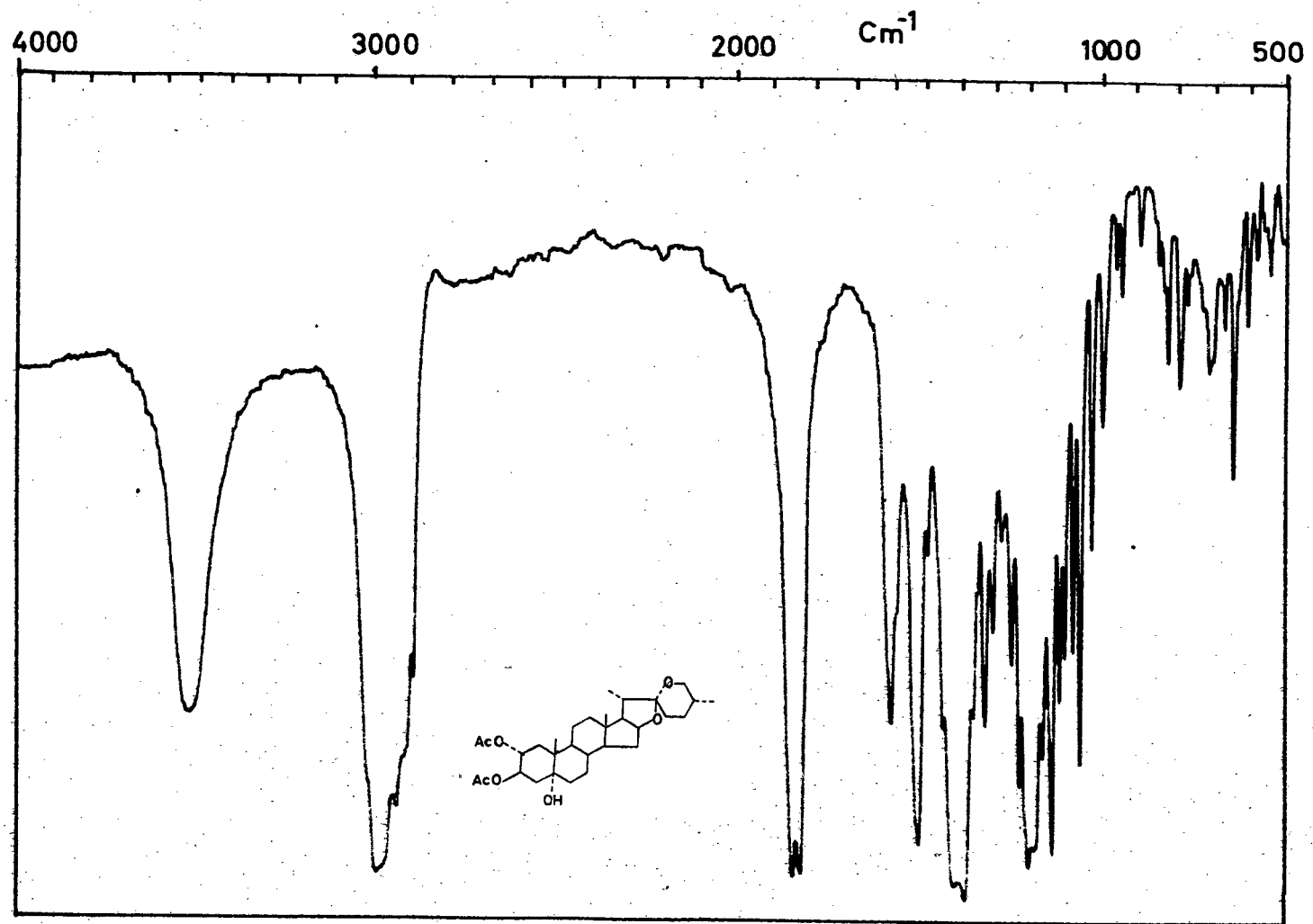


Fig. 6 I.R. spectrum of agapanthagenin diacetate

agapanthagenin is a steroidal sapogenin of the iso series.

Mass spectra

Budzikiewicz and co-workers (37) have shown that fragmentation of the spiroketal group gives rise to two ions at m/e 139 and m/e 115 which are specific to steroidal sapogenins and can be used for their characterisation. In most sapogenins, which have no strong charge localising groups in the rest of the molecule, the peak due to ion m/e 139 shows high intensity and is often the base peak. The mass spectrum of agapanthagenin (Fig. 24) gives evidence of these two characteristic peaks and confirms the presence of the spiroketal structure.

Nuclear magnetic resonance spectra

It has been shown by Kutney (38) that specific features, in the region of resonance due to the C-26 protons and also in the C-methyl region, can be used to distinguish between the normal and iso series.

Specific features of the 3.20-4.05 p.p.m. region

From a consideration of the configuration of the normal and iso sapogenins (Fig. 2) it can be seen that the C-26 protons are deshielded by the ring F oxygen atom and should, therefore, resonate at lower field giving a signal which is sufficiently removed from all other proton signals to be easily recognised. Also by virtue of differences in their chemical environment the splitting patterns of these protons should be different. The proton, in the normal series, at C-25 is in an equatorial conformation whereas in the iso series it is axially orientated. From a study of a number of sapogenins of both series Kutney noticed that in the iso series interaction of the C-26 protons with the equatorial methyl group at C-25 gave a broad multiplet with two main broad signals separated by 4 Hz. However in the normal series the methyl group at C-25 is in the axial position and splits the C-26 protons into a pattern which approximates a quartet. Spectra of smilagenin (25D), neotigogenin acetate (25L) and agapanthagenin diacetate reproduced in juxtaposition (Fig. 7) show the similarity between smilagenin and agapanthagenin diacetate.

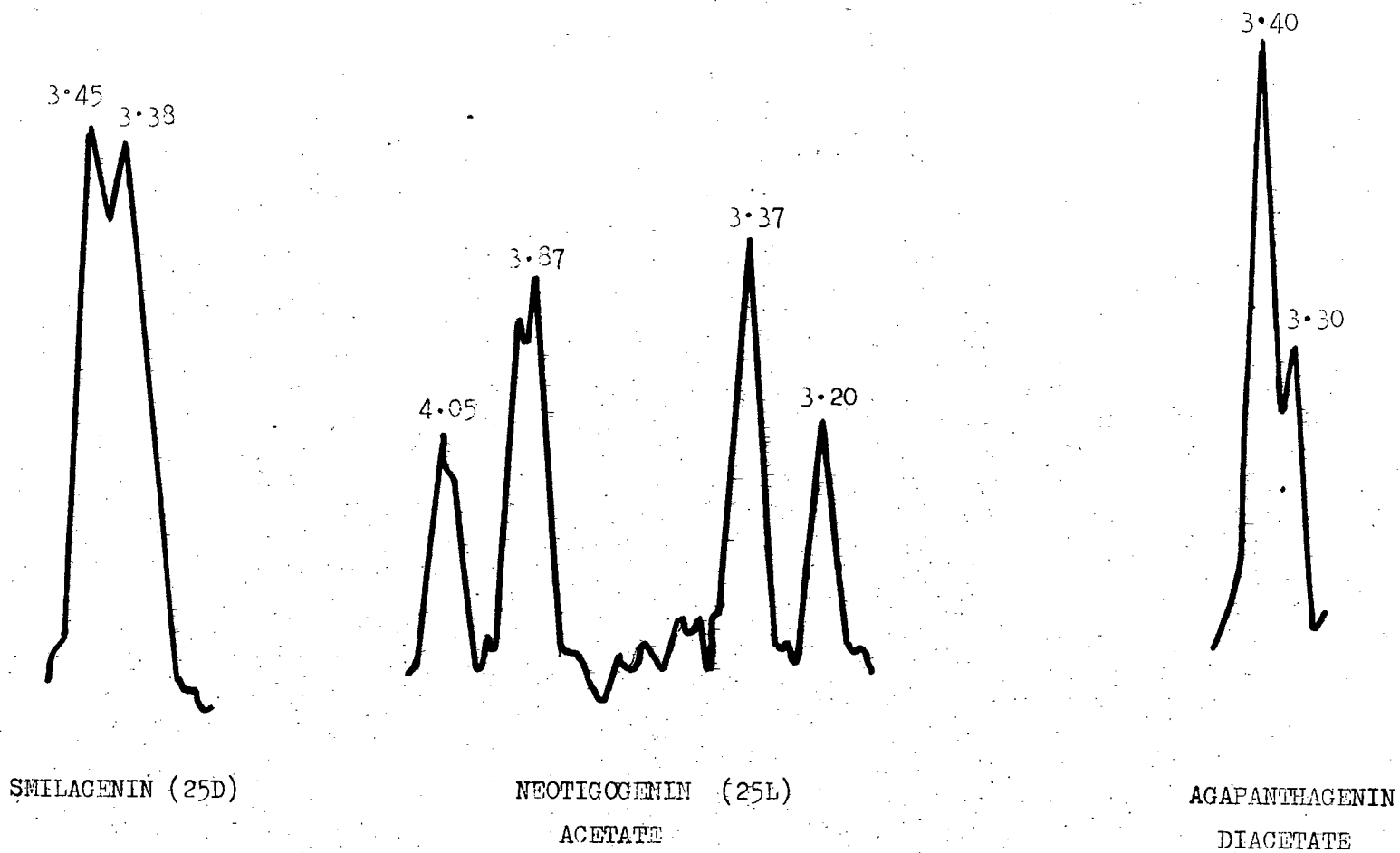


Fig7 NMR Spectra showing the C₂₆ proton signals in deuteriochloroform(p.p.m.)

Specific features of the C-methyl region

Signals for the C-21 and C-27 methyl groups which would be expected to be split into doublets overlap the angular methyl signals and are only visible as shoulders underlying these two sharp C-18 and C-19 methyl signals. This is shown in the spectra of smilagenin, neotigogenin acetate and agapanthagenin in deuteriochloroform (Fig. 8). When these spectra were run in pyridine (Fig. 9) a distinct solvent shift occurred. The sharp C-18 signal moved downfield in the iso sapogenins approximately 0.08 p.p.m. and the signal of the C-27 equatorial methyl group moved upfield to become the highest field signal in the spectrum showing as a doublet at 0.56 and 0.64 p.p.m. In the normal series the C-27 axial methyl signal moved downfield and the C-18 methyl signal still remained the highest field signal.

As can be seen (Fig. 9) in both smilagenin and agapanthagenin diacetate the C-27 methyl signal has moved upfield to become the highest field signal, whereas in neotigogenin acetate the sharp C-18 methyl signal still remains the highest field signal. It thus follows that in agapanthagenin the C-27 methyl

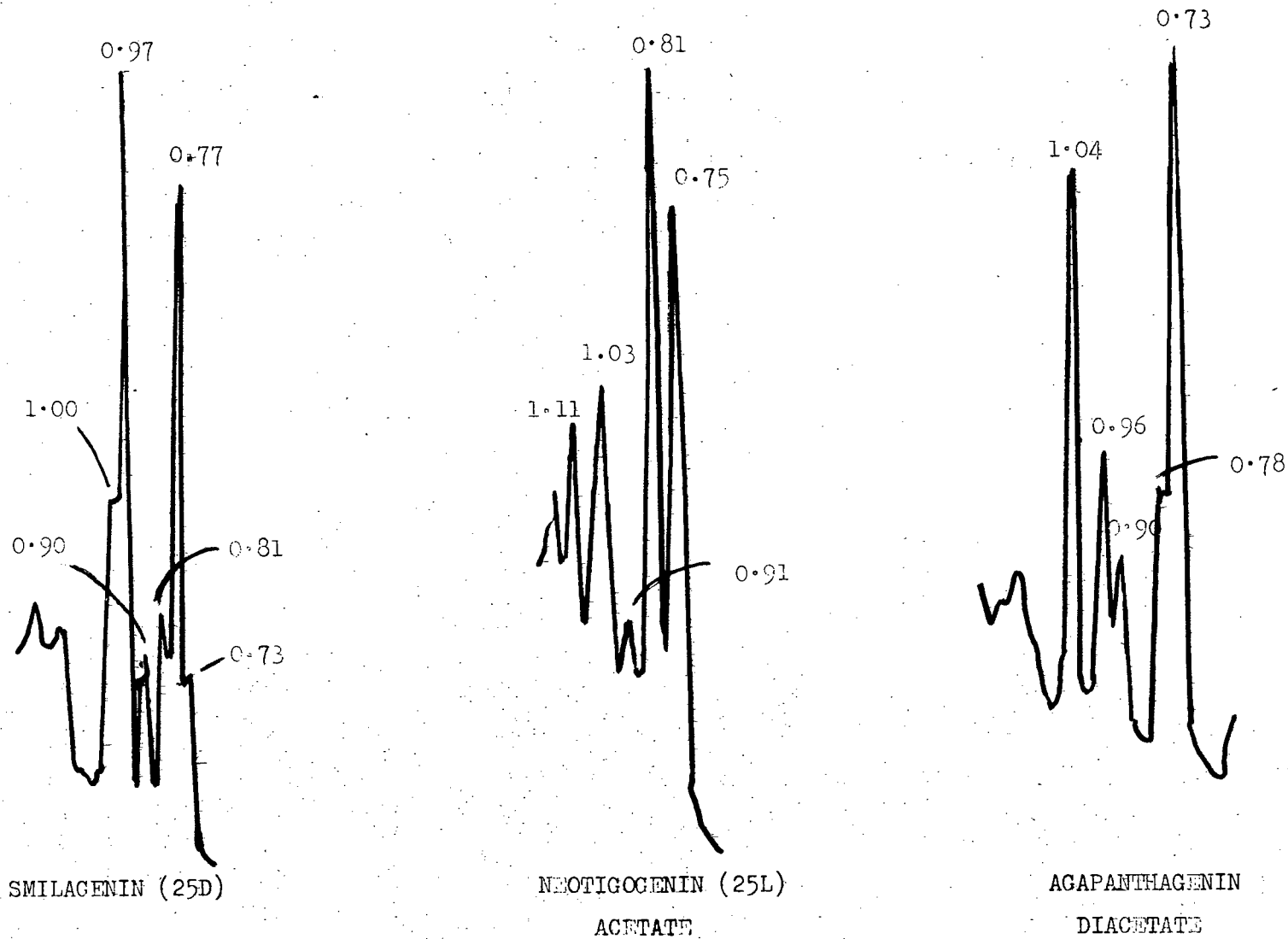
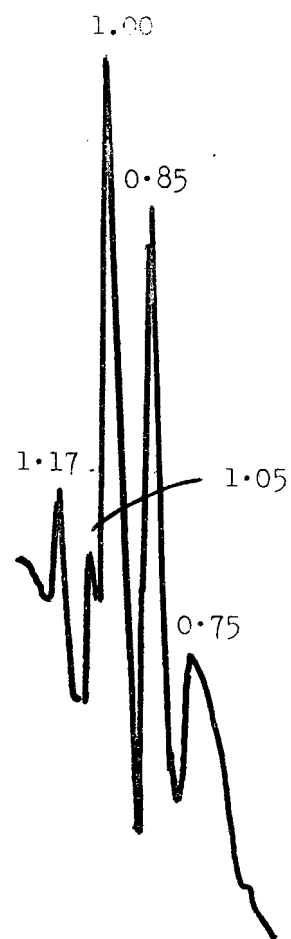
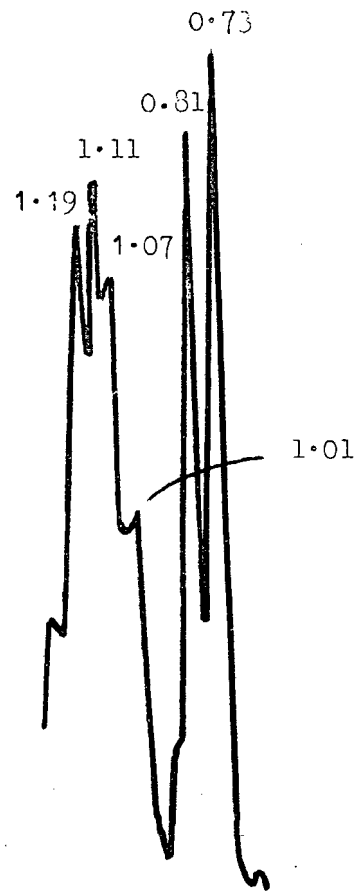


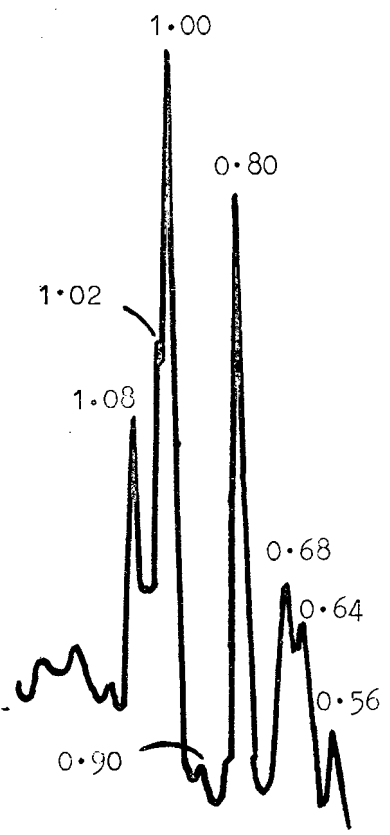
Fig. 8 NMR Spectra in the C-methyl region in deuteriochloroform (p.p.m.)



SMILAGENIN (25D)



NEOTIGOGENIN (25L)
ACETATE



AGAPANTHAGENIN
DIACETATE

Fig9 NMR Spectra in the C-methyl region in pyridine (p.p.m.)

group is equatorially orientated.

The spectral pattern of agapanthagenin diacetate in both the C-methyl region, and the low field region due to the resonance of the protons at C-26, gives strong support to it being an iso sapogenin.

4.2 The position and configuration of the secondary hydroxyl group in agapanthagenin

4.2.1 Reaction with lead tetraacetate

Agapanthagenin on treatment with lead tetraacetate gave a product with a molecular ion (m/e 446, Fig. 31) of 2 m.u. less than the corresponding molecular ion of agapanthagenin. This would be consistent with the loss of two hydrogen atoms as a result of the oxidative cleavage of the glycol function. The infrared spectrum of the reaction product (Fig. 10) showed an absorption band at 3580 cm^{-1} due to the O-H stretching vibrations of a hydroxyl group, a shoulder on the main C-H stretching band at 2720 cm^{-1} and a strong band at 1700 cm^{-1} indicative of C=O stretching absorption. The presence of these

bands are indicative of a hydroxyl and an aldehyde group.

Guthrie and Honeyman (39) have shown that dialdehydes formed by glycol cleavage can, under certain circumstances, undergo further reaction to give hemialdals with water, or, if there is a free hydroxyl group in a favourable position and conformation, could form cyclic hemiacetals. Thus if the hydroxyl groups in agapanthagenin were attached to C-2, C-3, and C-5 the possible products of lead tetraacetate oxidation could be the dialdehyde (XXIII), the hemialdal (XXIV), the hemiacetal (XII) or the aldol (XXII).

The absence of an ion m/e 464 in the mass spectrum coupled with bands in the infrared spectrum indicative of the $-CHO$ group eliminates XXIV. The presence of fragments at m/e 428 and m/e 403 can be readily explained by structures XII and XXIII.

A 5α -hydroxyl group would be in a favourable position and configuration to form a five membered cyclic hemiacetal with an aldehyde formed from a 2α -hydroxyl group and this would favour structure

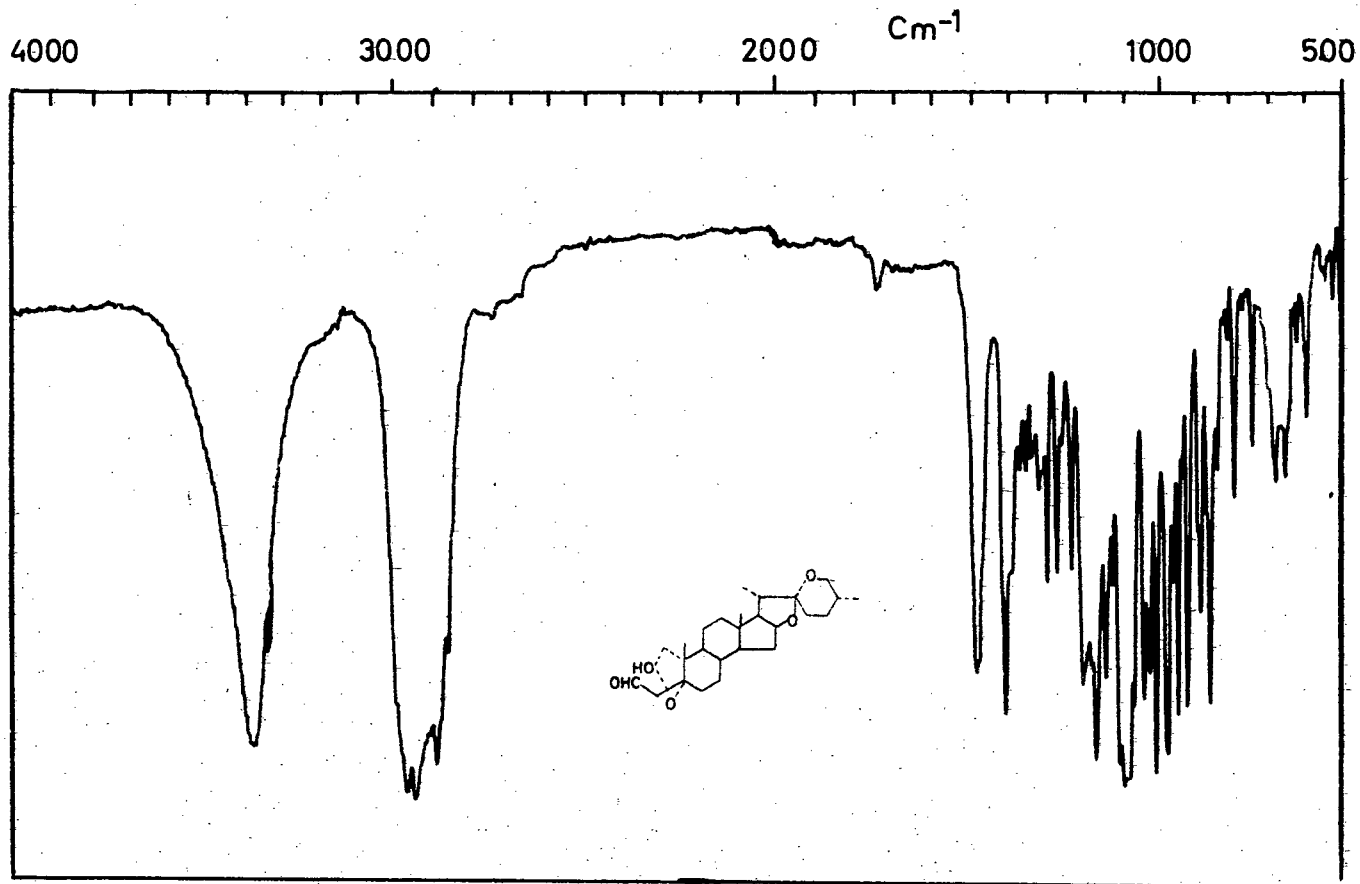


Fig.10 IR Spectrum of the lead tetraacetate oxidation product of agapanthagenin

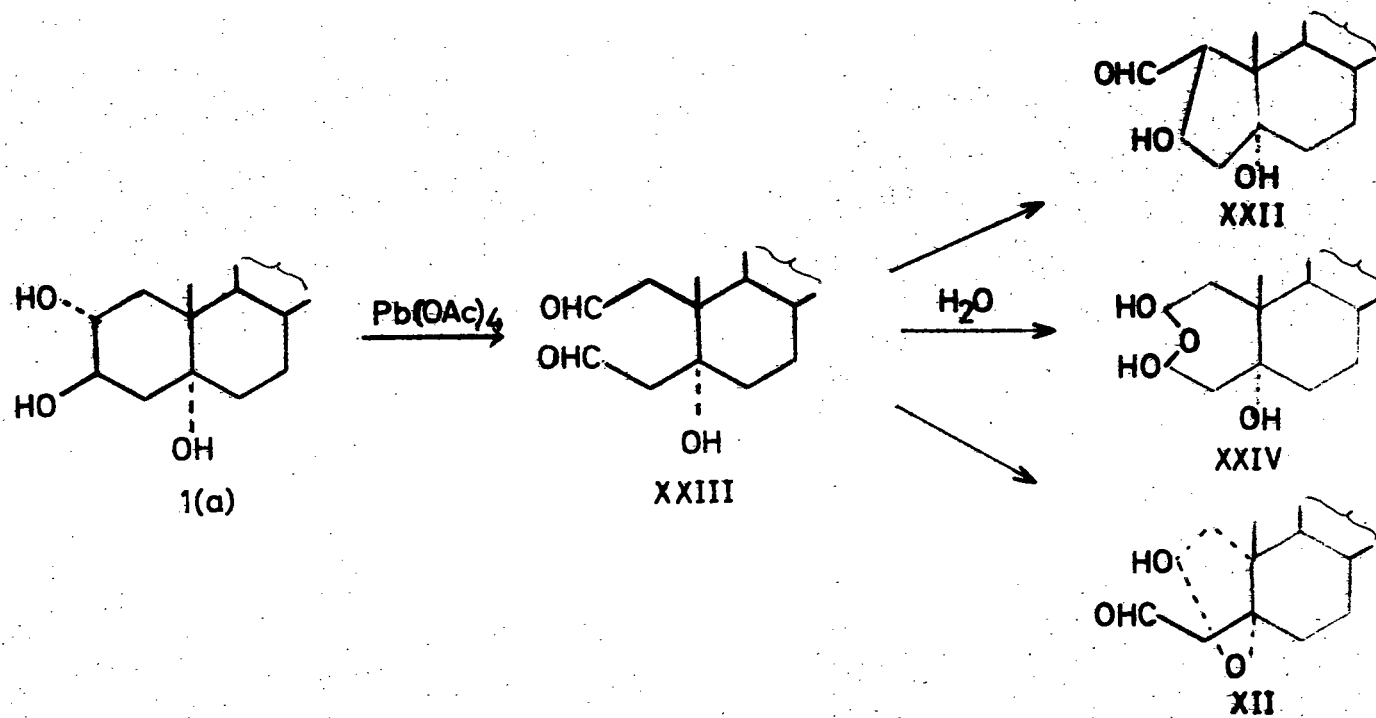
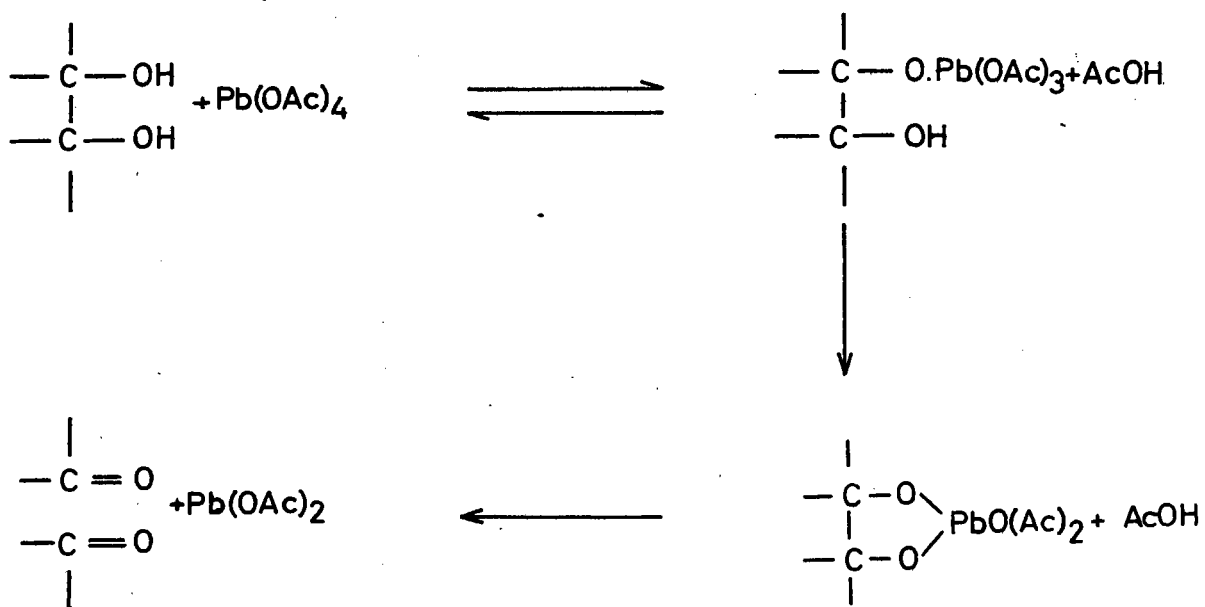


Fig.11 Possible lead tetraacetate oxidation products of agapanthagenin

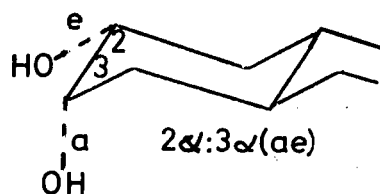
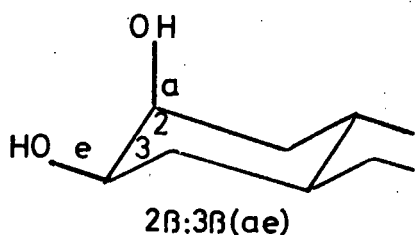
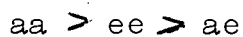
XII. This is supported by a peak in the mass spectrum (m/e 403) due to the fission of the fragment $\cdot\text{CH}_2\cdot\text{CHO}$.

4.2.2 The kinetics of oxidation by lead tetraacetate

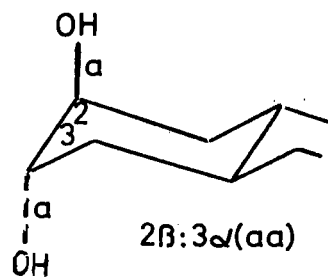
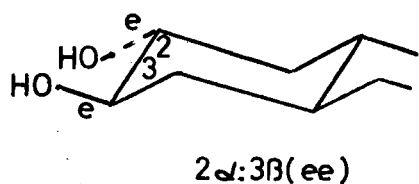
There is common assent to the view that no single mechanism prevails in the oxidation of vicinal diols with lead tetraacetate. The commonly accepted mechanism for the oxidative fission of disecundary glycols was proposed by Criegee et al.(40). This involves the initial reversible formation of a covalent O-Pb bond followed by a rate determining step requiring the formation of a five membered cyclic intermediate.



In order to form a cyclic complex the C-O bonds of the two hydroxyl groups must be rotated to a greater degree of coplanarity. Consideration of the four possible configurations of the spirostan 2:3-diols suggests that the energy required to force the two hydroxyl groups more nearly into the same plane should follow the order:



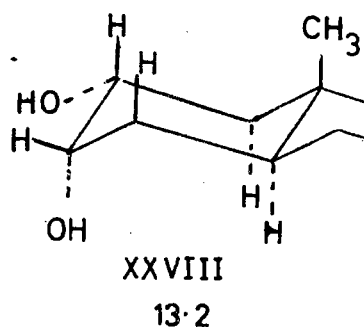
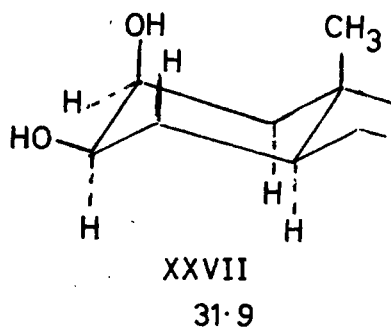
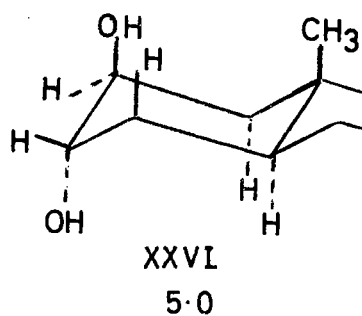
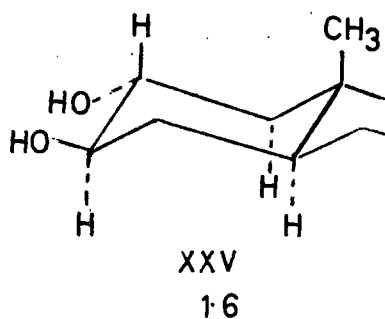
CIS



TRANS

In fact, forcing diaxial bonds more nearly into the same plane so as to permit cyclisation would

occur with great difficulty. On these premises the relative rates at which glycols undergo oxidative fission with lead tetraacetate should offer a means of determining the configuration of the two hydroxyl groups relative to each other. Djerassi and Ehrlich (41) investigated the rates of cleavage of gitogenin (XXV) and its three epimeric 2:3-hydroxy diols, XXVI, XXVII, XXVIII. The relative rates of glycol cleavage on oxidation with lead tetraacetate are indicated by the figures (expressed as $k \times 10^3$) under the formulas below.



As would be expected the two cis glycols (XXVII) and (XXVIII) were cleaved much faster than the two trans glycols (XXV) and (XXVI). The situation with the trans epimers is anomalous, in that the diaxial epimer XXVI was cleaved almost three times faster than the diequatorial epimer (XXV).

To explain the difference Fieser (14) suggested that in each series, cis and trans, a further determining factor may be the extent to which oxidation relieves steric strain. The greater reactivity of XXVII over XXVIII and of XXVI over XXV may be in part because of severe strain in XXVII and XXVI due to 1:3 interaction of axial methyl and hydroxyl groups.

The differences between the rates as recorded by Djerassi and Ehrlich were sufficiently large, however, to suggest that a comparative study of the rate of glycol fission of agapanthagenin and gitogenin (a $2\alpha:3\beta$ -diol of known configuration) could provide further information about the diol configuration. To this end a comparative rate study of these two saponinins was conducted the results of which are shown below.

sapogenin	kx10 ³
agapanthagenin	2.17
gitogenin	2.07

Although the figure for the rate constant for gitogenin was much higher than that recorded by Djerassi, it was similar enough to that of agapanthagenin to suggest that they both have the same glycol configuration.

4.2.3 The configuration of the glycol system from an evaluation of the hydroxyl absorption

It has been shown by Davies (42) that when the hydroxyl groups of a dihydroxy compound are sufficiently close together they will form an internal hydrogen bond. Kuhn (43), studied the infrared absorption spectrum, in the three micron region, of a number of cyclic dihydroxy compounds in dilute carbon tetrachloride solution. He found that, if the concentration of the solution did not exceed 0.005 molar, intra molecular bonding took place and that two absorption peaks occur with compounds in which the calculated length of the hydrogen bond between the two hydroxyl groups is

less than 3.3\AA . He defined the length of the hydrogen bond as the distance between the hydrogen atom of the one hydroxyl group and the oxygen atom of the other designated by the symbol H...O. The absorption peak at the higher frequency was due to the free hydroxyl group and the one at lower frequency due to the bonded hydroxyl. The spread between the two bonds, $\Delta\nu$, increases as the length of the hydrogen bond decreases and hence with related diols the observed values of $\Delta\nu$ should provide a measure of the relative proximity of the hydroxyl groups. Badger (44) has also shown that the stronger the hydrogen bond the greater is $\Delta\nu$.

In picturing the H...O distances in compounds containing hydroxyl groups on adjacent carbon atoms it is convenient to consider the dihedral angle θ which is defined as the angle that one observes bounded by the two C-O bonds when the eye of the observer and the two carbon atoms are in a straight line. This can be shown pictorially in what are called Newman projections. In cycloHexane-cis-1:2-diol, one hydroxyl group occupies an axial position and the other an equatorial. In the trans compound there are two possibilities; both hydroxyl substituents

may be axial or both may be equatorial. If the two hydroxyl groups are axial they would be too far apart for hydrogen bonding to occur.

The relationship between the calculated H...O distance and Δv for cis- and trans-cycloHexane-1,2-diol is shown below (Table 9).

Table 9. Relationship between H...O distance and Δv

Compound	Δv	Calculated H...O distance \AA°
cis-cycloHexane-1,2-diol	39	2.34
trans-cycloHexane-1,2-diol	32	2.34

To explain the fact that both cis and trans diols have the same calculated H...O distance and yet the cis compound has a larger Δv value, Kuhn (45) put forward the theory that the attraction between the hydroxyl groups in forming a hydrogen bond will tend to produce a rotation around the C-C bond thus reducing the dihedral angle (Chart 4).

As can be seen cis substituents are much closer together than trans substituents and would have a

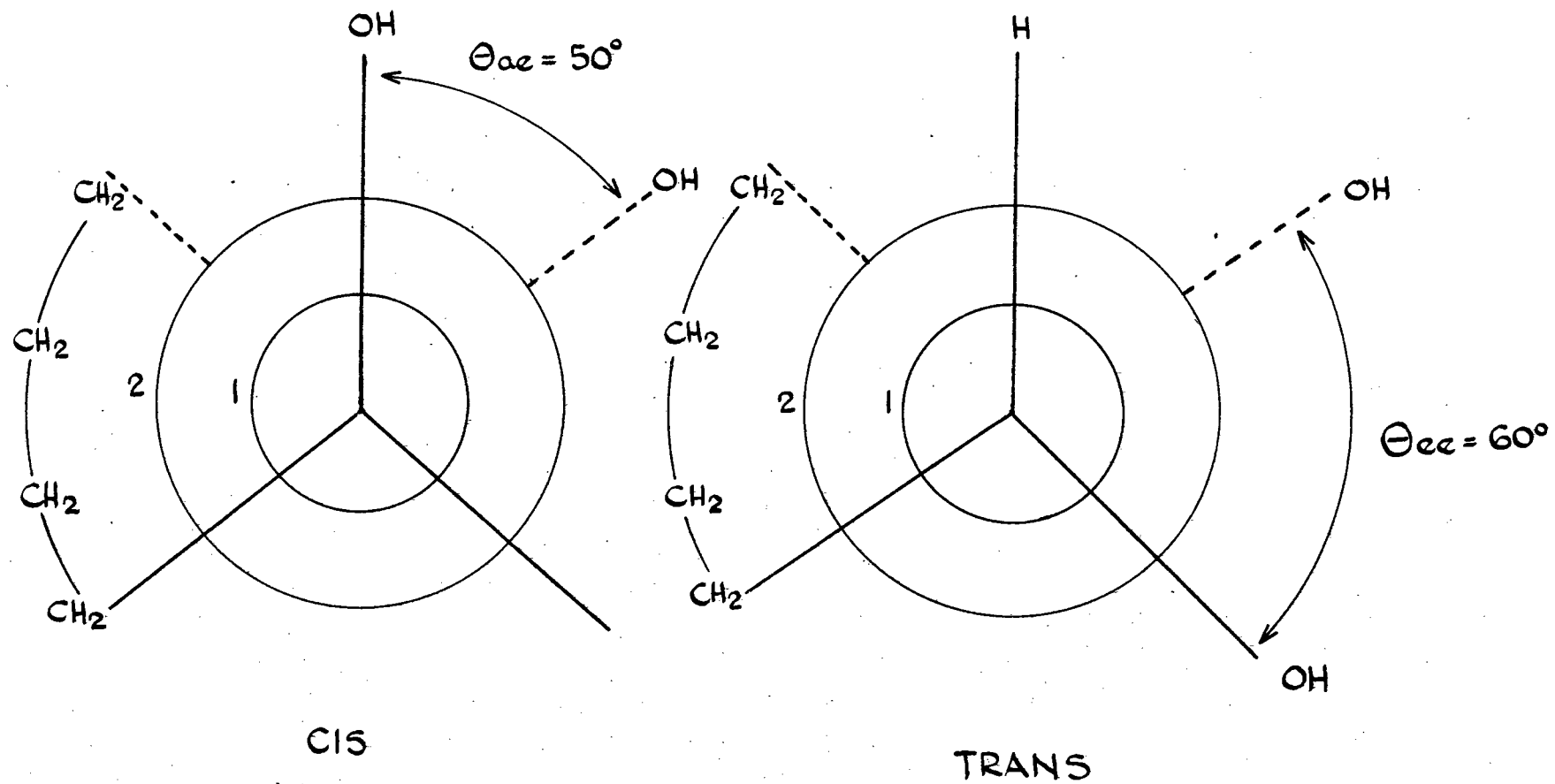


Chart 4 Newman projections of CIS and TRANS cycloHexane diols

larger value for $\Delta\nu$. The $\Delta\nu$ for the trans-Decalin-2:3-diols measured by Ali and Owen (46) are shown in Table 10.

Table 10. Infrared absorption of trans-Decalin-2:3-diols in carbon tetrachloride

Diol	Infrared absorption cm^{-1}		
	Free OH	Bonded OH	$\Delta\nu$
trans-Decalin-2 α :3 α	3625	3588	37
trans-Decalin-2 α :3 β	3630	3600	30
trans-Decalin-2 β :3 α	3630	-	0

It was not possible to obtain spectral information on agapanthagenin due to its insolubility in carbon tetrachloride. High resolution spectra of its Δ^5 , and 5 α -acetoxy derivatives in dilute carbon tetrachloride were used instead. These derivatives gave the following two peaks in the three micron region due to free and bonded hydroxyl absorption (Fig. 13).

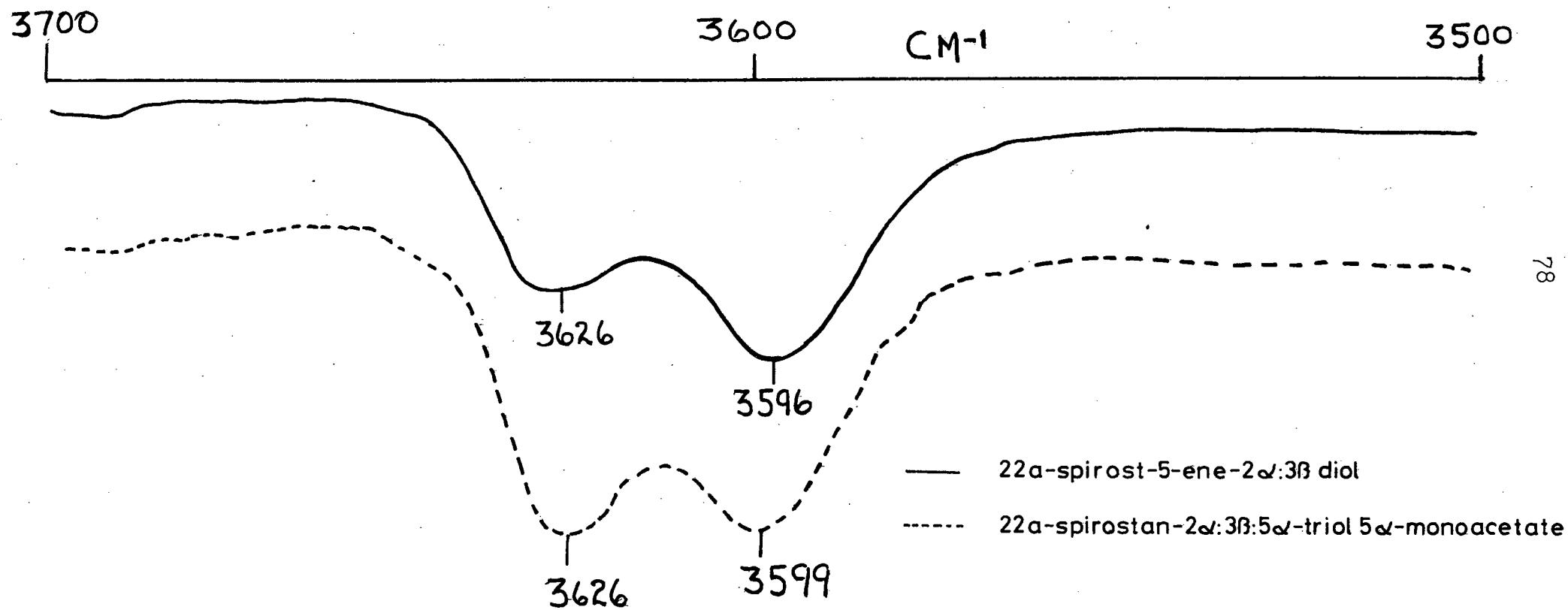


Fig.13 IR Spectra in the three micron region of 22 α -spirost-5-ene-2 α :3 β -diol and 22 α -spirostan-2 α :3 β :5 α -triol 5 α -monoacetate

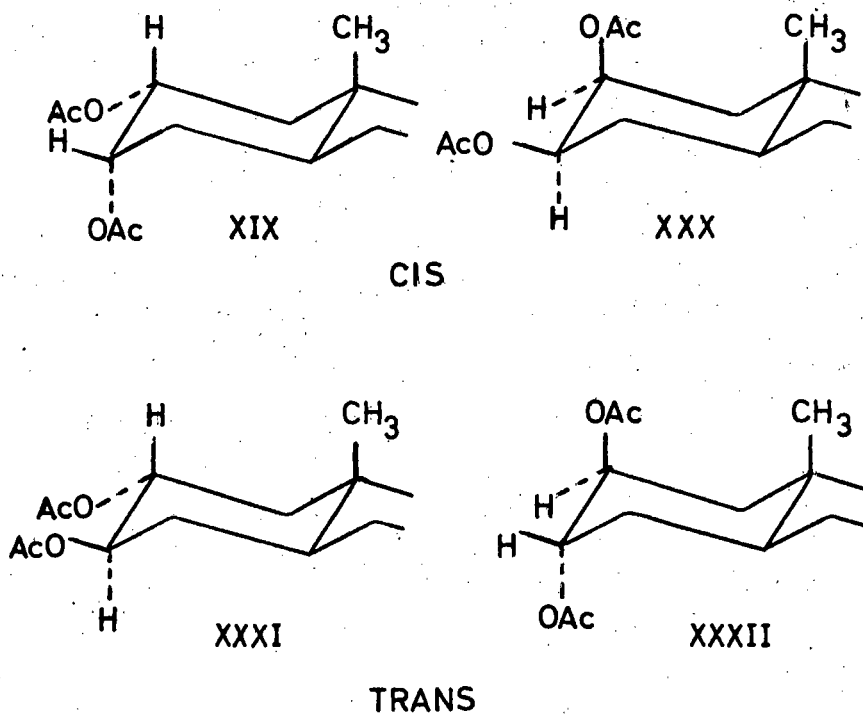
<u>Compound</u>	<u>Free OH</u>	<u>Bonded OH</u>	$\Delta\nu$
Δ^5 -derivative of agapanthagenin (IXa)	3626	3596	30
5 α -mono acetate of agapanthagenin (VII)	3626	3599	26

The spread, $\Delta\nu$, of the Δ^5 -derivative agrees favourably with that of trans-Decalin-2 α :3 β -diol (Table 10) and supports the diequatorial structure of its 2:3-dihydroxy groups. The result obtained with the 5 α -monoacetate derivative cannot be used to prove the diequatorial configuration but is in good agreement with it. As these derivatives were prepared from agapanthagenin by methods known to cause no configurational changes in the diol structure it would be reasonable to conclude that these results support the assignment of the glycol structure of agapanthagenin as a 2 α :3 β -diol.

4.2.4 The nuclear magnetic resonance of agapanthagenin diacetate in the low field region

An examination of the four possible epimeric 2:3-diacetoxy spirostans XXIX, XXX, XXXI, XXXII,

shows that the two protons attached to the vicinal carbon atoms C-2 and C-3 in the cis derivatives are subjected to different deshielding effects.



The equatorial proton being strongly deshielded by the carbonyl of the neighbouring acetoxy group should resonate at much lower field than the axial proton. This should lead to two separate proton signals in the low field region for XXIX and XXX.

The position with the trans derivatives is quite different. In this case, whether the protons are diaxial or diequatorial, being in similar chemical environments they show similar chemical shifts and thus give similar signals in the low field region of the spectrum. Thus XXXI and XXXII should give signals for the two protons which are either superimposed or near to each other.

An examination of the n.m.r. spectrum of agapanthagenin diacetate in deuteriochloroform (Fig. 14) shows a single unresolved two proton multiplet centred at 5.22 p.p.m. This could be due to the overlapping C-2 and C-3 proton signals and would thus indicate that they are either both diaxial or both diequatorial as in XXXI and XXXII respectively.

It has been shown by Bhacca and Williams (58) that the half band width of the signal can be used as a means of distinguishing between trans diaxial and trans diequatorial substituents on vicinal carbon atoms. Trans diequatorial protons give a signal with a half band width of approximately 2.5 Hz, whereas in the case of diaxial protons the half band width of the signal is approximately 15 Hz. The half band width

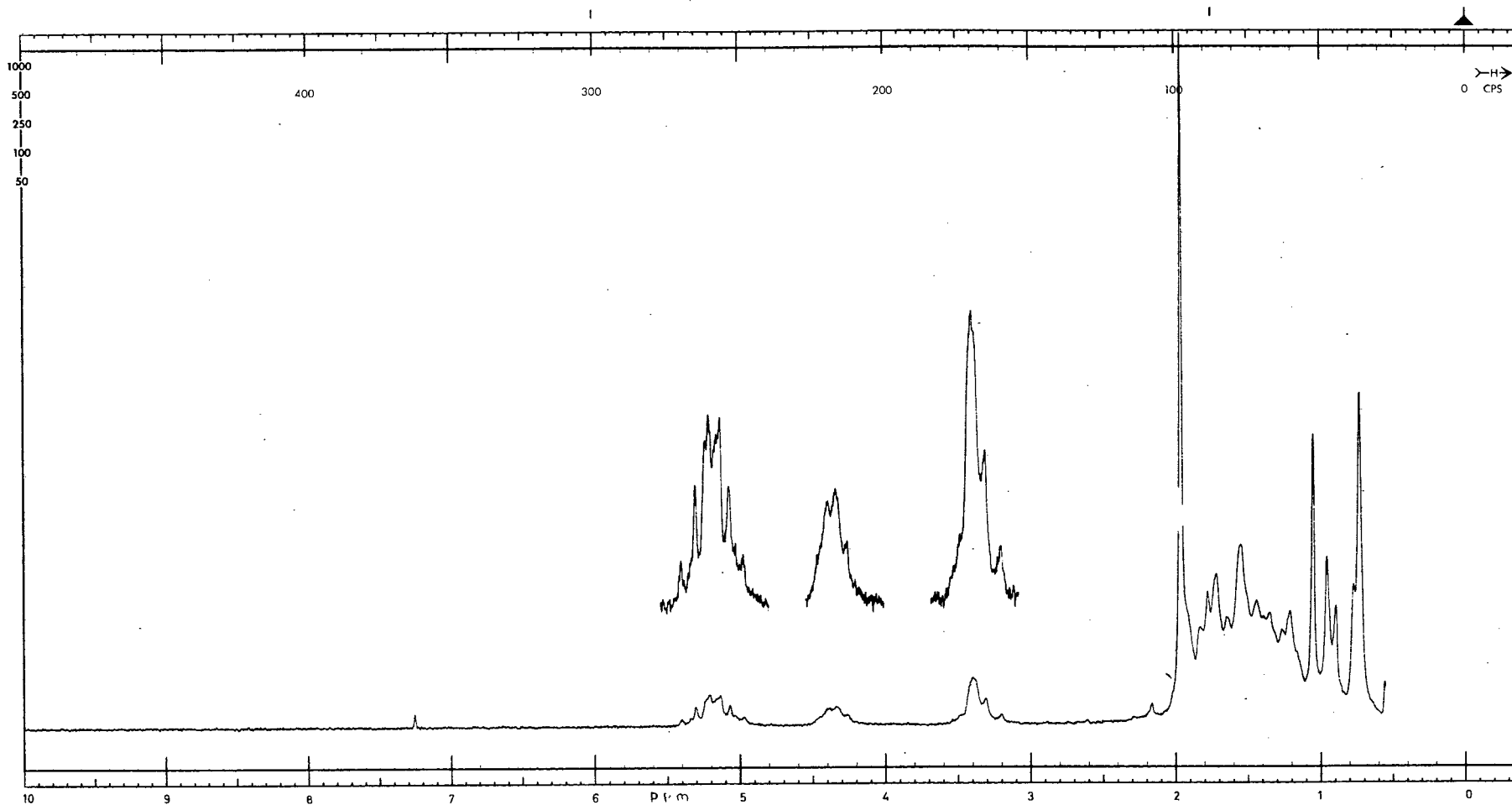
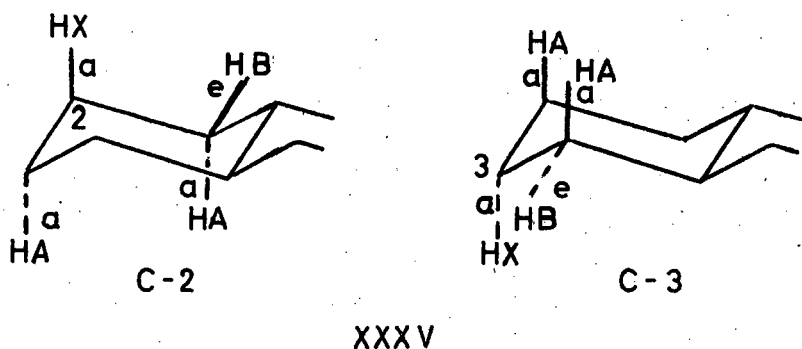


Fig.14 NMR Spectrum of agapanthagenin diacetate in deuteriochloroform

of the signal in agapanthagenin diacetate is 13 Hz which suggests that the C-2, C-3 protons are diaxial as in XXXI.

As a result of solvent shift the signals for the C-2 and C-3 protons were separated when the spectrum was run in pyridine (Fig. 15). They appeared as two sextets centred at 5.44 and 5.85 p.p.m. conforming to a A_2BX system XXXV.



The splitting pattern (Fig. 16) gives coupling constants

$$J_{2\beta,3\alpha} = J_{2\beta,1\alpha} = 10 \text{ Hz}$$

$$J_{2\beta,1\beta} = 4 \text{ Hz}$$

$$J_{3\alpha,2\beta} = J_{3\alpha,4\beta} = 10 \text{ Hz}$$

$$J_{3\alpha,4\alpha} = 4 \text{ Hz}$$

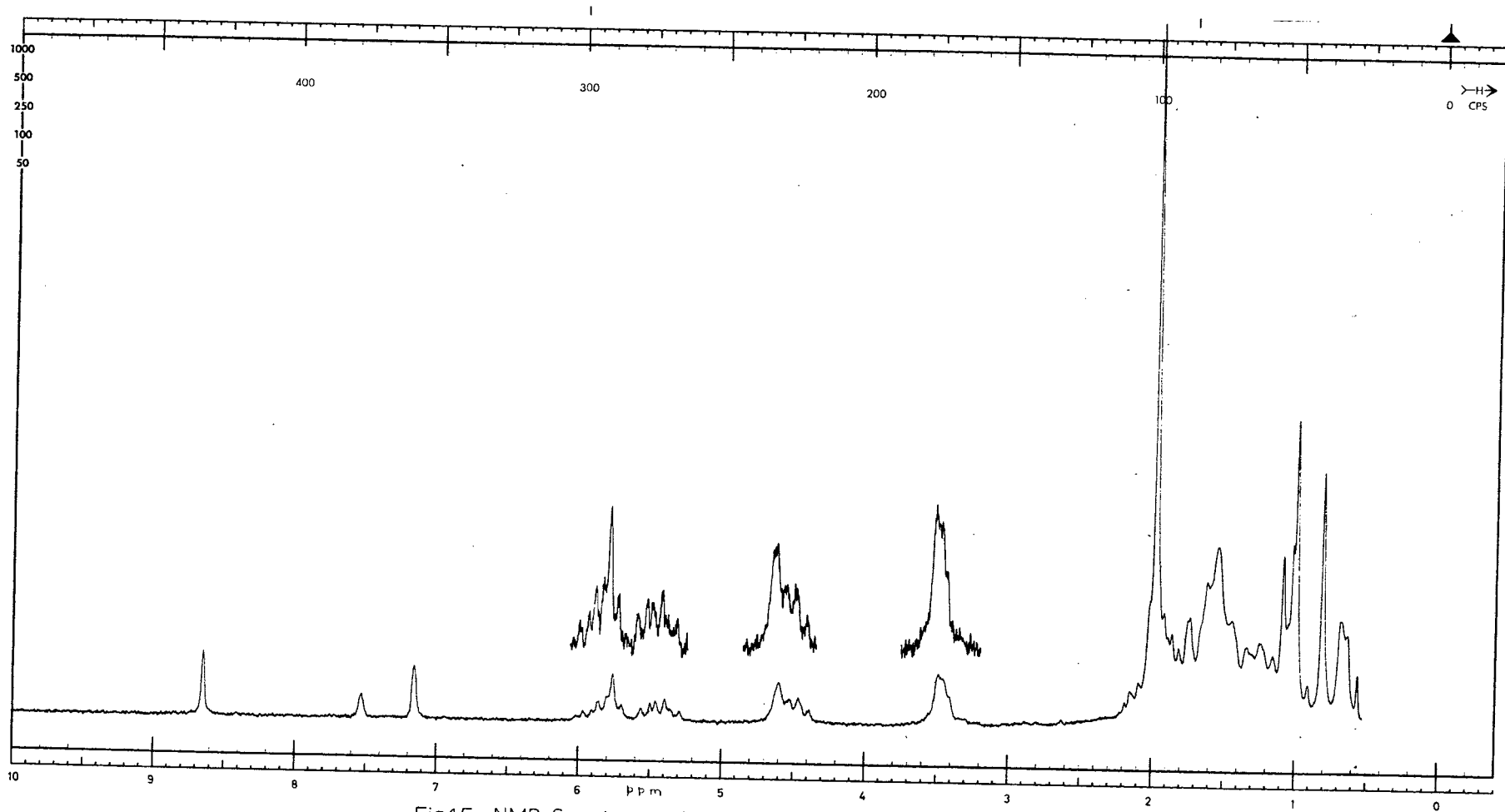


Fig.15 NMR Spectrum of agapanthagenin diacetate in pyridine

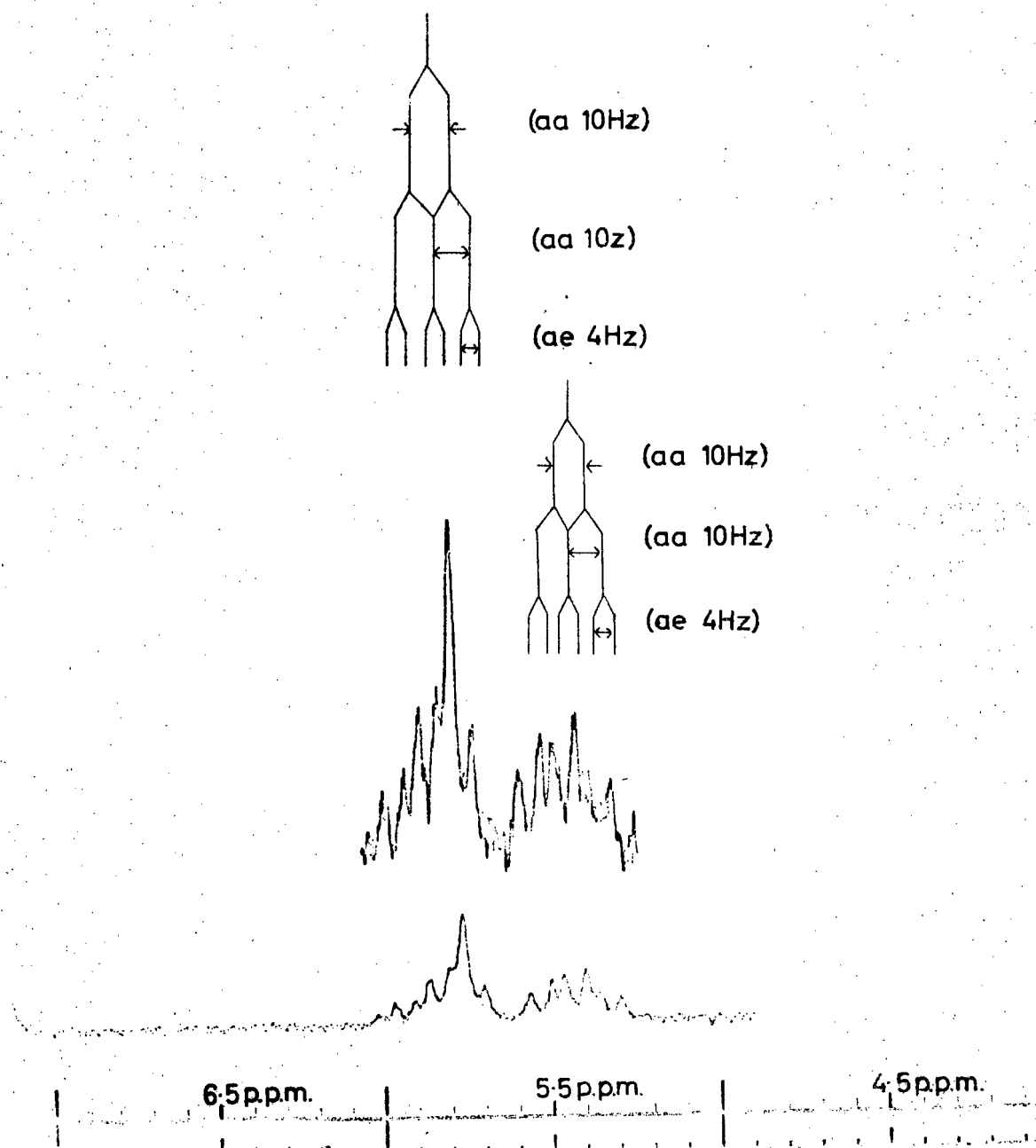


Fig.16 The C-2 and C-3 proton signals in the NMR spectrum of agapanthagenin diacetate in pyridine

It has been shown by Williams and Bhacca (59) that the coupling constants between axial-axial protons on adjacent carbon atoms, J_{aa} , range from 9.5 - 13.4 Hz and values for the coupling constants between axial and equatorial protons on adjacent carbon atoms bearing an equatorial substituent, J_{ae} , from 4.5 to 6.5 Hz. The splitting patterns shown by the C-2 and C-3 protons suggest a diaxial configuration which would confirm the diequatorial nature of the two acetoxy substituents.

4.2.5 Chromic acid oxidation of agapanthagenin

Oxidation of vicinal diols with chromic acid should result in ring fission with the formation of a dicarboxylic acid. The product of chromic acid oxidation of agapanthagenin gave a monobasic acid on the basis of calculations of its equivalent weight, which was confirmed by a peak at m/e 460 in the mass spectrum due to the molecular ion.

The infrared spectrum (Fig. 17) gave a broad absorption band between 2500-3000 cm^{-1} which is typical of the O-H stretching vibrations of a carboxyl group.

Two peaks in the C=O stretching region, at 1695 cm^{-1} and 1786 cm^{-1} would be consistent with the absorption of the C=O groups of a carboxylic acid and a five membered lactone. On methylation the peak at 1695 cm^{-1} shifted to 1739 cm^{-1} as would be expected when a carboxyl group is methylated. The peak at 1695 cm^{-1} remained unchanged in the spectrum of the methyl ester (Fig. 18). This evidence would be consistent with the reaction sequence by which agapanthagenin (Ia) was oxidised to give a diacid (XXXIV) which then lactonises to form a monoacid lactone (II) by the elimination of water from the carboxyl group at C-2 and the C-5 hydroxyl group. These reactions confirm the relative positions of the three hydroxyl groups at C-2, C-3 and C-5, and also provide evidence in support of the 2α - and 5α -configuration of the hydroxyl groups in agapanthagenin.

4.2.6 Acetonide formation

It has been shown by Pataki et al. (47) that cis-cycloHexane-1,2-diols form acetonide derivatives whereas the trans isomers do not. It was not possible to prepare an acetonide of agapanthagenin which

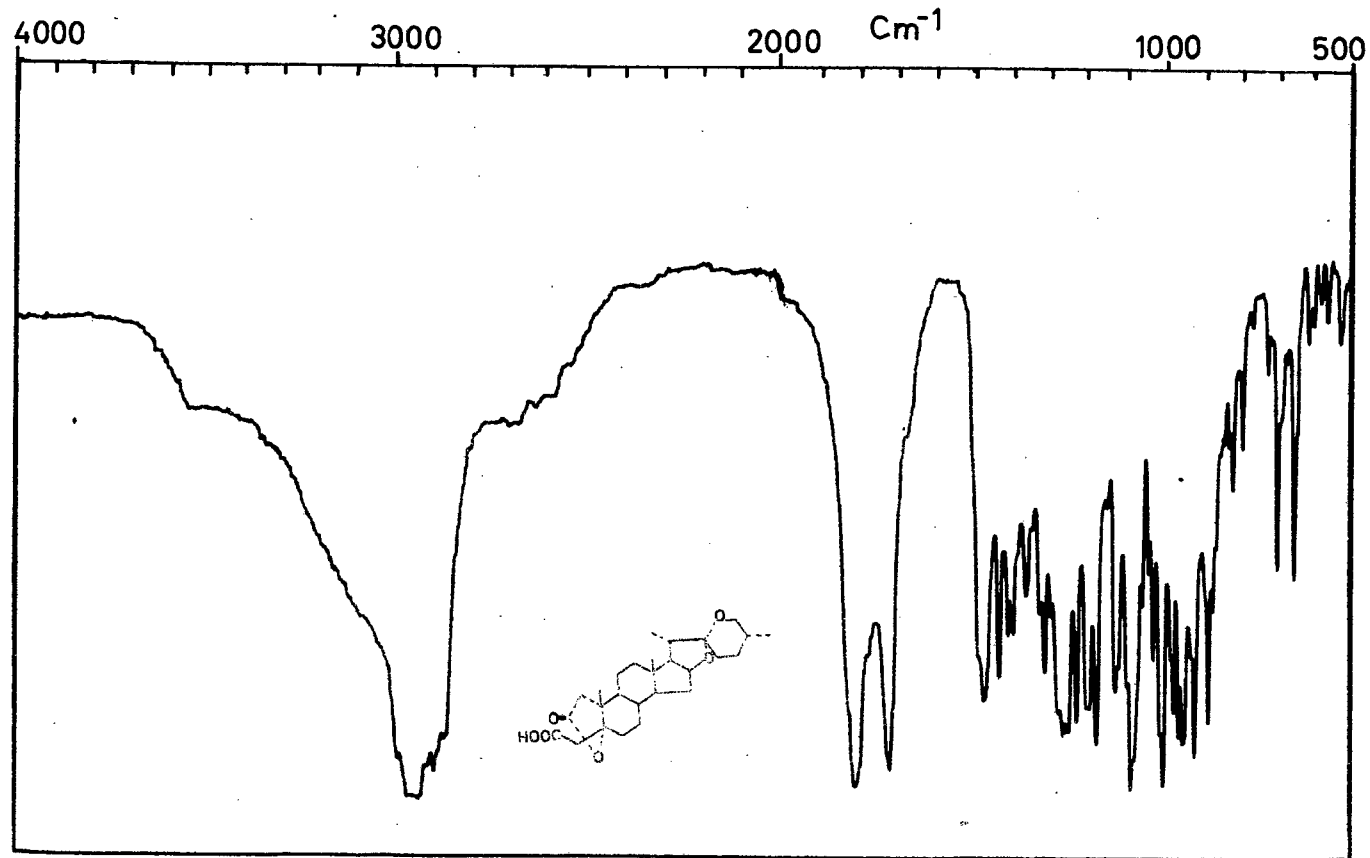


Fig.17 I.R. spectrum of 5 α -Hydroxy-2:3-seco-22 α -spirostan-2:3 dioic lactone

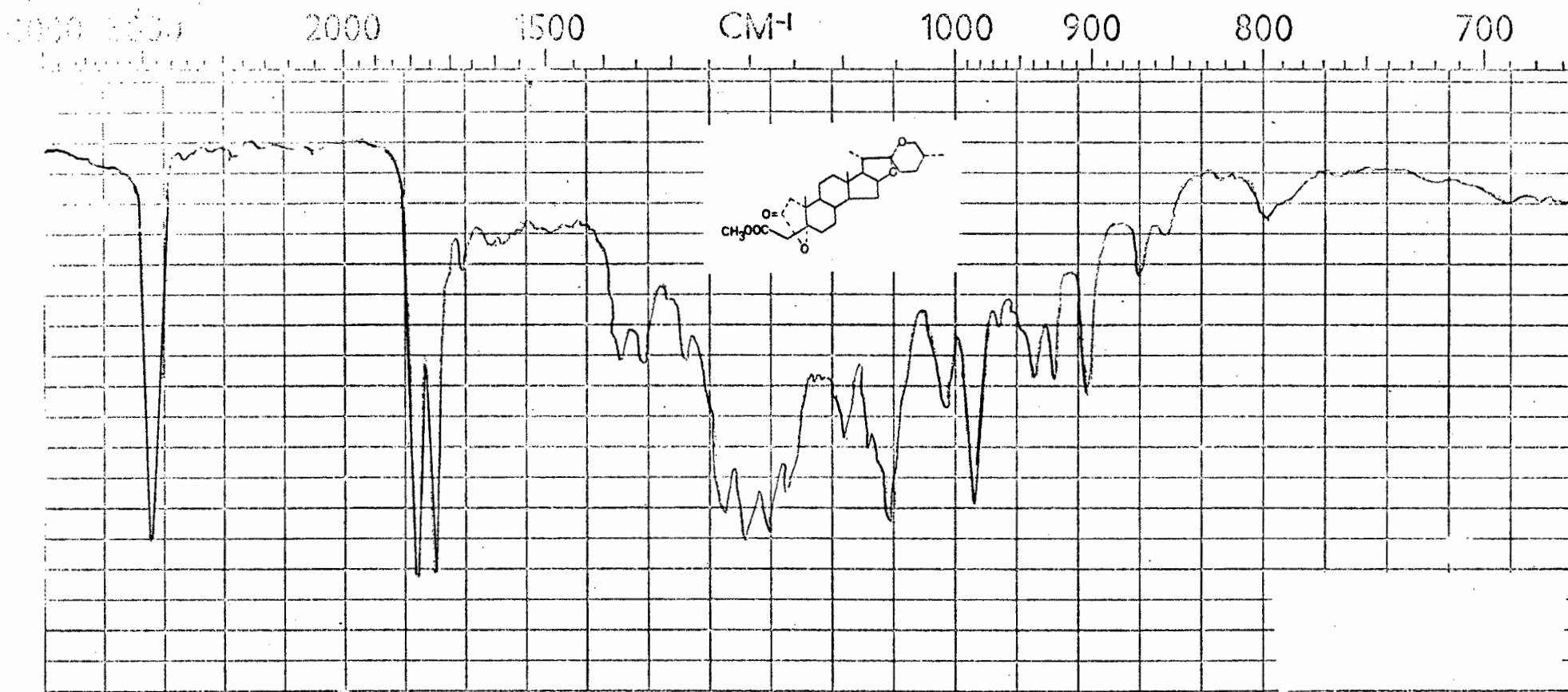
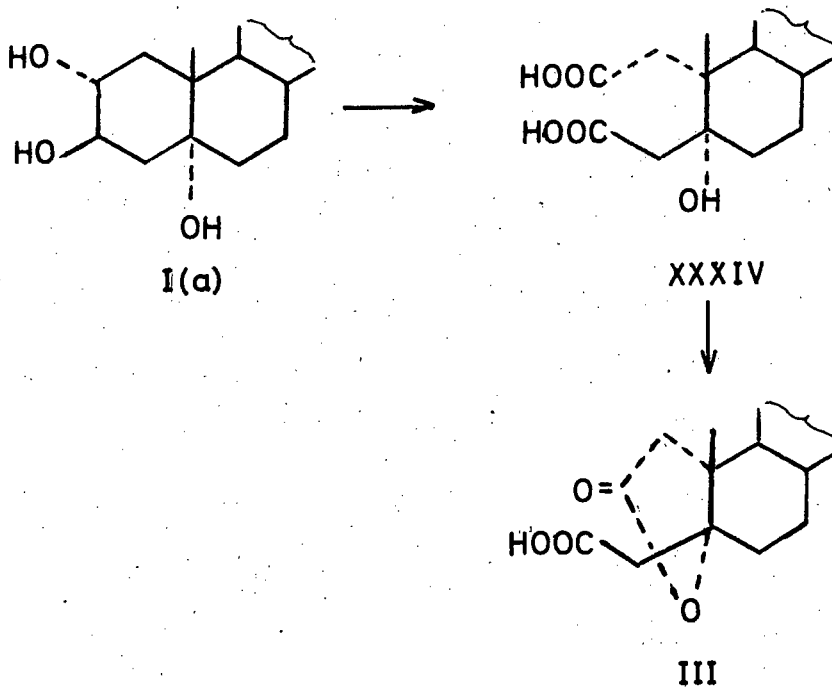


Fig.18 IR Spectrum of the methyl ester of 5 α -hydroxy-2:3-seco-22a-2:3-dioic lactone



suggested that the glycol function was trans.

4.2.7 Mesylation of agapanthagenin and solvolysis of the dimesylate

In an investigation of the corresponding four 2:3 diols derived from 5α -spirostan-12-one, Slates and Wendler (48) made the surprising discovery that the dimesylates of the $2\alpha:3\alpha$, and $2\alpha:3\beta$ diols on reaction with sodium iodide in acetone at 100° were converted in high yield into the Δ^2 olefine whereas

under the same conditions the dimesylates of the $2\beta:3\beta$ and $2\beta:3\alpha$ diols remained unchanged.

On treatment with methanesulphonylchloride agapanthagenin formed a dimesylate. This was confirmed by a molecular ion in the mass spectrum (Fig. 28) which was 156 m.u. more than that of agapanthagenin corresponding with two mesyl groups. The infrared spectrum (Fig. 19) gave bands at 1323 and 1167 cm^{-1} indicative of the mesyl group. This dimesylate readily underwent solvolysis with sodium iodide in acetone to give the Δ^2 olefine (XIII). This was confirmed by an ion in the mass spectrum (Fig. 29) at m/e 414 due to the molecular ion which was 34 m.u. less than in the corresponding ion in the spectrum of agapanthagenin due to the elimination of the two secondary hydroxyl groups. On the basis of the findings of Pataki, and Slates and Wendler, therefore, these results confirm the diequatorial configuration of the two secondary hydroxyl groups in agapanthagenin.

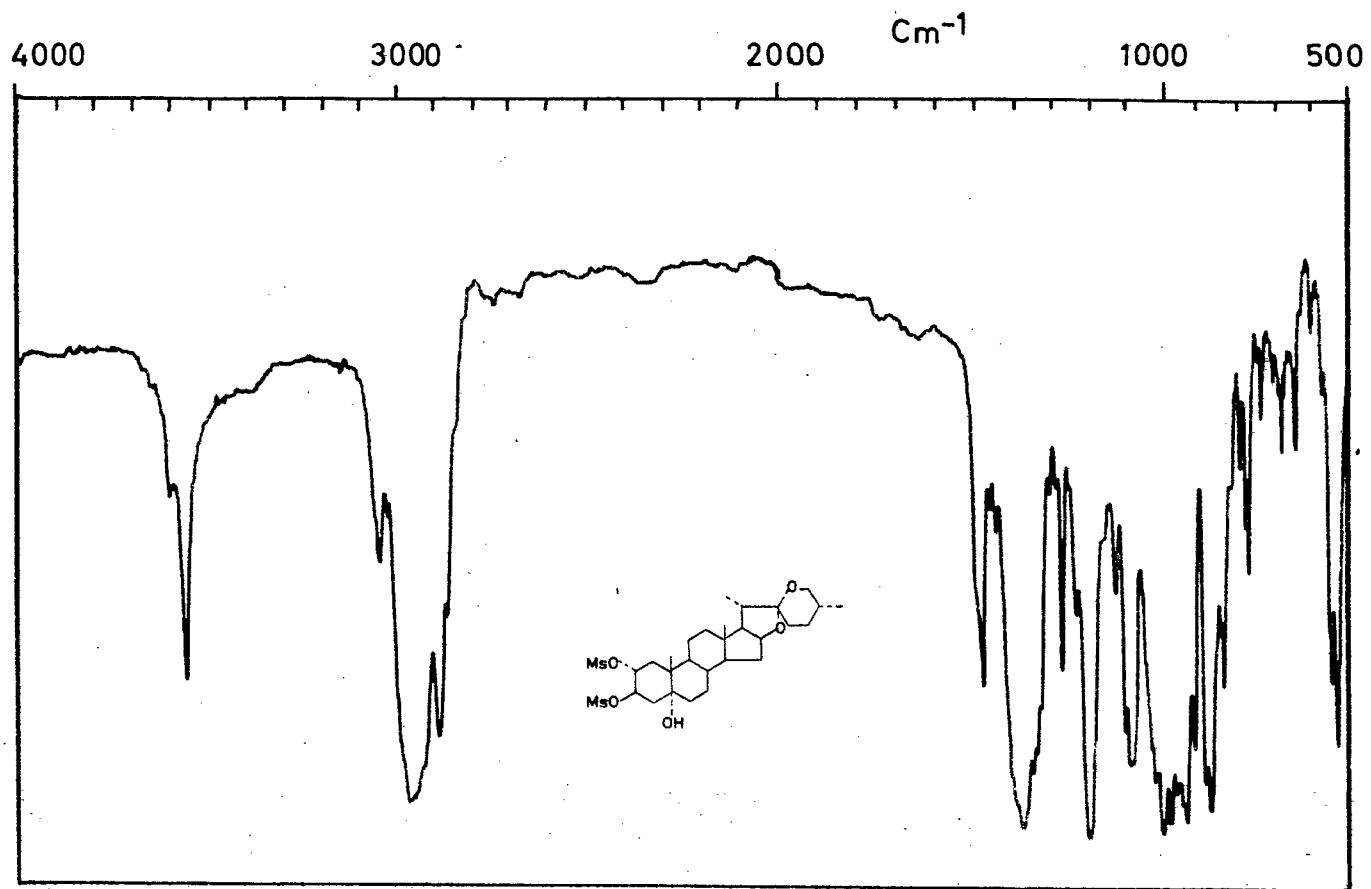


Fig.19 IR Spectrum of the dimesylate of agapanthagenin

4.3 The position and configuration of the tertiary hydroxyl group

4.3.1 Acetylation of the tertiary hydroxyl group

It has been shown by Bladon (49) that a tertiary hydroxyl group at C-5 can be acetylated only if it is in the α -configuration.

Models of the two configurations show quite plainly that the β -position would be shielded by the axial methyl group at C-10 and so would hinder the approach of the reagent and thus prevent acetylation. Although the 5α -hydroxyl group is still hindered, rear attack would be possible under vigorous conditions.

Acetylation of the tertiary hydroxyl group in agapanthagenin to give a 2,3,5-triacetoxy derivative was accomplished. This derivative showed no hydroxyl absorption in the infrared (Fig. 20) and the mass spectrum gave a peak at m/e 574 due to a molecular ion which would be in agreement with its formulation VI.

On mild hydrolysis this triacetate (VI) gave a

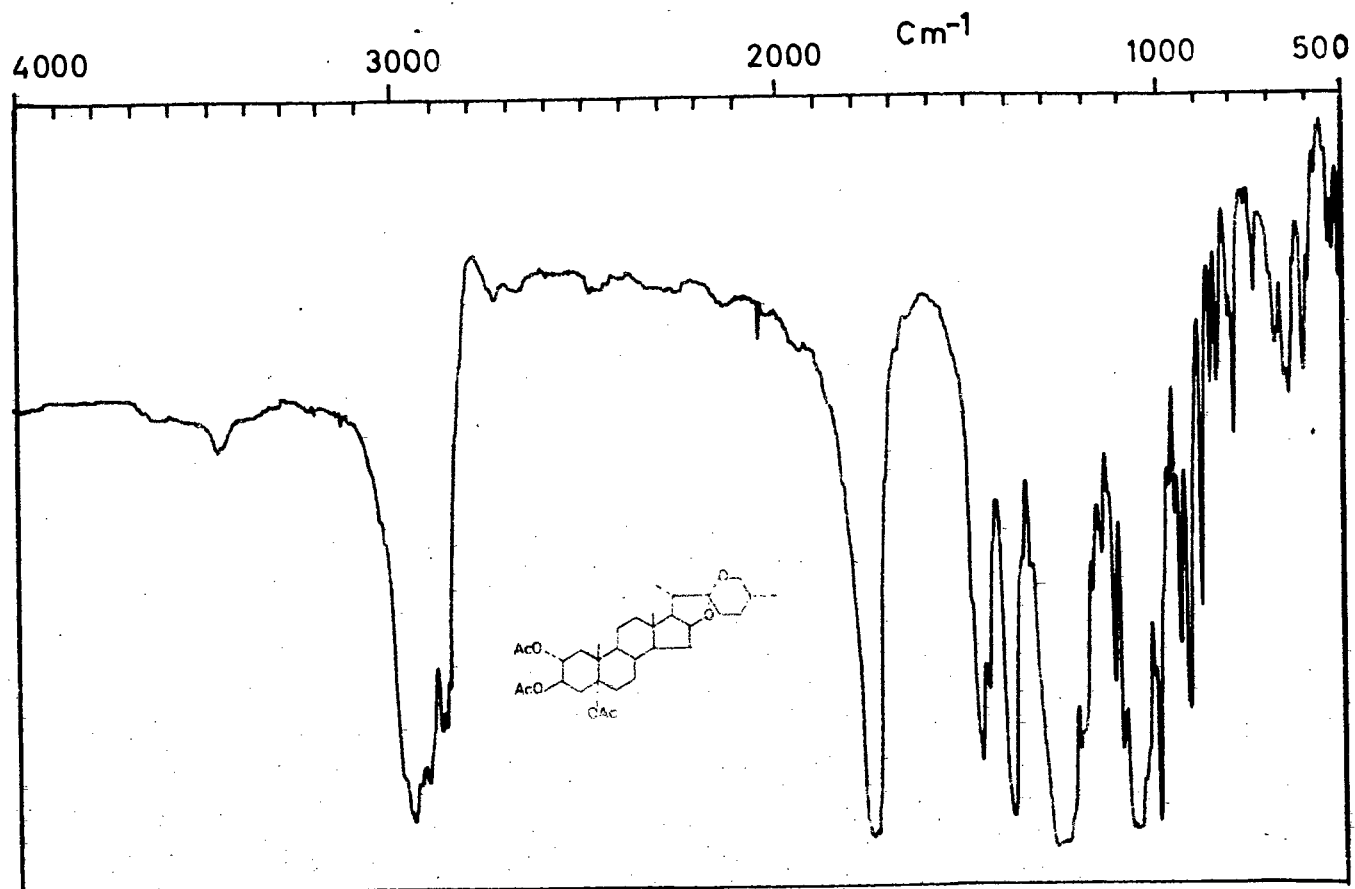


Fig.20 IR Spectrum of 2 α :3 β :5-Triacetoxy-22 α -spirostan

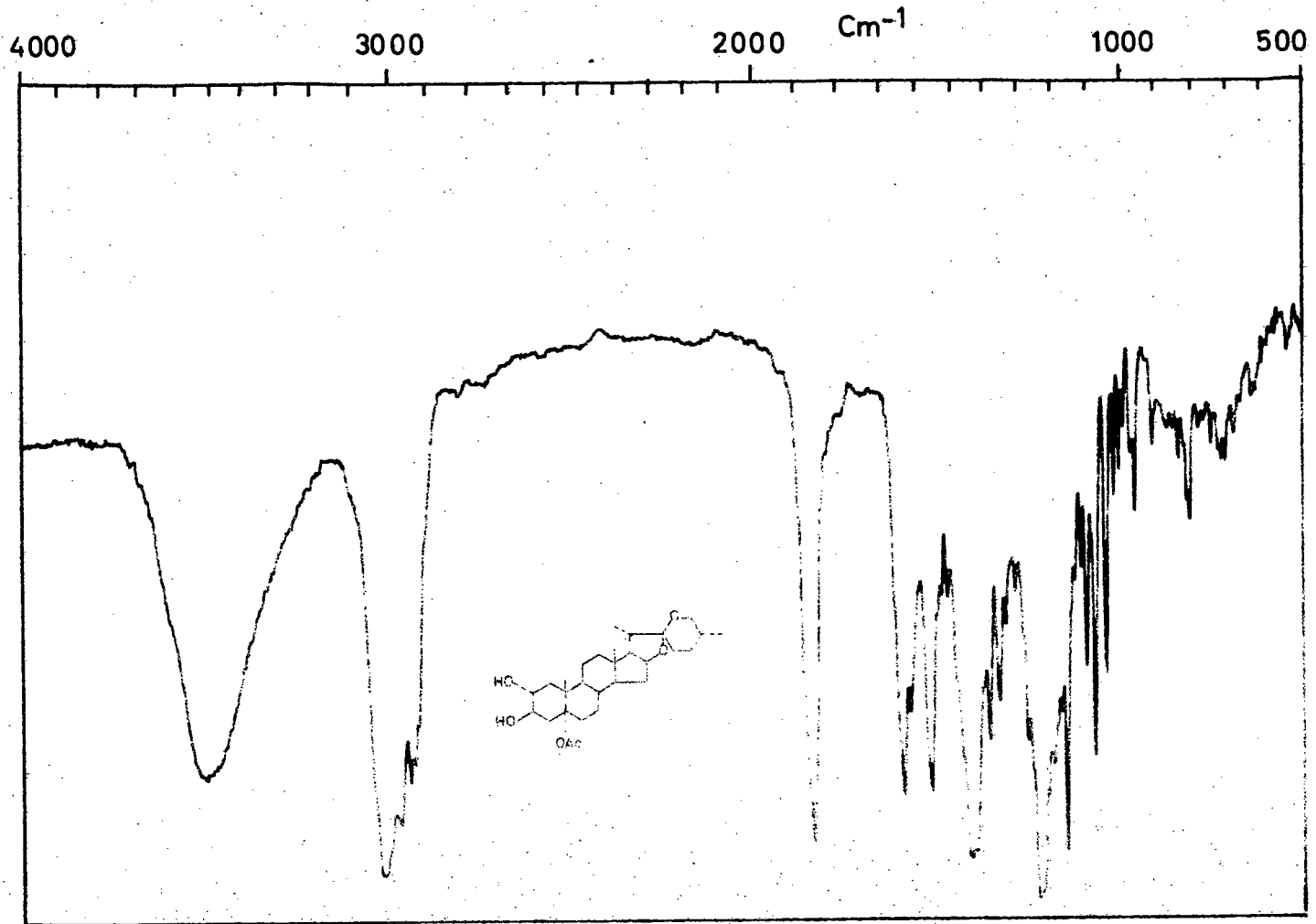


Fig.21 IR Spectrum of 22 α -spirostan-2 α ,3 β ,5 α -triol 5 α -monacetate

product which showed an absorption band at 3470 cm^{-1} due to O-H stretching vibrations of a hydroxyl group and typical bands at 1720 and 1250 cm^{-1} due to the acetate group (Fig. 21). The mass spectrum showed a peak at m/e 490 due to the molecular ion which would be in agreement with its formulation as 22a-spirostan-2 α :3 β :5 α triol-5 α -monoacetate (VII).

Thus on the basis of the finding of Bladon the tertiary hydroxyl group should have the 5 α -configuration.

4.3.2 Dehydration of the tertiary hydroxyl group

Ionic elimination of water from a cyclic alcohol in which free rotation about a single bond is not possible can be effected smoothly only if the groups to be eliminated are trans to each other. Elimination of the tertiary hydroxyl group at C-5 involves the transition state where the C-4 or the C-6 hydrogen bond enters the C-5 octet from the front (β) as the hydroxyl group departs from the rear (α) with the shared electron pair. Since the hydrogen atoms at both C-4 and C-6 are secondary there should be no particular prefe-

rence for either elimination and thus the Δ^4 and Δ^5 dehydration products should be formed in equal proportions.

The influence of substituents on the nature of the products formed by ionic elimination of the 5 α -hydroxyl group in compounds has been the subject for investigation by a few workers. Fudge (50) reported that cholestan-5-ol on dehydration with thionyl chloride in pyridine gave a mixture of cholest-4 and -5 enes in approximately equal proportions. This suggests that the hydroxyl group at position 3 has no influence on the direction of dehydration. Cholestan-3 β :5-diol and 3 α :5-diol as their monoacetates with the same reagent gave cholesteryl acetate and epicholesteryl acetate respectively unaccompanied by the Δ^4 -compound. These latter findings suggest that an acetoxy group in either the 3 β or the 3 α position directs the elimination to the Δ^5 compound. In contrast with the observations of Fudge it was found that agapanthagenin diacetate on dehydration gave rise to both the Δ^4 and Δ^5 products. Furthermore the Δ^4 product was the predominant product if not more than 2 moles of thionyl chloride was used at 0°, whereas at 10° in the presence of a large excess of thionyl

chloride the proportion of the Δ^5 product increased but the overall yield of Δ^4 and Δ^5 isomers was reduced by the formation of a high percentage of decomposition products.

Partial dehydration of agapanthagenin was also effected by treatment with 13% alcoholic potassium hydroxide at 20° to give a mixture of the Δ^5 dehydration product and unchanged agapanthagenin.

4.3.3 Epoxidation of the dehydration products and their reduction with lithium aluminium hydride

Epoxide formation is stereospecific and in cyclic olefines the approach of the peracid is predominantly from the less hindered side. In the 5α -spirostanes the β -face is shielded by the two angular methyl groups and the spiroketal E, F rings, whereas the α -face, or rear side, is relatively flat. These spacial differences account for a general tendency for perphthalic acid to attack from the rear to produce an α -epoxide. It has been confirmed by Plattner (51) that 3-acetoxy cholesterol on oxidation with peracids gives a $5\alpha:6\alpha$ epoxide. Heilbron (52) also

showed that cholest-4-ene on epoxidation gave the 4 α :5 α epoxide. Therefore by analogy it would be consistent to assume that the products formed by the epoxidation of the Δ^4 and Δ^5 derivatives of agapanthagenin should give the corresponding α -epoxides.

Epoxides on treatment with lithium aluminium hydride are cleaved with the formation of the corresponding alcohol. Attack by the nucleophilic AlH_4^- anion is considered to be the chief driving force in the opening of the epoxide ring. Attack of the nucleophile would take place on the less hindered carbon atom followed by inversion at this site of attack with the formation of a trans diaxial reaction product. Models show that the hydride ion would preferably attack at C-4 in the 4 α :5 α epoxides and at C-6 in the 5 α :6 α epoxides and in each case should lead to the 5 α alcohol.

The sequence of these reactions is shown in Chart 5.

On the basis of these stereospecific reactions the dehydration products of a 5 β -hydroxy compound would form β -epoxides which on reduction would under-

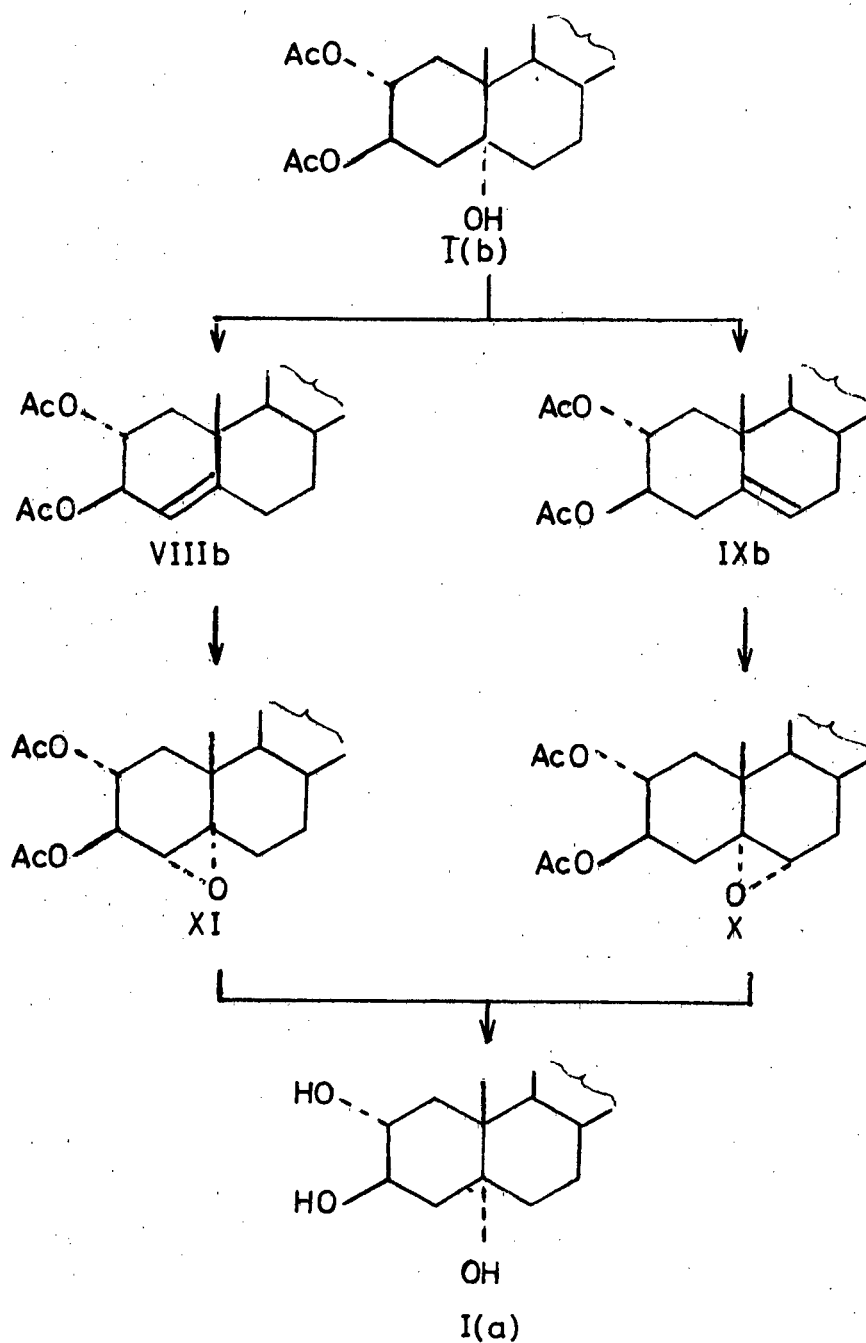


CHART 5 The reaction products derived from agapanthagenin

go diaxial fission to give a mixture of the 5β - and the 6β -hydroxy derivatives. Dehydration of a 5α -hydroxy compound on the other hand would form predominantly α -epoxides which on reduction by diaxial fission should give the same 5α -hydroxyl reduction product.

It was found that the Δ^5 diacetate of agapanthagenin (IXb) with monopero-phthalic acid gave a single epoxide X which is analogous with the major product of peroxidation of cholesterol obtained by Plattner (51). Reduction of this epoxide with lithium aluminium hydride yielded agapanthagenin (Ia) and a trace of an unidentified product. The Δ^4 -diacetate (VIIIb) on epoxidation also yielded a single epoxide and in conformity with the investigations of Roberts (53) can be considered an α -epoxide (XI). Reduction of this epoxide with lithium aluminium hydride also yielded agapanthagenin as the only product.

Reduction of these two epoxides to agapanthagenin conclusively establishes the position and configuration of the tertiary hydroxyl as 5α .

5. The mass spectra of agapanthagenin and its derivatives

The presence of the spiroketal system in steroidal sapogenins, should strongly direct fragmentation of the sapogenin nucleus. Budzikiewicz and co-workers (37) postulated that electron attack on the spiroketal ring system will result in cleavage of the $C_{22} - C_{23}$ or $C_{22} - O$ bonds to provide transitions c, d, e, and f (Chart 6). This theory is strongly supported by the fact that the most abundant fragments in the mass spectra of agapanthagenin and its derivatives can be formulated as decomposition products of these transition ions formed by further α -cleavage or fission of a carbon-oxygen bond to give the ions g, h, i, j, k, l, m, and n (Chart 7). The fragments m and n, in particular, are characteristic fragments of ring F and can be used for the identification of steroidal sapogenins.

The mass spectra of gitogenin, a $2\alpha:3\beta$ -dihydroxy sapogenin of known structure and its diacetate have been included to facilitate the interpretation of the fragmentation patterns of agapanthagenin and its derivatives.

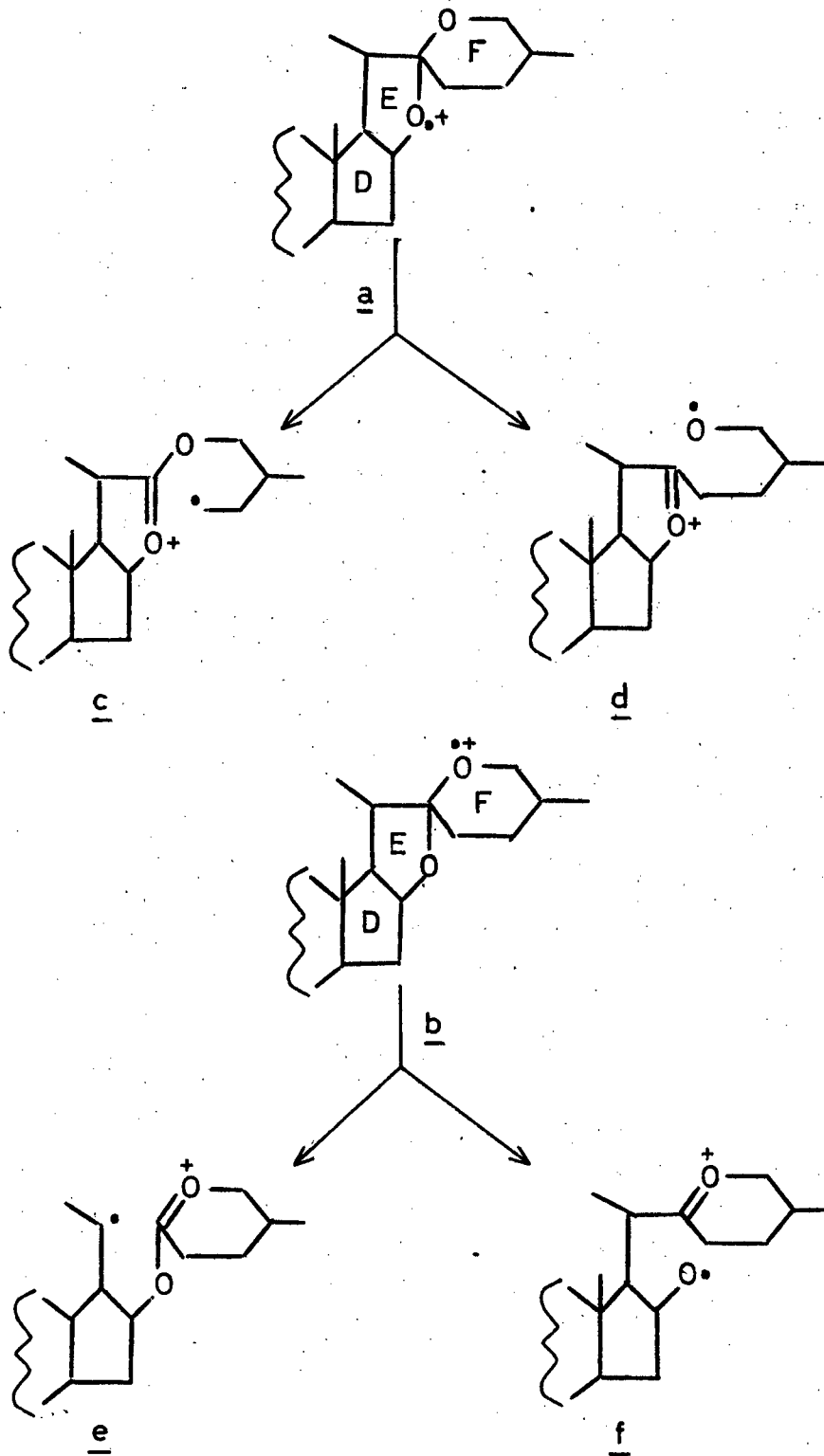


Chart 6 Primary fragmentation of the spiroketal system
in steroidal sapogenins

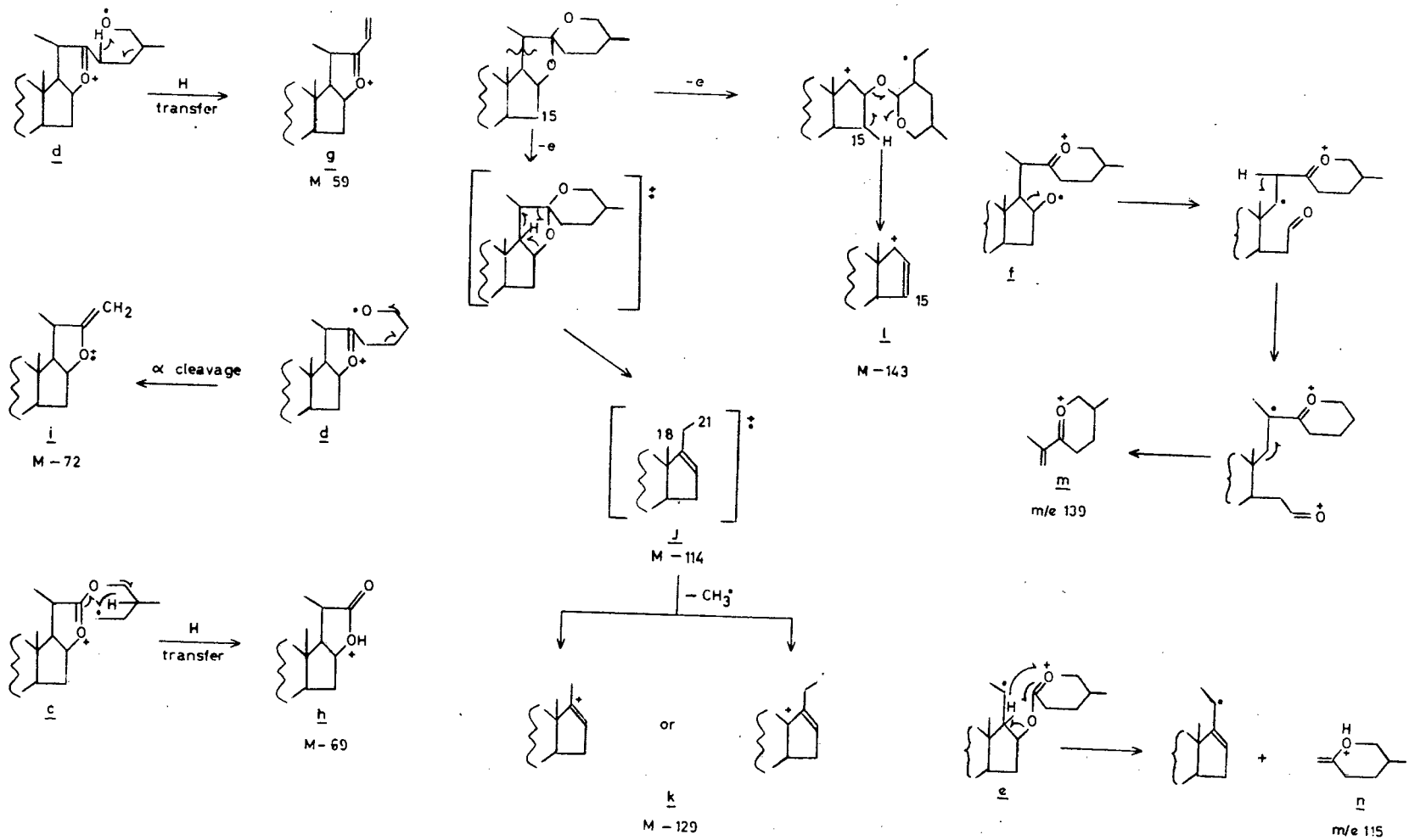


Chart 7 Secondary fragmentation of the spiroketal system in steroidal sapogenins

Table 11. The most abundant ions in the mass spectra of sapogenins

Compound	Ions m/e												
	g	h	i	j	k	l	m	n	o	p	q	r	y
gitogenin	373	363	360	318	303	289	139	115	-	-	-	-	-
agapanthagenin	389	379	376	334	319	305	139	115	316	301	287	269	280
yuccagenin	371	361	358	316	301	287	139	115	298	283	269	-	-
5 α -hydroxy-22a-spirost-2-en (XIII)	355	345	342	300	285	271	139	115	282	267	253	-	-

The positions of the peaks due to the most abundant ions in the spectra of sapogenins and their acetates are shown in Tables 11 and 12.

5.1 The mass spectra of gitogenin and its diacetate

A peak in the mass spectrum of gitogenin (Fig. 22) at m/e 432 was due to the molecular ion M. The base peak was due to the fragment m from ring F and the abundance of peaks at g, h, i, j and n were all due to fragments formed by a number of closely related fragmentations of the spiroketal system (Charts 6 and 7). As can be seen from the fragmentation pattern, the spiroketal system localises the charge and undergoes fragmentation. The steroidal ring system appeared to be stable and did not undergo eliminations or fragmentations. The mass spectrum of gitogenin diacetate (Fig. 23) showed a similar fragmentation pattern to that of gitogenin except that the presence of peaks due to ions m/e 342 and 282 indicated that an acetoxy group can be more easily eliminated than a hydroxyl group.

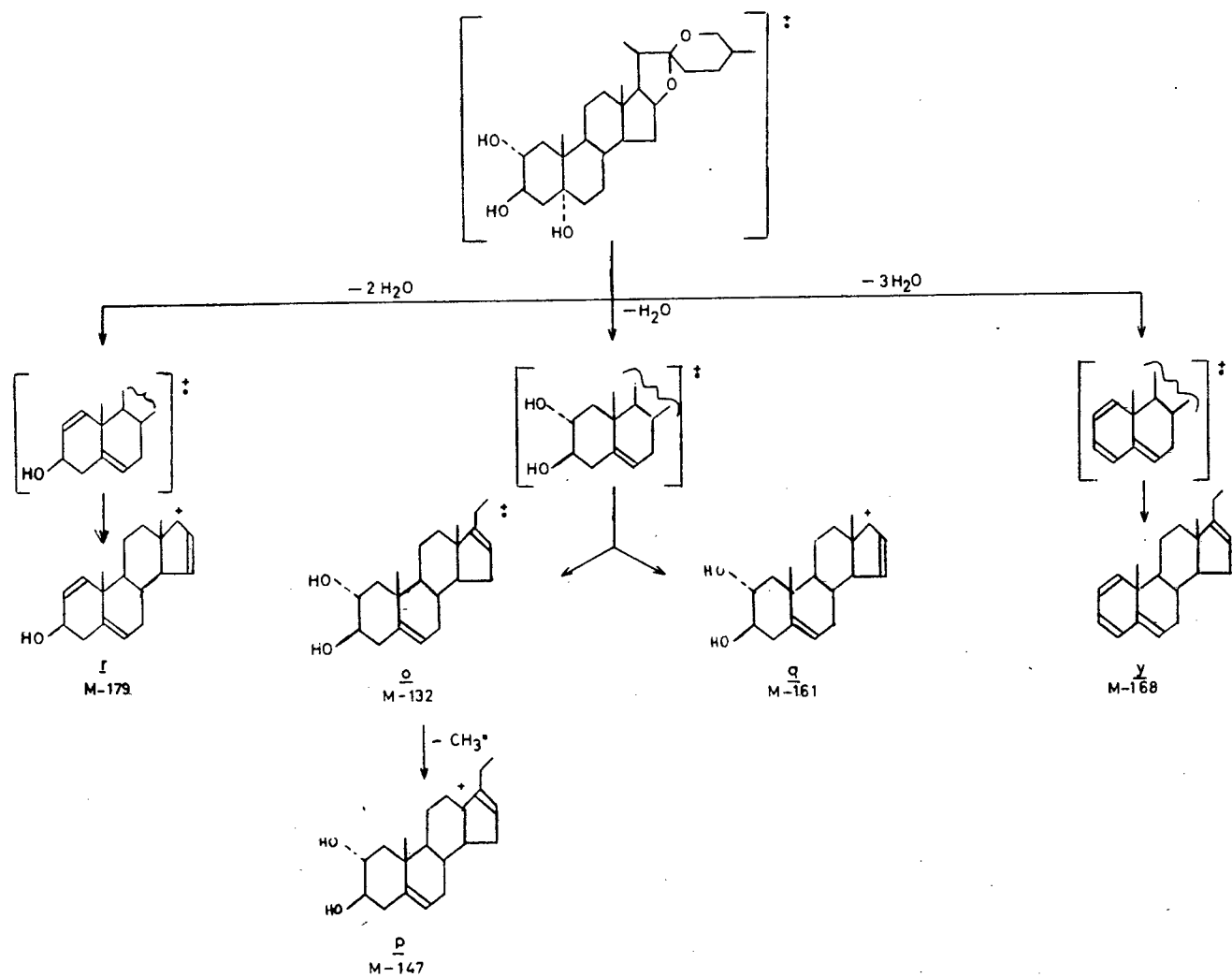


Chart 8 Fragments of the mass spectrum of agapanthagenin

5.2 The mass spectrum of agapanthagenin

The mass spectrum (Fig. 24) showed a peak due to the molecular ion at m/e 448 which is 16 m.u. higher than that in the spectrum of gitogenin and would account for the third hydroxyl group in this compound. The base peak was due to ion m (m/e 139) which confirmed the presence of ring F of the spiroketal system. A feature of the spectrum was the presence of additional peaks at o, p, q and y of high intensity. These ions can be considered as being formed from the molecular ion by the elimination of a molecule of water followed by fragmentation of the spiroketal system. The abundance of ion o ($M-132$, 40%) indicated the ease with which the tertiary hydroxyl group is eliminated. The absence of this ion in the spectrum of gitogenin suggests that it can act as a diagnostic feature for the identification of easily dehydrated tertiary hydroxyl groups in steroidal sapogenins. The course of fragmentation is outlined in Chart 8.

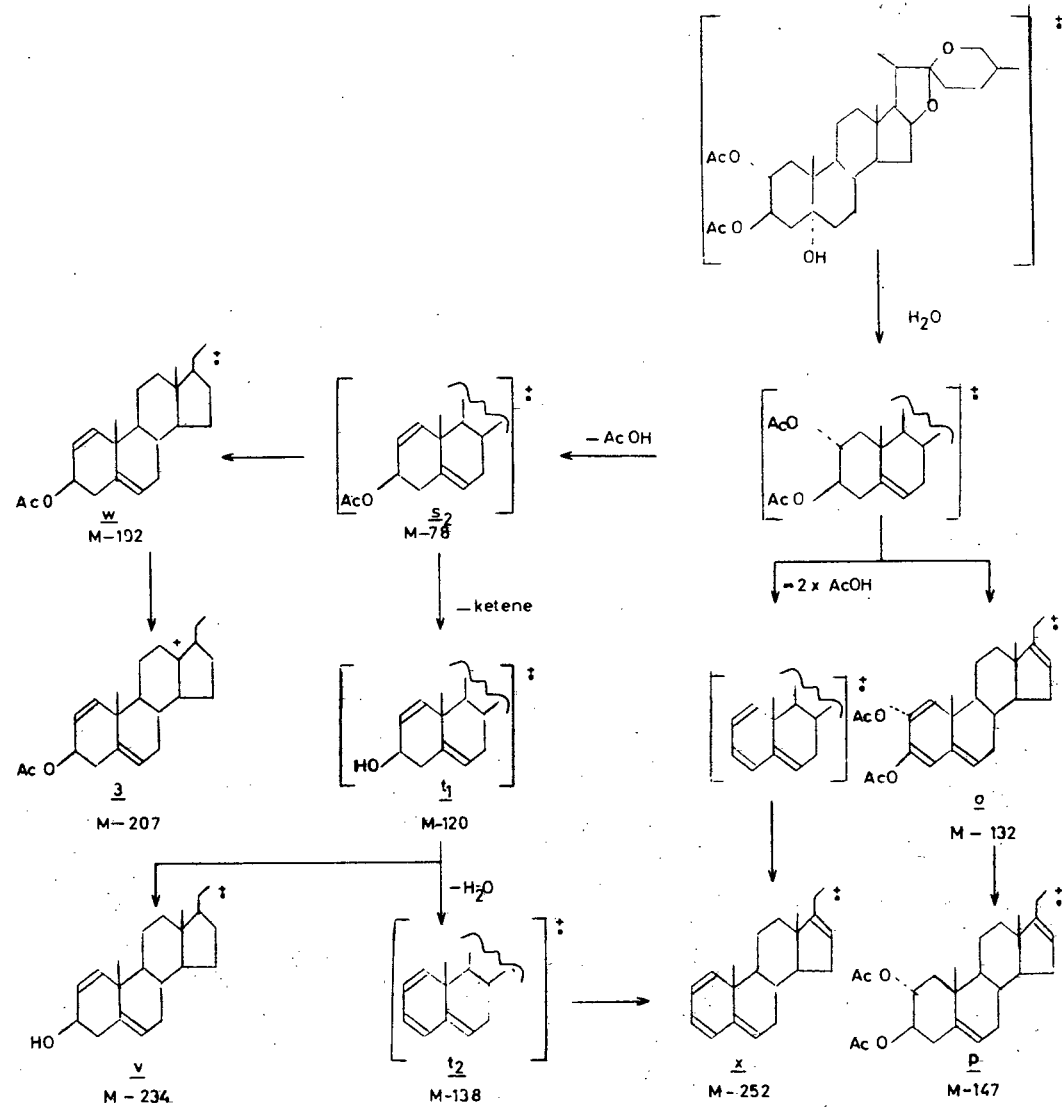


Chart9 Fragments of the mass spectrum of agapanthagenin diacetate

5.3 The mass spectrum of agapanthagenin diacetate
(Fig. 25)

The peak at m/e 532 was due to the molecular ion and was 84 m.u. higher than the corresponding molecular ion peak in agapanthagenin indicating that the two secondary hydroxyl groups had undergone acetylation to give a 2:3-diacetoxy derivative. The origin of the major peaks due to fragmentation of the spiroketal system to give ions g, h, i, j, k, l, m and n occurred in a similar manner to that of gitogenin diacetate. The other major peaks can be considered as having arisen by the elimination of ring A substituents followed by fragmentation of the spiroketal system. A possible explanation for the ion t (m/e 412) is that it was formed (from ion s₂) by the elimination of ketene from the C-3 acetoxy group instead of the elimination of acetic acid. It is well known that phenyl acetates preferentially lose ketene rather than acetic acid so that under the influence of the Δ^1 and Δ^5 double bonds the C-3 acetoxy group might be expected to behave in a similar manner. The ion o (M-132) also appeared as a peak in this spectrum. The intensity of this peak was reduced due to the formation of u (48%) which resulted from the loss of both water and

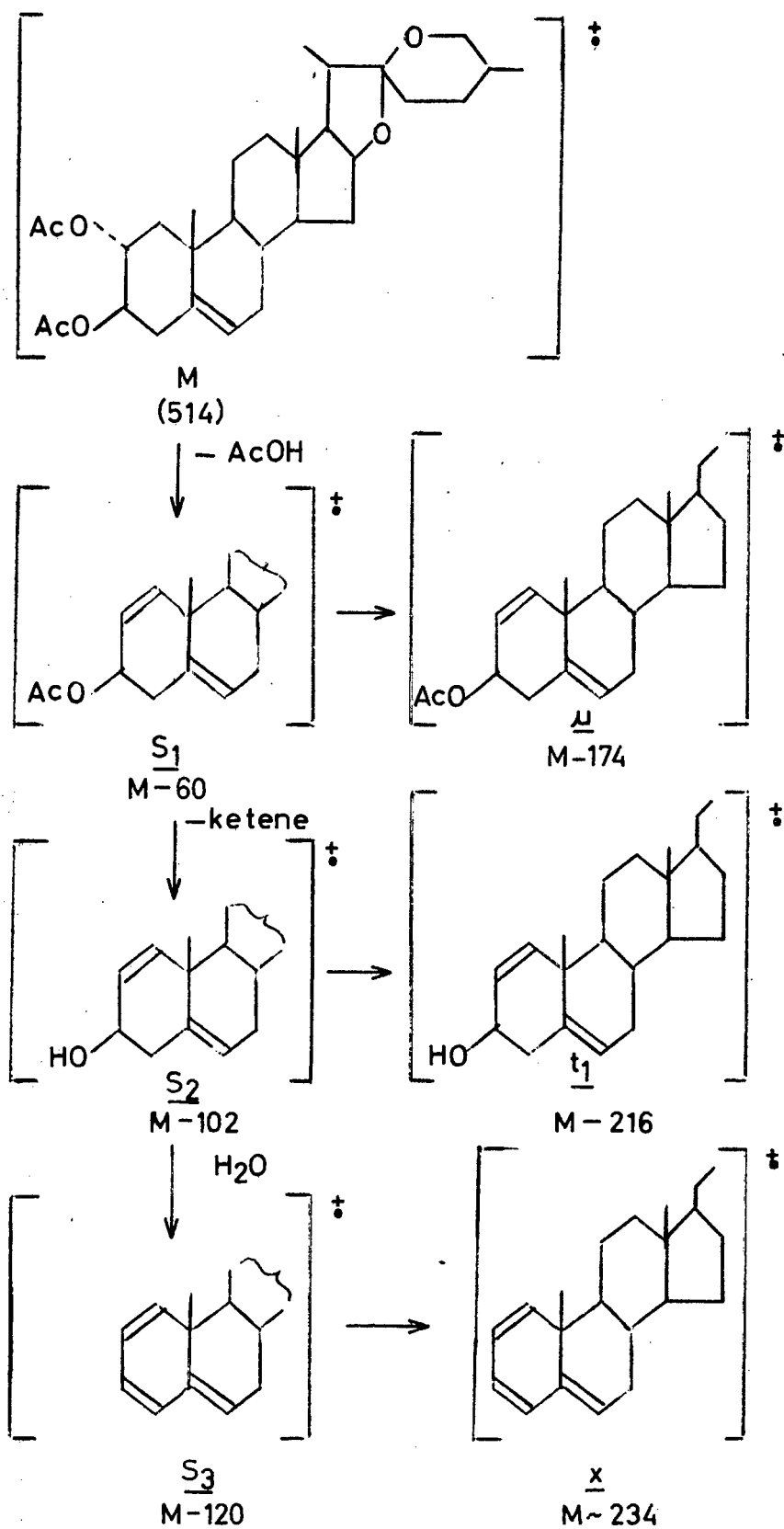


CHART 10 Fragment of mass spectrum of yuccagenin diacetate

acetic acid from the molecular ion prior to fragmentation of the spiroketal system. In this case the abundance of peaks o and u are indicative of the presence of the tertiary hydroxyl group. These fragmentations are outlined in Chart 9.

5.4 The mass spectrum of yuccagenin (IXa)

This spectrum (Fig. 26) showed a peak at m/e 430, due to the molecular ion, which is 18 m.u. less than in agapanthagenin as a result of the elimination of the tertiary hydroxyl group. This is also shown by the abundance of ions which are 18 m.u. less than corresponding ions in agapanthagenin.

5.5 The mass spectrum of yuccagenin diacetate (IXb)

Apart from the base peak (m/e 139) this spectrum (Fig. 27) showed four predominant peaks s₁ (M-60, 48%), u (M-174, 48%), x (M-234, 42%) and t₁ (M-216, 30%). A possible explanation for the formation of these ions is shown in Chart 10. Elimination of the C-2 acetoxy

group from the molecular ion as acetic acid gave ion s_1 (m/e 454) which then underwent ring E,F fragmentation to give ion u . Ketene was lost from the C-3 acetoxy group of ion s_1 (as was the case with agapanthagenin diacetate) to give ion s_2 (m/e 412) which then underwent fragmentation of the spiroketal system to give ion t_1 (m/e 298). Further loss of the C-3 hydroxyl group (as water) from ion s_2 gave ion s_3 (m/e 394) which also underwent fragmentation of the spiroketal system to give ion x (m/e 280). These fragmentations show the close relationship between the fragmentation pattern of yuccagenin diacetate and agapanthagenin diacetate suggesting similarity in the diacetate structure and configuration.

5.6 The mass spectrum of the dimesylate of agapanthagenin (V)

This spectrum (Fig. 28) gave a peak of low intensity (m/e 604, 0.5%) due to the molecular ion which is 156 m.u. more than the corresponding peak in agapanthagenin confirming the formation of a dimesylate. The concerted elimination of the two mesyl group at C-2 and C-3 together with the hydroxyl group at C-5

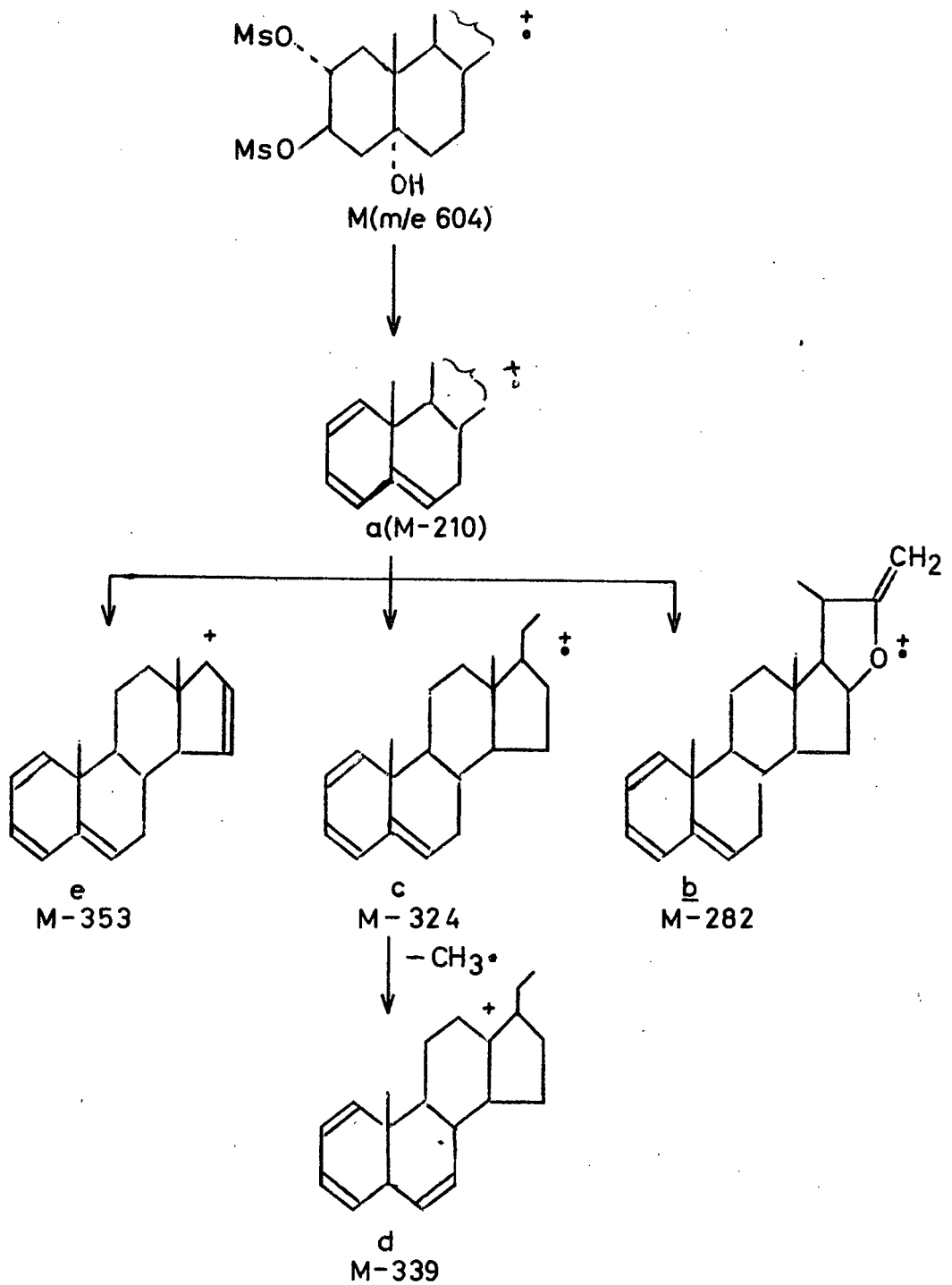


CHART 11 Fragmentation pattern of the dimesylate of agapanthagenin

gave ion a (m/e 394, 20%). This ion then underwent further fragmentations of the spiroketal system to give ion b (m/e 322, 5%), c (m/e 280, 10%), d (m/e 265, 8%) and e (m/e 251, 70%) as shown in Chart 11. The base peak f (m/e 96) was possibly due to the ion $\text{CH}_3\text{SO}_2\text{OH}^+$.

5.7 The mass spectrum of the product of solvolysis of the dimesylate of agapanthagenin

This spectrum (Fig. 29) gave a peak at m/e 414 due to the molecular ion which was 190 m.u. less than that of the dimesylate(V). This would be consistent with the elimination of two methanesulphonate ions as a result of solvolysis to yield 5 α -hydroxy-22a-spirost-2-en(XIII). The peak at m/e 396 is related to the molecular ion by a metastable peak at m/e 378.7 and could have been formed by the elimination of the C-5 hydroxyl group as water to give ion a. The peak due to ion o (m/e 282) could have been formed from ion a by fragmentation of the spiroketal system.

A metastable peak at 192 relates ion o with the molecular ion. The loss of water from the molecular

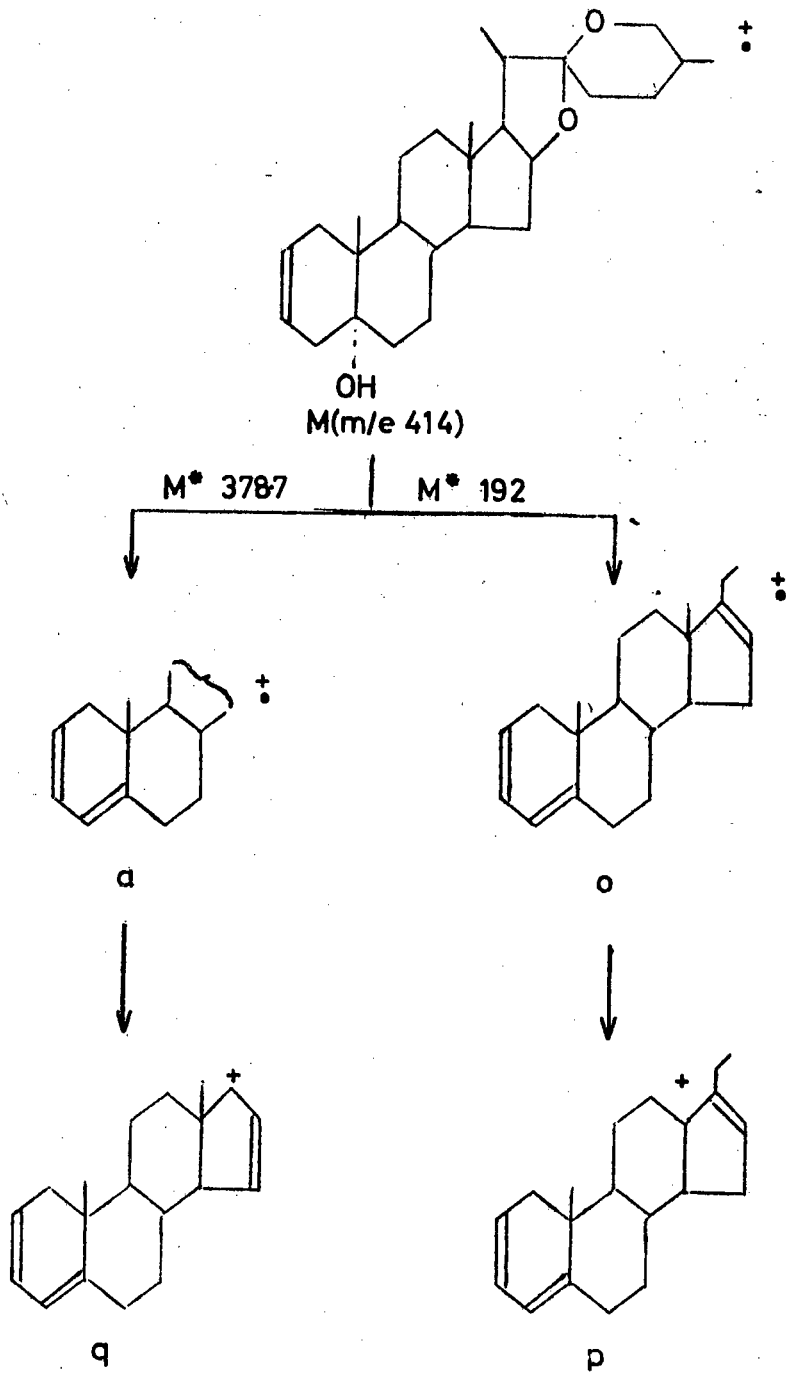


Chart 12 Fragmentation pattern of 5 α -Hydroxy-
22 α -spirost-2-en

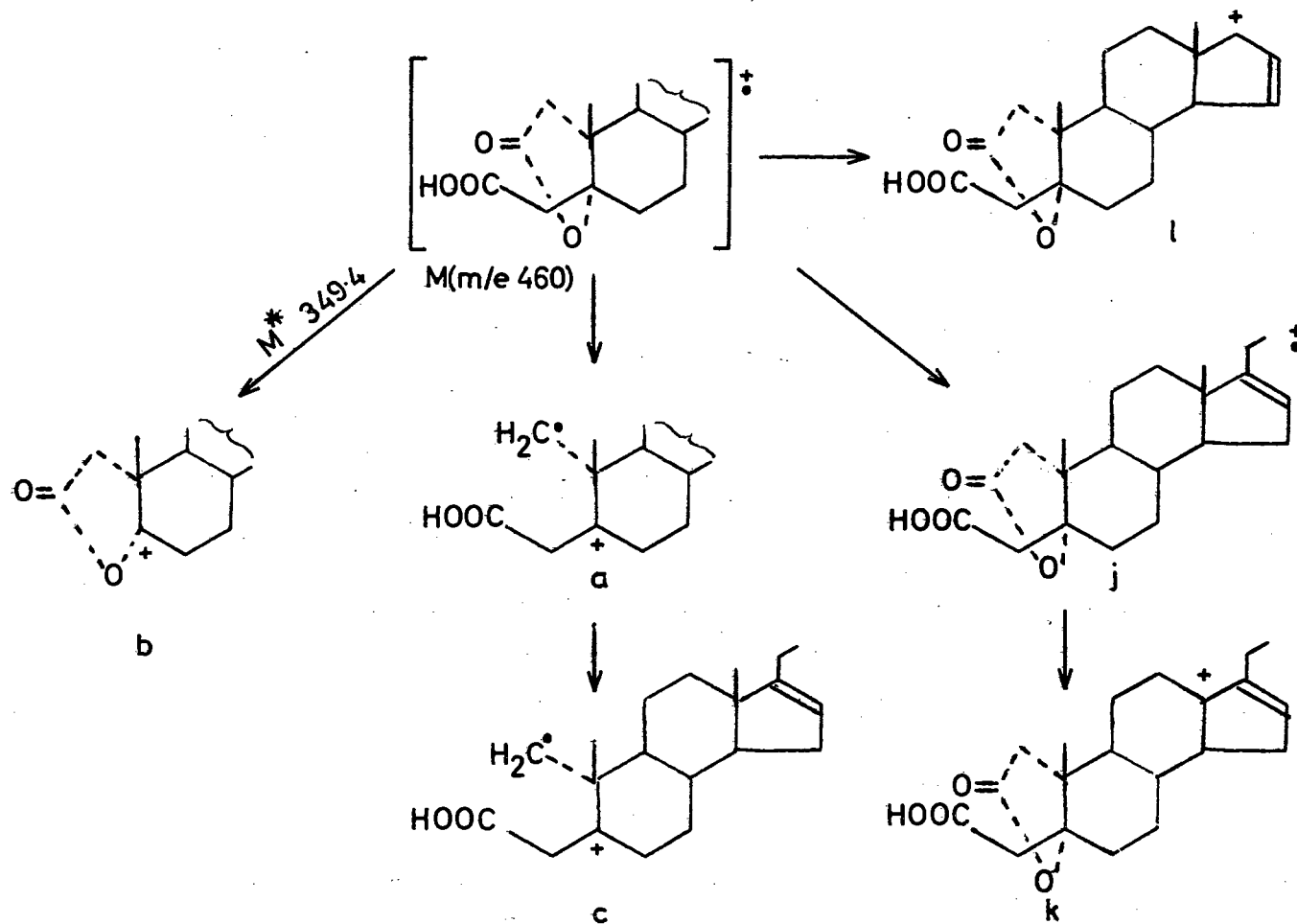


CHART 13 Fragmentation pattern of 5 α -Hydroxy-2:3-seco-22 α -spirostan-2:3-dioic lactone

ion and fragmentation of the spiroketal system to give ion o might have occurred as a concerted process. Ion p was possibly formed by the elimination of the methyl group from C-13 by allylic activation. Cleavage of the bond between C-17 and C-20 of ring E of ion a would account for the formation of ion q. The base peak was due to the usual ring F fragment (m/e 139). The fragmentation pattern is shown in Chart 12.

5.8 The mass spectrum of 5 α -Hydroxy-2:3-seco-22 α -spirostan-2:3-dioic lactone (III).

The peak at m/e 460 in this spectrum (Fig. 30) was due to the molecular ion and the base peak at m/e 139 to the typical ring F fragment. The peaks i, j, k, and l were formed from the molecular ion by the usual fragmentations of the spiroketal system. A feature of the spectrum was the appearance of peaks a (m/e 416) and b (m/e 401). Peak a was almost certainly due to the elimination of carbon dioxide from the lactone ring. The peak b at m/e 401 was related to the molecular ion by a metastable peak at m/e 394.4 and could have been formed by the loss of the

e and d. The fragmentation pattern is outlined in Chart 14 and provides evidence for the formulation of this product as a hemiacetal (XII) (Fig. 11).

5.10 The mass spectra of agapanthagenin triacetate (VI) and its hydrolysis product (VII)

A peak at m/e 574 (due to the molecular ion) in the mass spectrum (Fig. 32A) of the product obtained by the treatment of agapanthagenin diacetate with acetyl chloride in dimethylaniline was 42 m.u. higher than that in the mass spectrum of ion r (m/e 298) in a similar way agapanthagenin diacetate indicating the tertiary hydroxyl group had undergone acetylation. A peak s (m/e 514) was possibly formed by the elimination of this tertiary acetoxy group (as acetic acid) from the molecular ion. This ion then lost 114 m.u. by fragmentation of the spiroketal system to give ion u (m/e 400). The base peak, m/e 454, could have been formed by the loss of 120 m.u. due to the elimination of two acetoxy groups (as acetic acid) from the molecular ion. This ion again lost a further 114 m.u. due to ring E,F fragmentation to give ion v (m/e 340). The loss of three acetoxy groups (as acetic acid) from the molecular ion gave ion w (m/e 394).

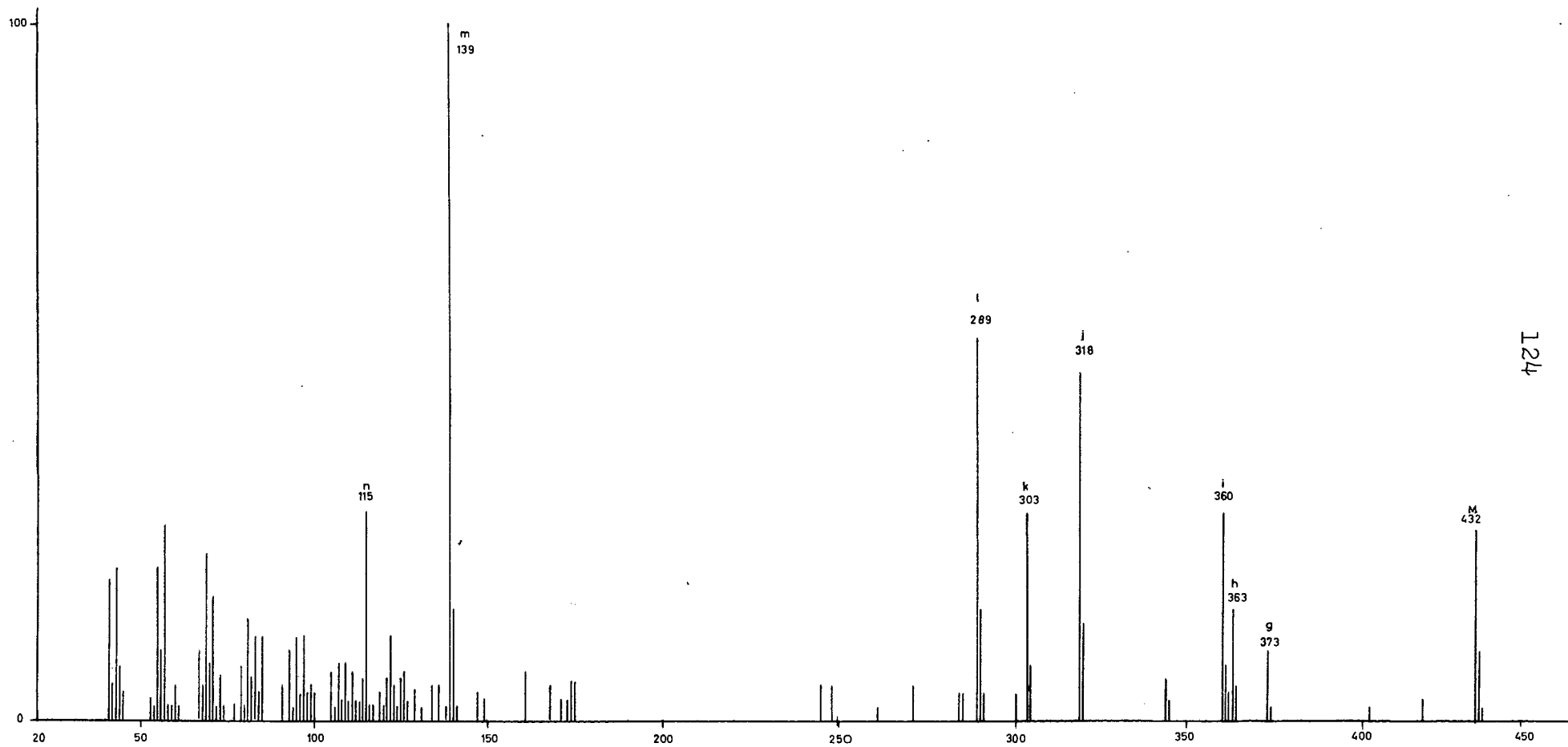


Fig. 22 Mass spectrum of gitogenin

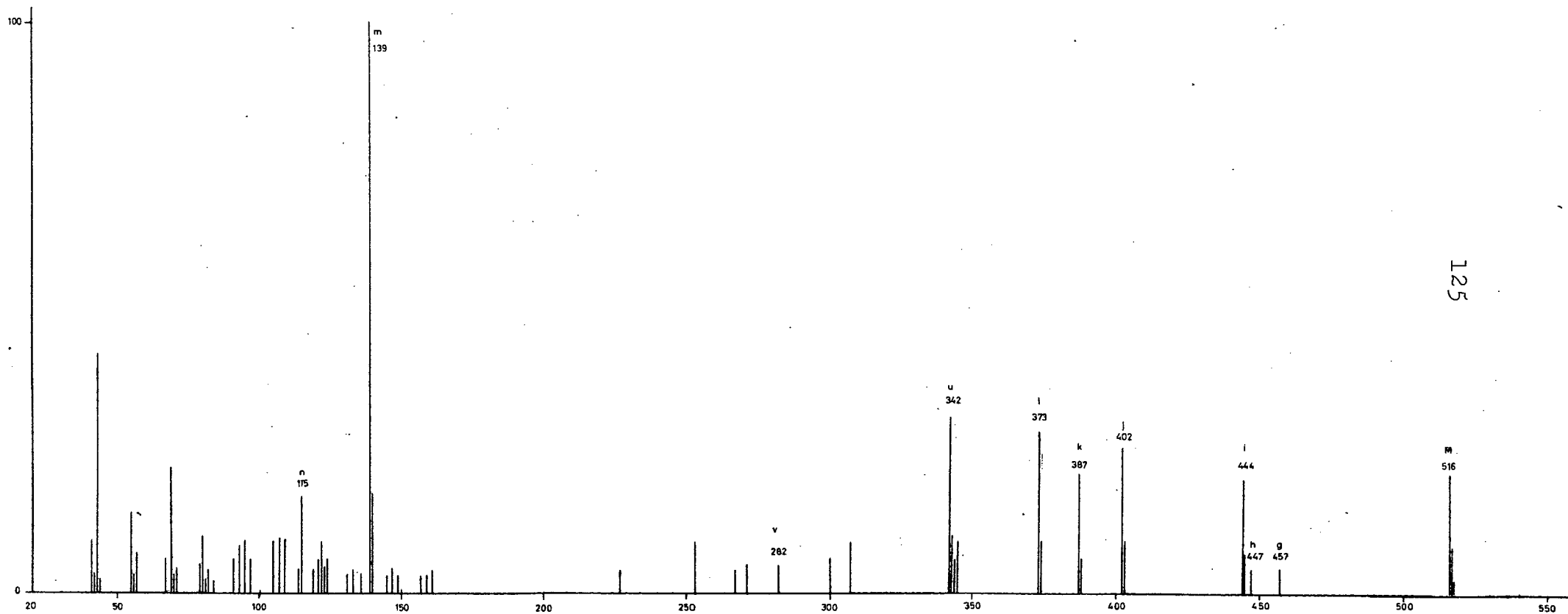


Fig. 23 Mass spectrum of gitogenin diacetate

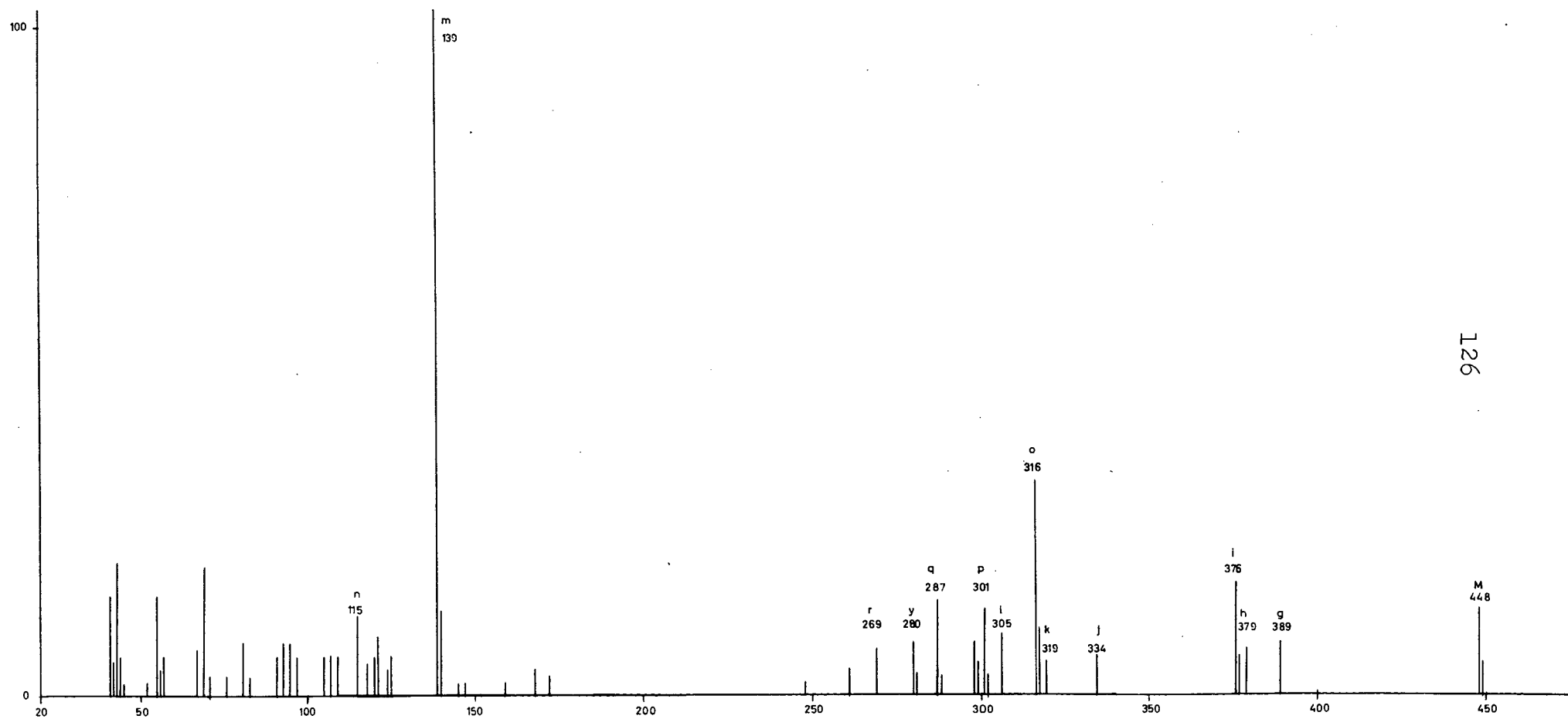


Fig. 24 Mass spectrum of agapanthagenin (22 α -Spirost-2 α :3 β :5 α -triol)

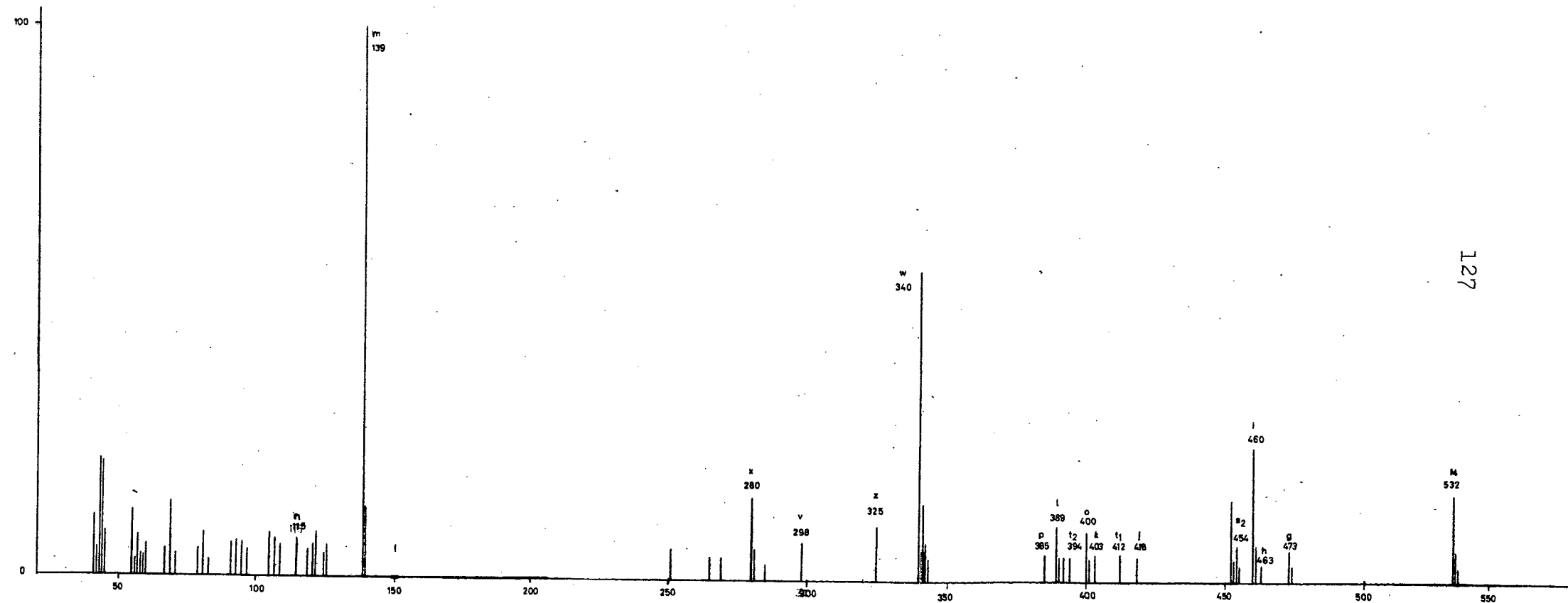


Fig.25 Mass spectrum of agapanthagenin diacetate

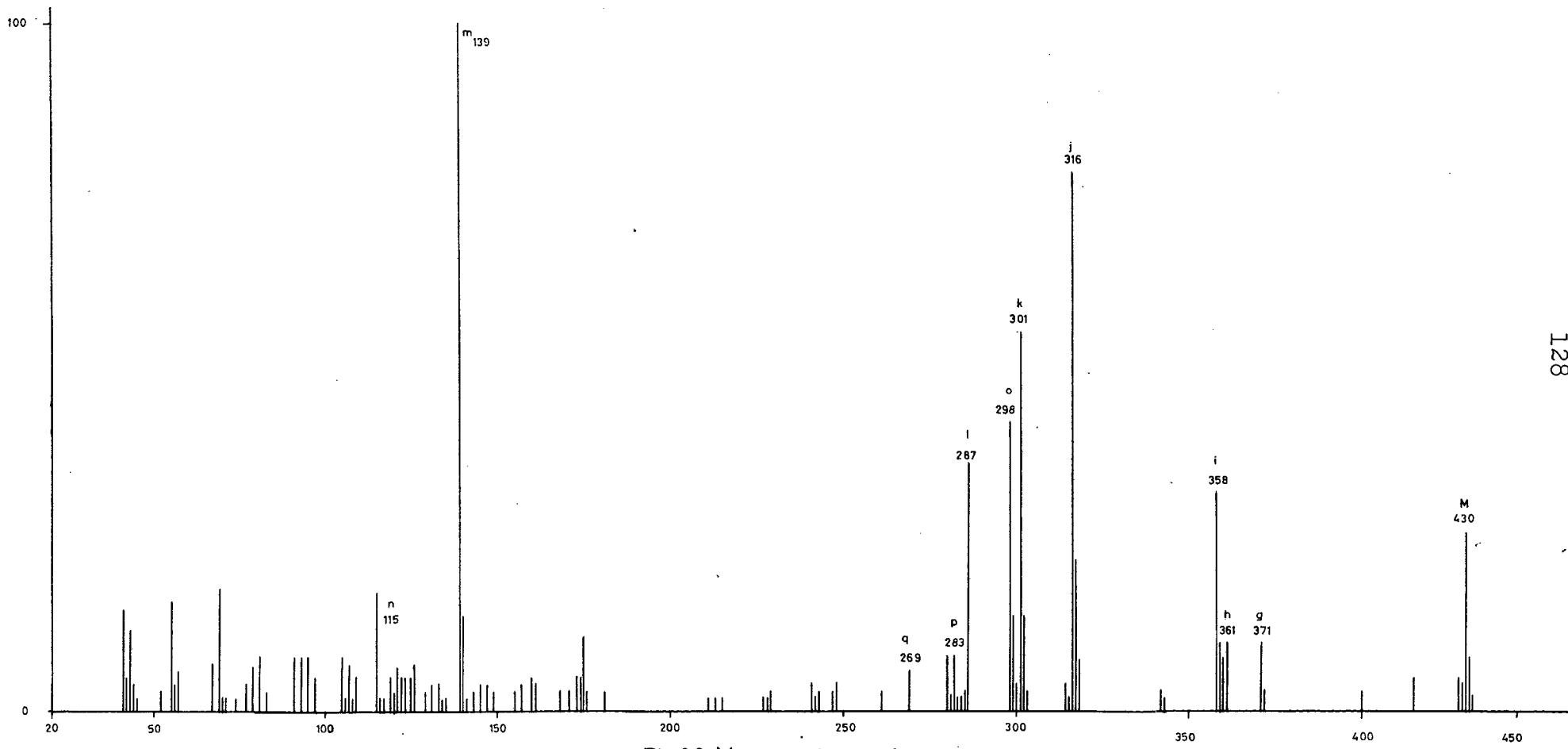


Fig.26 Mass spectrum of yuccagenin

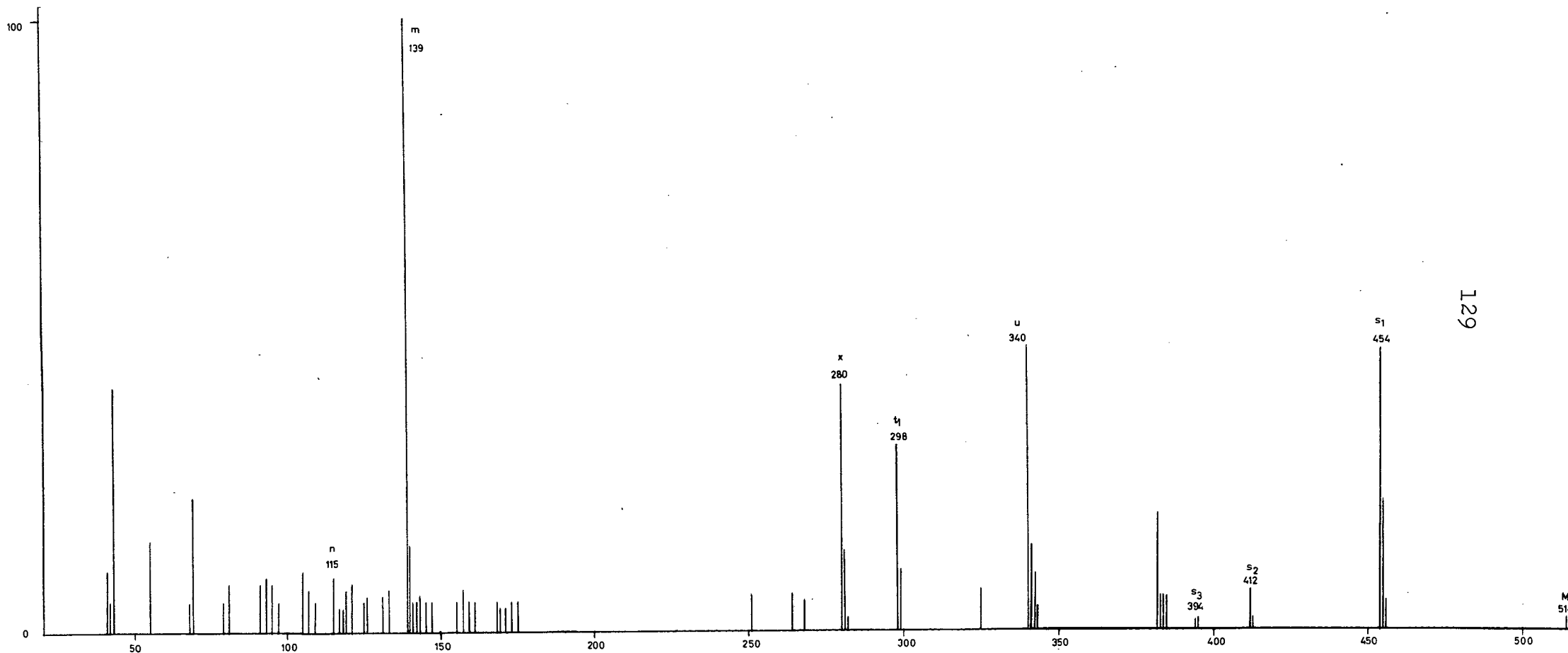


Fig.27 Mass spectrum of yuccagenin diacetate

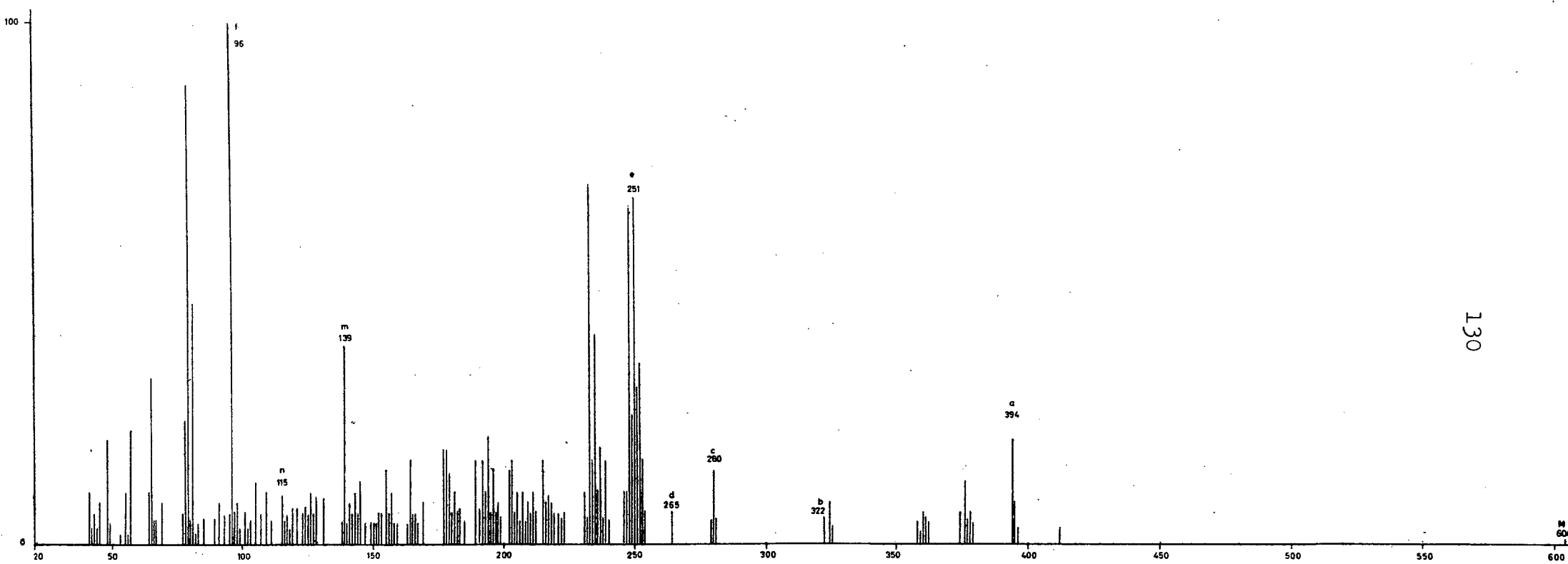


Fig.28 Mass spectrum of 2 α :3 β -dimesylate of agapanthagenin

130

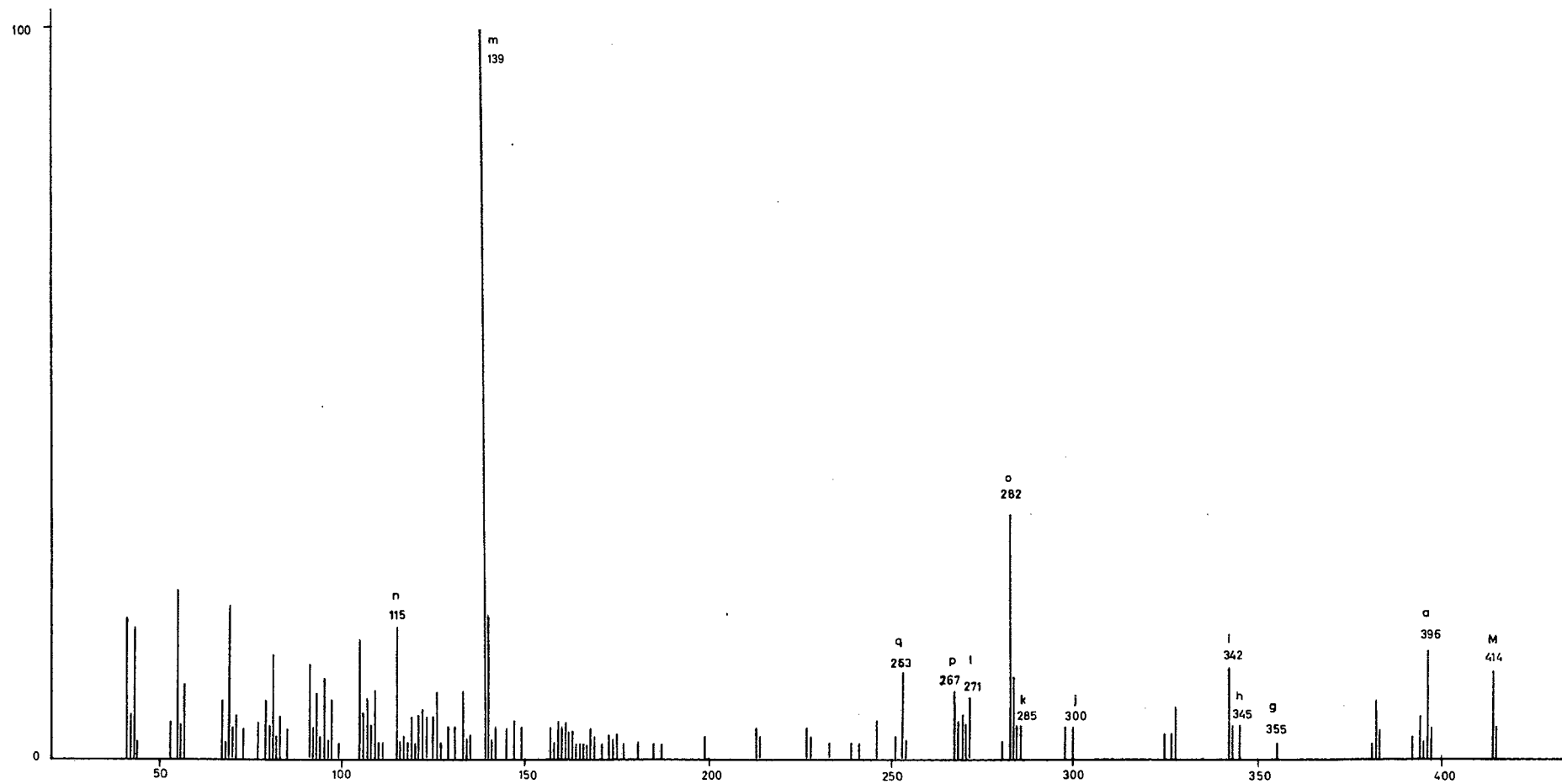


Fig.29 Mass spectrum of 5 α -Hydroxy-22 α -spirost-2-en

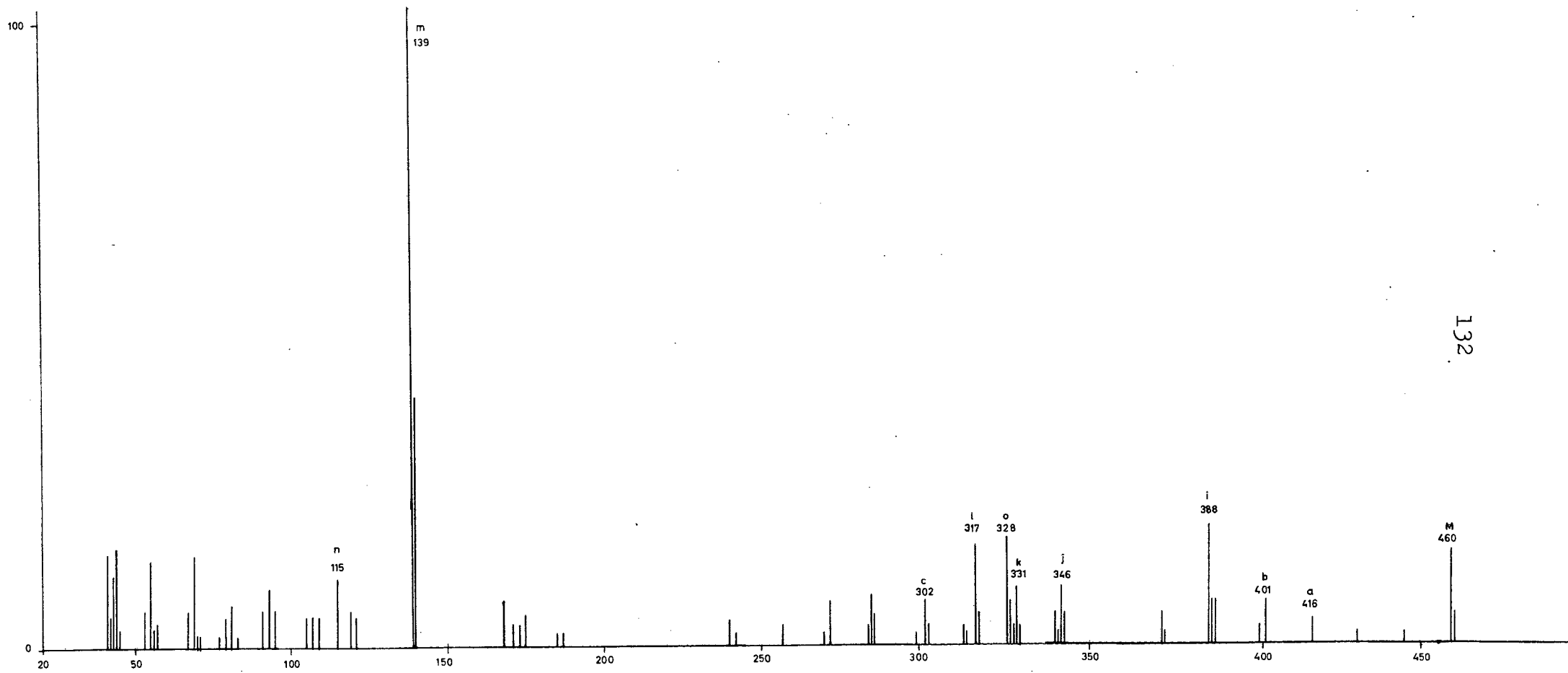


Fig.30 Mass spectrum of 5 α -Hydroxy-2:3-seco-22 α -spirostan-23-dioic lactone

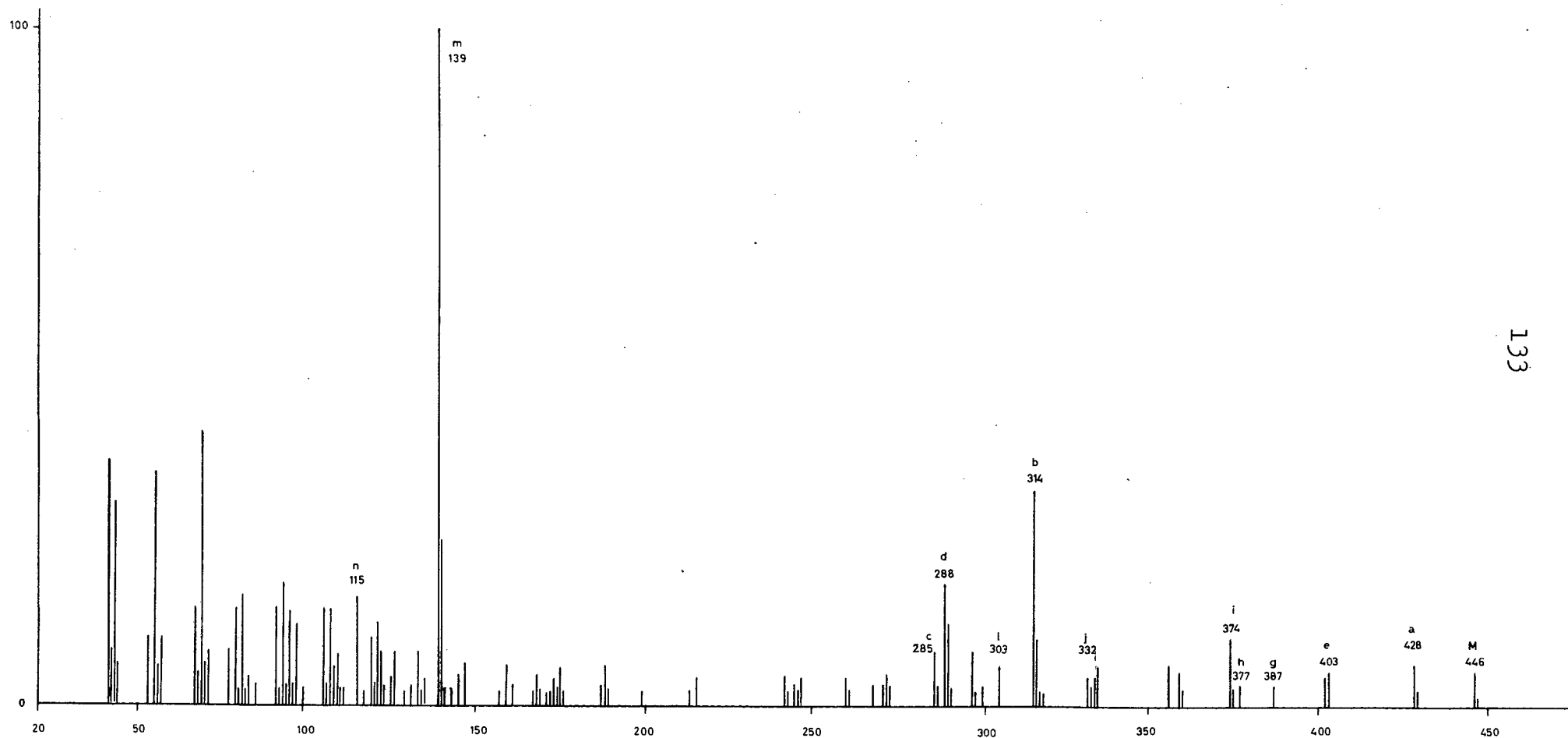


Fig. 31 Mass spectrum of oxidation product of agapanthagenin with lead tetraacetate

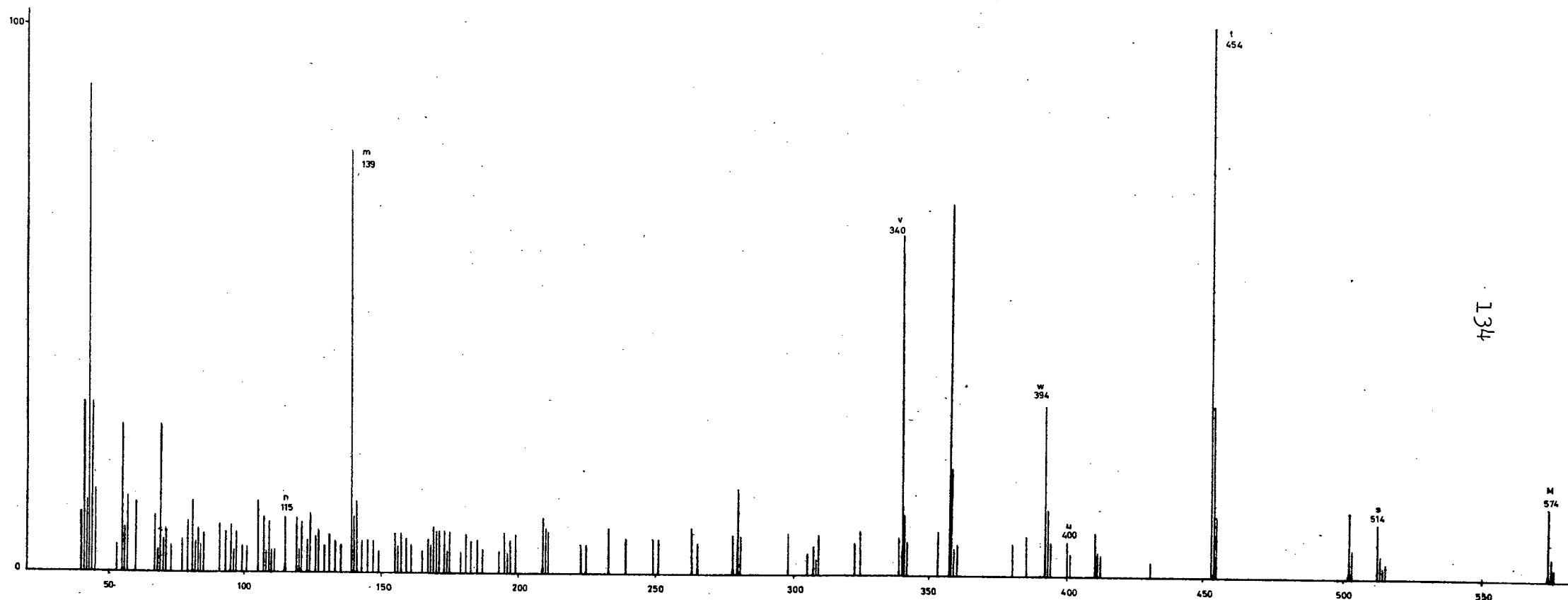


Fig. 32A Mass spectrum of 2 α :3 β :5-Triacetoxy-22 α -spirostan

134

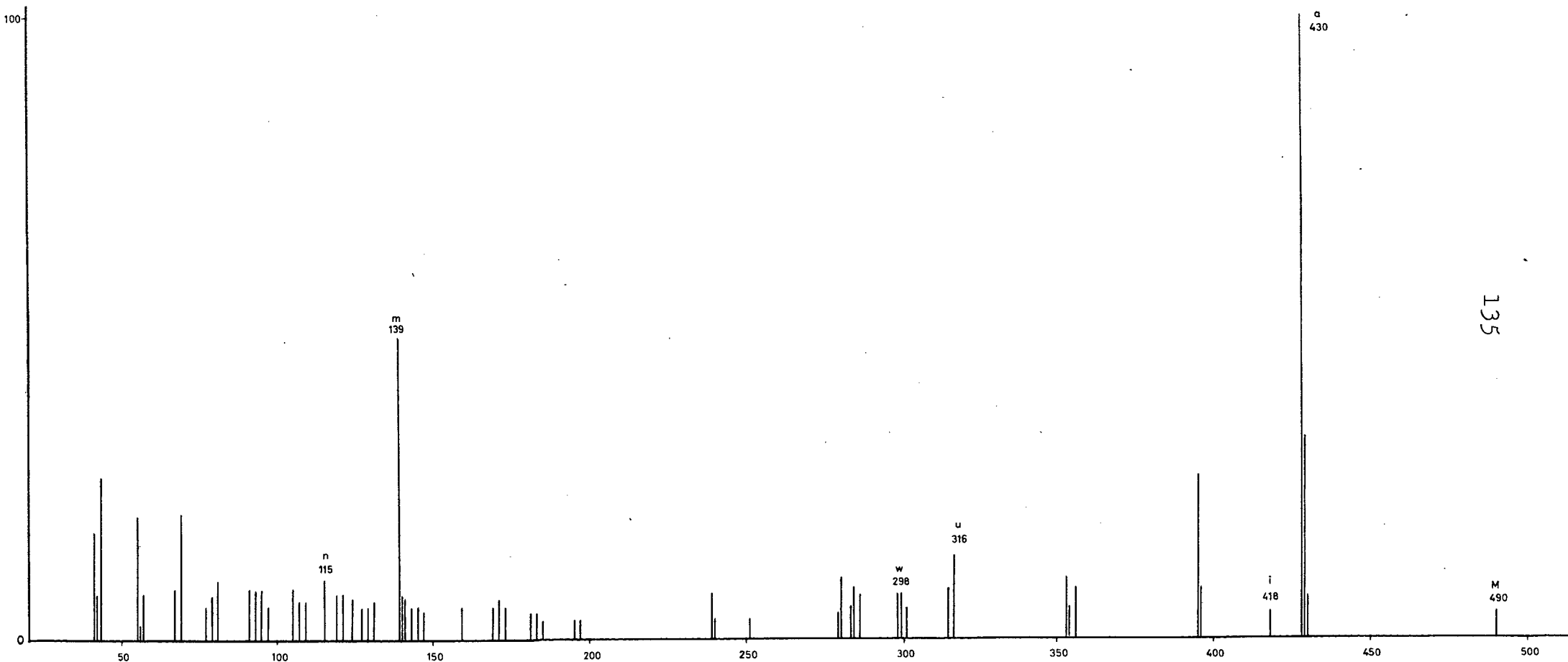


Fig 32B Mass spectrum of 22 α -Spirostan-2 α :3 β :5 α -triol5 α -monoacetate

6. Some preliminary investigations into the structure of praecoxigenin

On the basis of its active mass praecoxigenin corresponds with the formula $C_{27}H_{40}O_4$. Peaks at m/e 115 and m/e 139 in its mass spectrum due to ions resulting from fragments of ring F of the spiroketal system provide evidence for its designation as a steroidal sapogenin. This was confirmed by the presence of absorption bands in the infrared spectrum at 860, 900, 920 and 980 cm^{-1} due to the characteristic ring F vibrations of steroidal sapogenins. The intensity of the band at 900 cm^{-1} was approximately double that of the band at 920 cm^{-1} which is a characteristic of sapogenins of the iso or 25D series. This is confirmed by the signal of the two protons at C_{26} which resembled a broad multiplet centred at 3.40 p.p.m. showing two main broad peaks separated by 4.5 Hz. This pattern is very typical of spectra of sapogenins which have an equatorial methyl group at C_{25} .

Two possible structures correspond to this formulation, one a dihydroxy-diene and the other a monohydroxy-enone.

The oxygen functions

The infrared spectrum (Fig. 33) shows a strong absorption band at 3400 cm^{-1} due to the O-H stretching vibrations of a hydroxyl group. The absence of an absorption band at $1700\text{-}1720\text{ cm}^{-1}$ due to the C=O stretching vibrations of a ketone excludes the possibility of pradoxigenin being a monohydroxy-enone. The infrared spectrum of the acetylated derivative (Fig. 34) shows no absorption bands indicative of a hydroxy group.

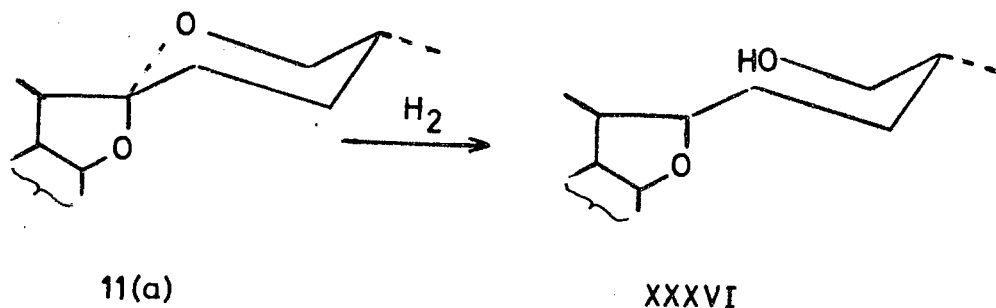
The molecular formula of the acetyl derivative, on the basis of its active mass, was $\text{C}_{31}\text{H}_{44}\text{O}_6$ which indicates that it has two acetyl groups. This is confirmed by a six proton signal in the nuclear magnetic resonance spectrum (Fig. 35) at 2.01 p.p.m. due to the acetoxy methyl groups. The signals due to the protons attached to the same carbon atoms as the acetoxy groups appeared as a superimposed multiplet at low field centred at 5.20 p.p.m. This indicates that these two protons are in similar chemical environments and are therefore either both axial or both equatorial. The six line pattern of the signal is

similar to that of agapanthagenin diacetate and would suggest that both protons are axial (59). This evidence would suggest that the two hydroxyl groups in praecoxigenin are diequatorial having a $2\alpha:3\beta$ configuration as is the case with agapanthagenin.

The double bonds

Evidence has been lead to show that praecoxigenin is a diene. There is no region of maximal absorption in the UV, therefore, these two double bonds are unconjugated. A signal at 5.08 p.p.m. in the n.m.r. spectrum is due to a single olefinic proton and suggests that one of the double bonds is trisubstituted and the other tetrasubstituted. As the fragmentation in the mass spectrum indicates the absence of olefinic centres in the spiroketal ring system, the two double bonds are therefore associated with the steroidal ring system. The position of the tetrasubstituted double bond would thus be limited to either the 8,9- or the 8,14-positions. If it were in the 8,9-position it would exclude the trisubstituted double bond from occupying the 6,7-, the 11,12-, and the 14,15-positions and if it were in the 8,14-position it would exclude

the trisubstituted double bond from occupying the 6,7-position. This leaves the possible positions for the trisubstituted double bond as 4,5- or 5,6-. On catalytic hydrogenation with platinum in acetic acid one molecule of hydrogen was consumed. In the A/B-trans series, double bonds at the 7,8-, 8,9-, and 8,14-positions are not hydrogenable. It was, therefore, expected that hydrogenation of the trisubstituted double bond had taken place. The four typical absorption bands indicative of ring F were absent in infrared spectrum (Fig. 36) and the mass spectrum (Fig. 39) showed no characteristic peaks at m/e 115 and m/e 139 due to fragmentation of ring F. It was therefore suspected that hydrogenolysis of praecoxigenin (IIa) had taken place with the opening of ring F to give the dehydro derivative (XXXVI).



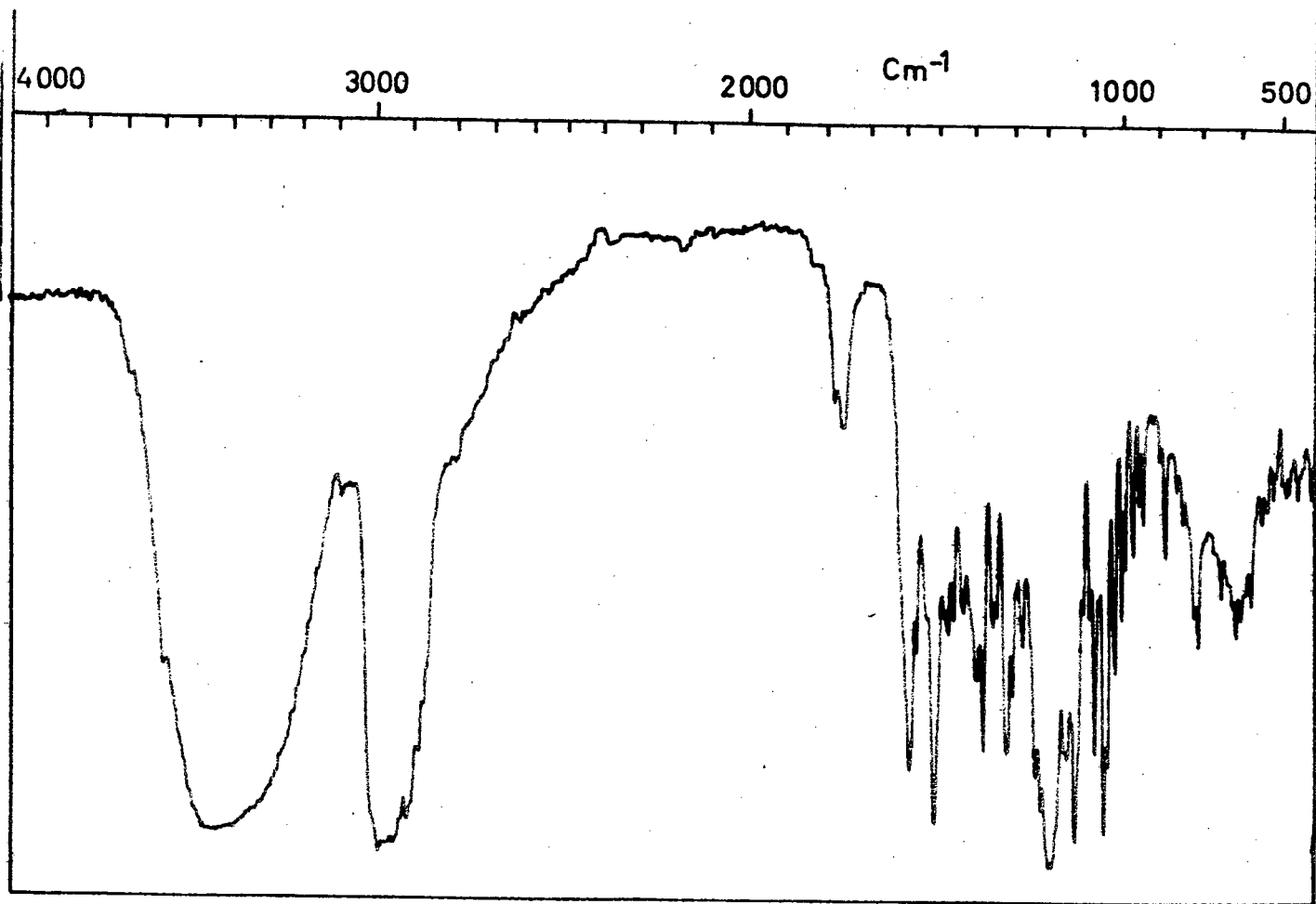


Fig. 33 I.R. spectrum of praecoxigenin

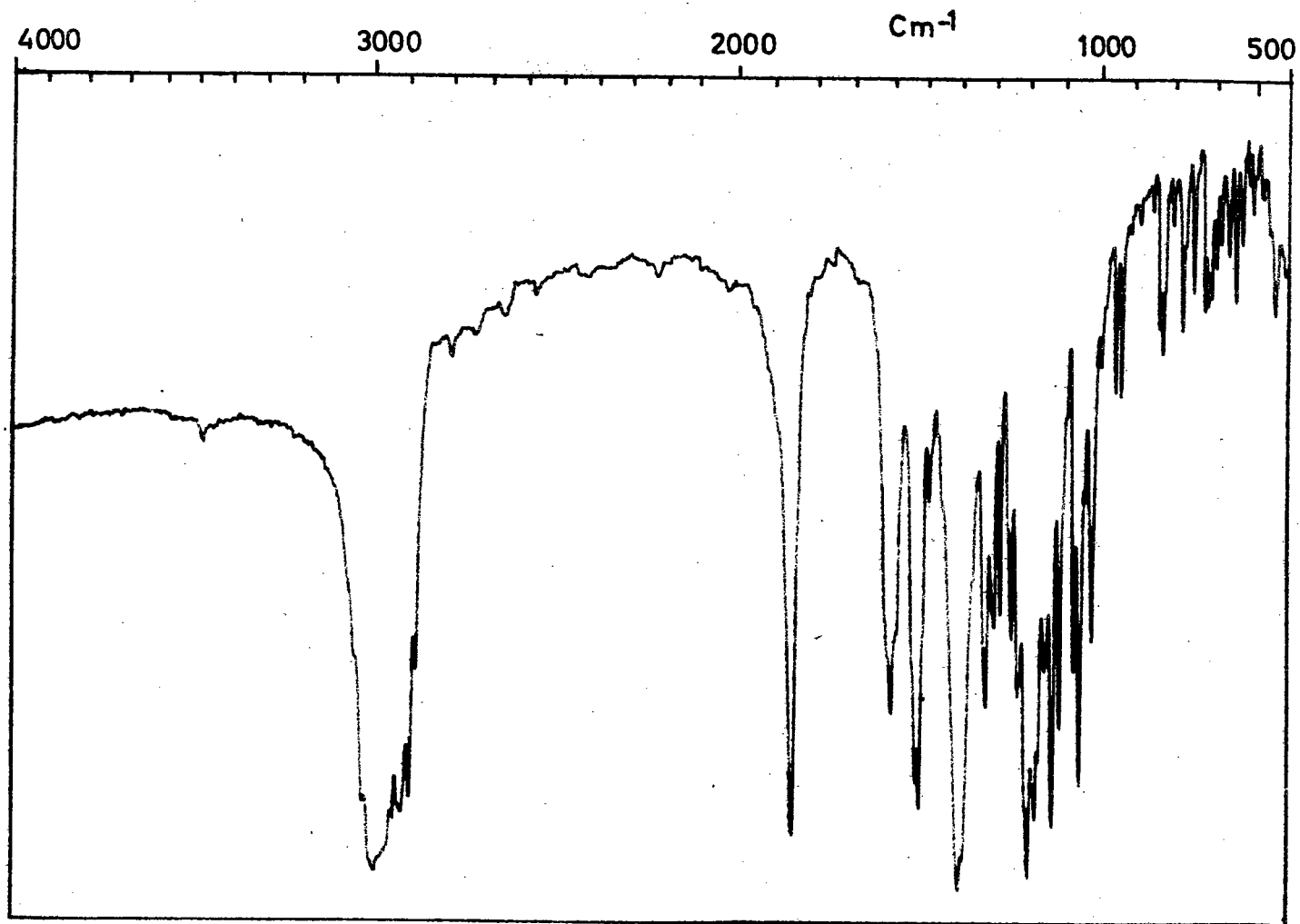


Fig.34 I.R. spectrum of praecoxigenin diacetate

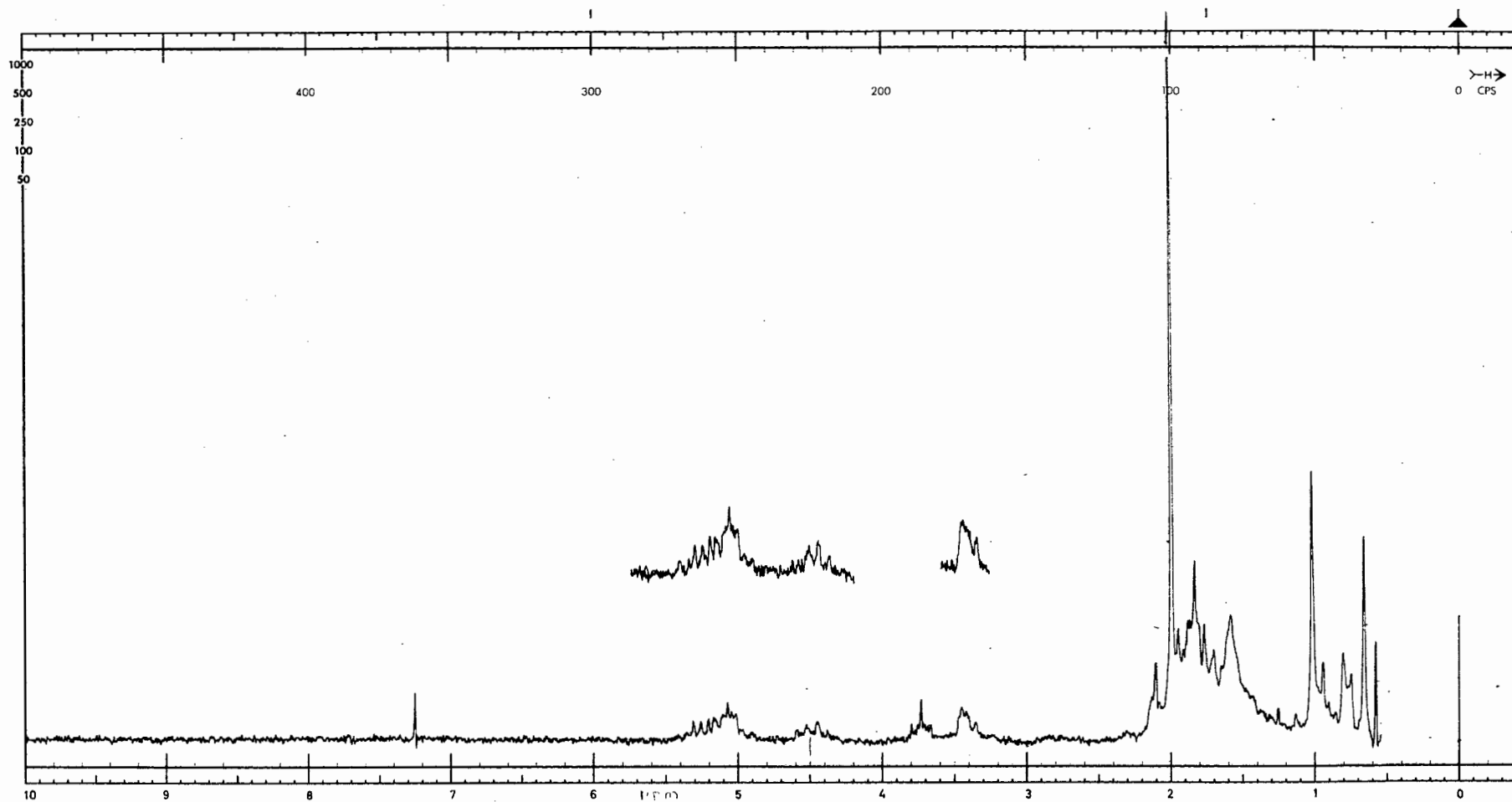


Fig.35 NMR spectrum of praecoxigenin diacetate (CDCl₃)

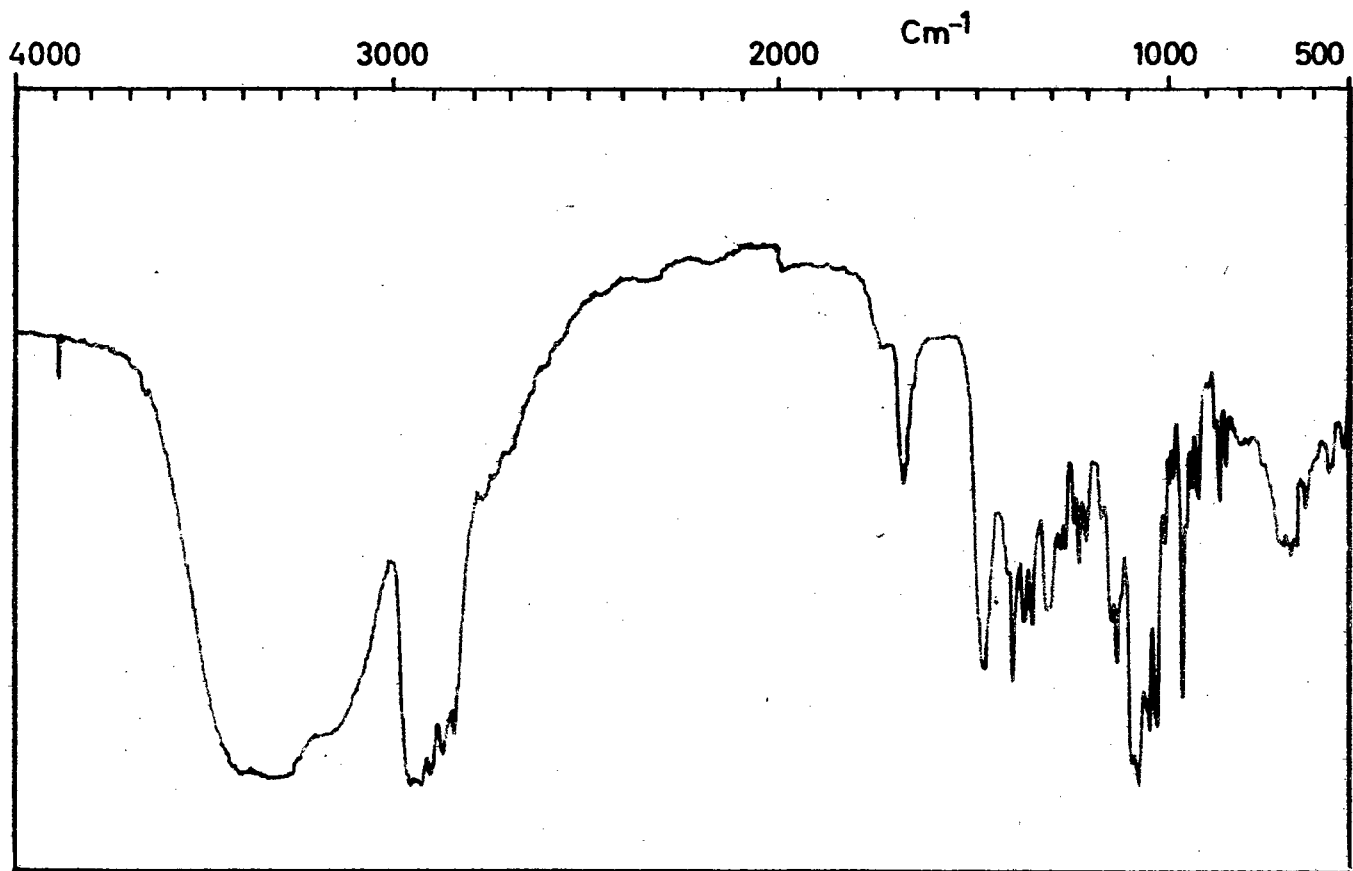


Fig.36 I.R. spectrum of the product of catalytic hydrogenation of praecoxigenin

The fact that hydrogenolysis of the spiroketal system occurs preferentially to hydrogenation of the double bonds suggests that this trisubstituted double bond must be sterically hindered. The normal process of cis hydrogenation probably involves intermediate ring formation. Thus the substrate lies on its flattest side on the catalyst and two active sites at the surface form a quasi-ring with the unsaturated carbon atoms and two hydrogens originally dissolved in the catalyst. An unsaturated A/B-trans steroid should be absorbed on the catalyst on the less hindered rear side, and hence hydrogenation should occur by rear attack. It is possible that the C-2 acetoxy group hinders rear attack at the Δ^4 or Δ^5 positions.

The positions of the two double bonds in praecoxigenin thus remains unresolved. Further experimental work to this end was hampered by insufficient pure material.

Mass spectrum of praecoxigenin

The base peak M (m/e 428) in the spectrum (Fig. 37) due to the molecular ion was 4 m.u. less than that of

gitogenin suggesting that there are two double bonds in the molecule. The major contributor to the peak (M-15) was almost certainly the fragment which may be formulated as ion a which could have been formed by the loss of the C-10 methyl group by allylic activation by double bonds in the Δ^5 and $\Delta^{8(9)}$ positions, proposed. Another peak (b) of low intensity (1%) at M-18 could have been due to the loss of a hydroxyl group as water. An intense peak (c) (M-33, 57%) could have occurred by the loss of a methyl group and elimination of a hydroxyl group (as water) in a concerted process. It is related to the molecular ion by a metastable peak at m/e 364.6. This ion then undergoes spiroketal fragmentation with the loss of 114 m.u. to give p. The peak (m/e 353) possibly arose by fragmentation of ring A with the loss of fragment $\text{CH}_3\text{.CHOH.CHOH.}$ of 75 m.u. to give ion d. This ion then loses 114 m.u. by fragmentation of the spiroketal system to give ion e (M-189). A proposed relationship between these ions, on the assumption that the double bonds are Δ^5 and $\Delta^{8(9)}$ is represented in Chart 15. The peaks i, j, k, l, q are formed from the molecular ion by the usual type of spiroketal fragmentation. The characteristic peaks m (m/e 139) and n (115) indicate that this compound has the usual spiroketal ring system.

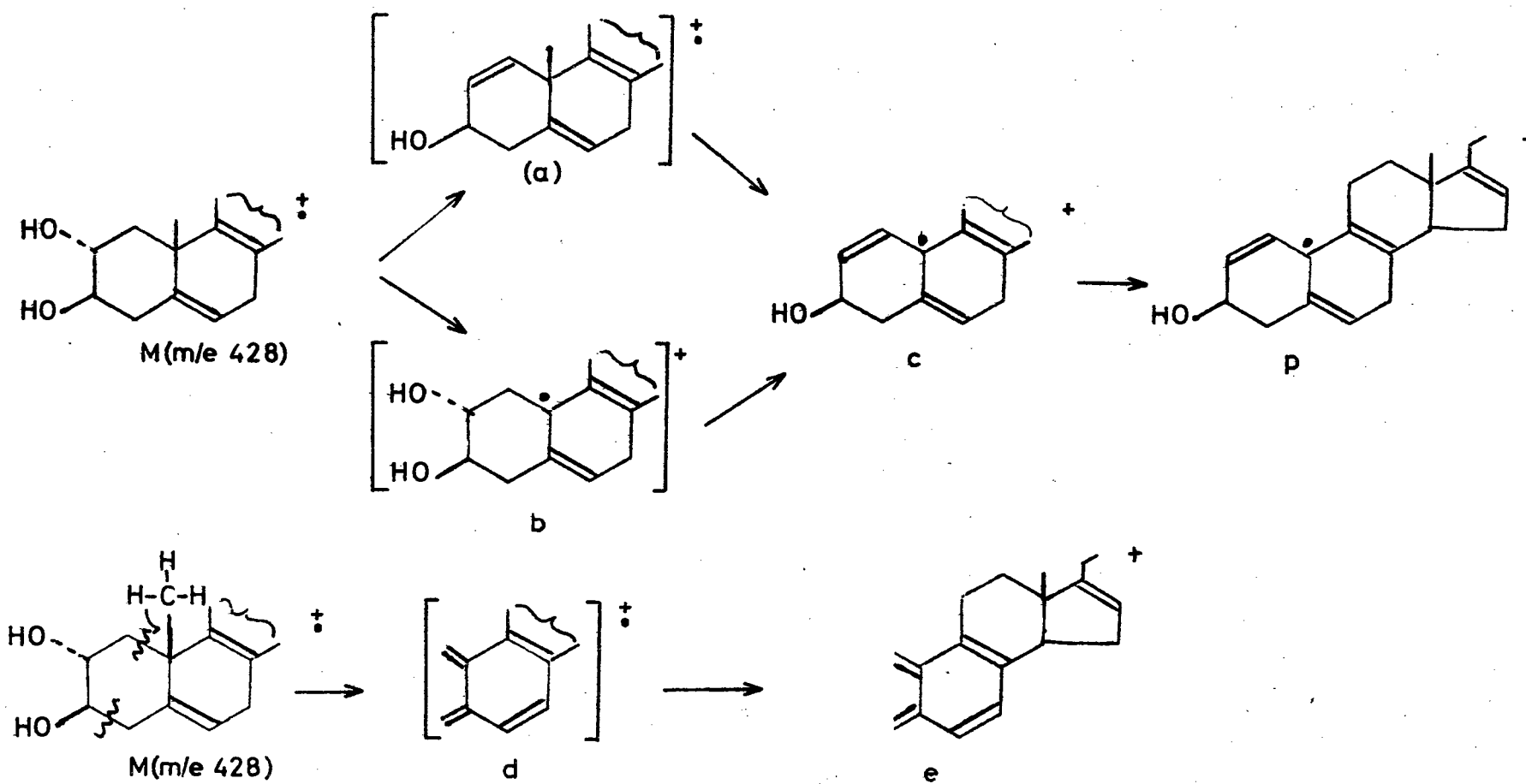


CHART 15 Proposed fragmentation of praecoxigenin

Mass spectrum of praecoxigenin diacetate

The mass spectrum (Fig. 38) shows a peak of low intensity at m/e 512 due to the molecular ion which is 84 m.u. more than praecoxigenin indicating that both hydroxyl groups had been acetylated. The base peak M is due to the typical ring F fragment m/e 139. The major peaks in the spectrum arose by an interrelated series of eliminations from ring A and fragmentation of the spiroketal ring system as shown in Chart 16.

Mass spectrum of the product of catalytic hydrogenation of praecoxigenin

The mass spectrum (Fig. 39) shows a base peak at m/e 430 which is due to the molecular ion and is 2 m.u. more than the molecular ion of praecoxigenin indicating that one molecule of hydrogen had been taken up. A significant feature of the spectrum is the absence of peaks at m/e 139 and m/e 115 due to the typical fragments formed by ring F fragmentation. The absence of these fragments indicates that there is no ring F structure in this compound.

Peaks a (m/e 415), b (m/e 412), c (m/e 397) and d (m/e 355) could have been due to ions formed by ring A elimination and cleavages by the same process as was the case with praecoxigenin (Chart 15) and thus suggests that rings A and B in these two compounds have the same structure.

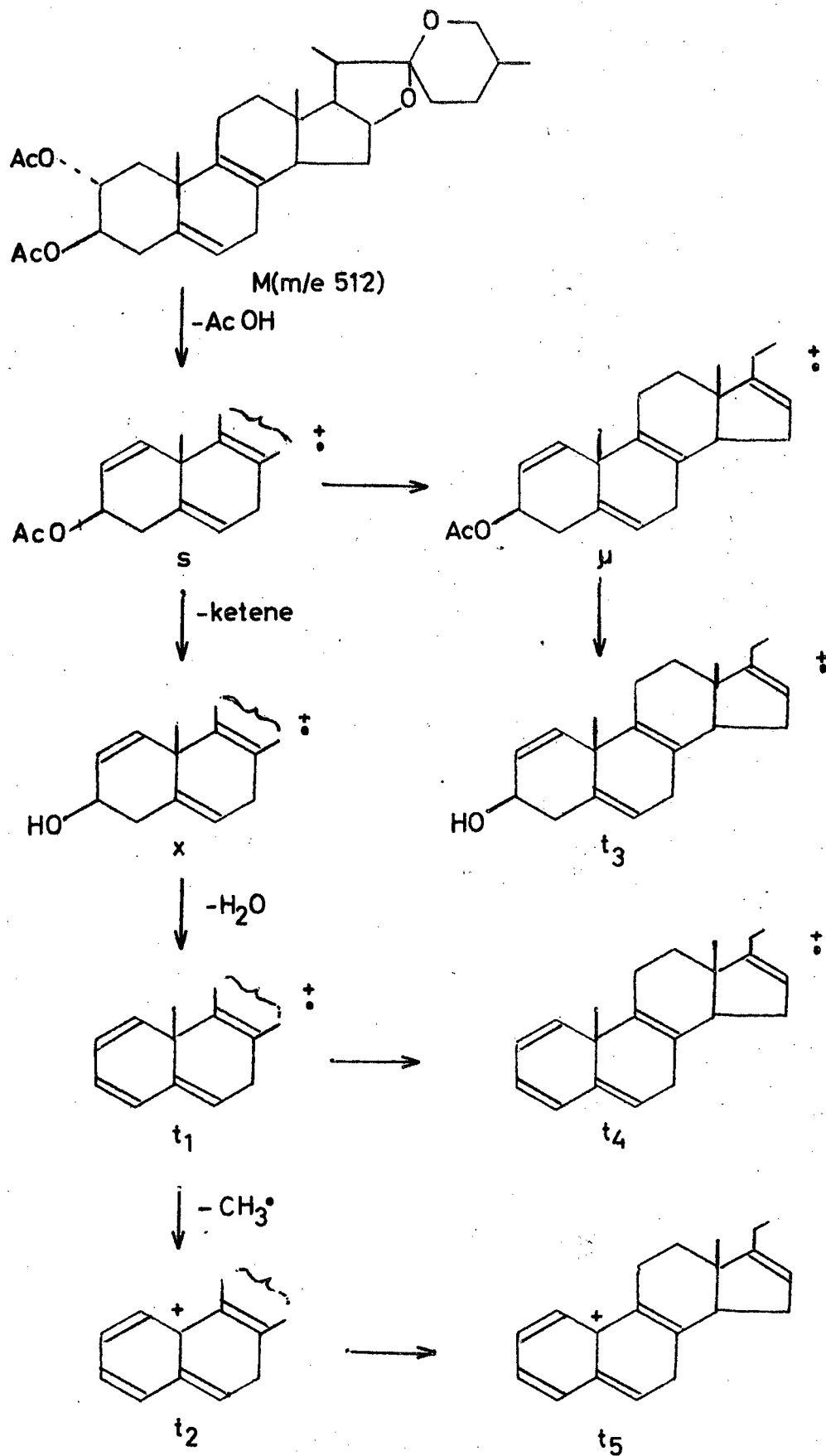


Chart 16 Proposed fragmentation of praecoxigenin diacetate

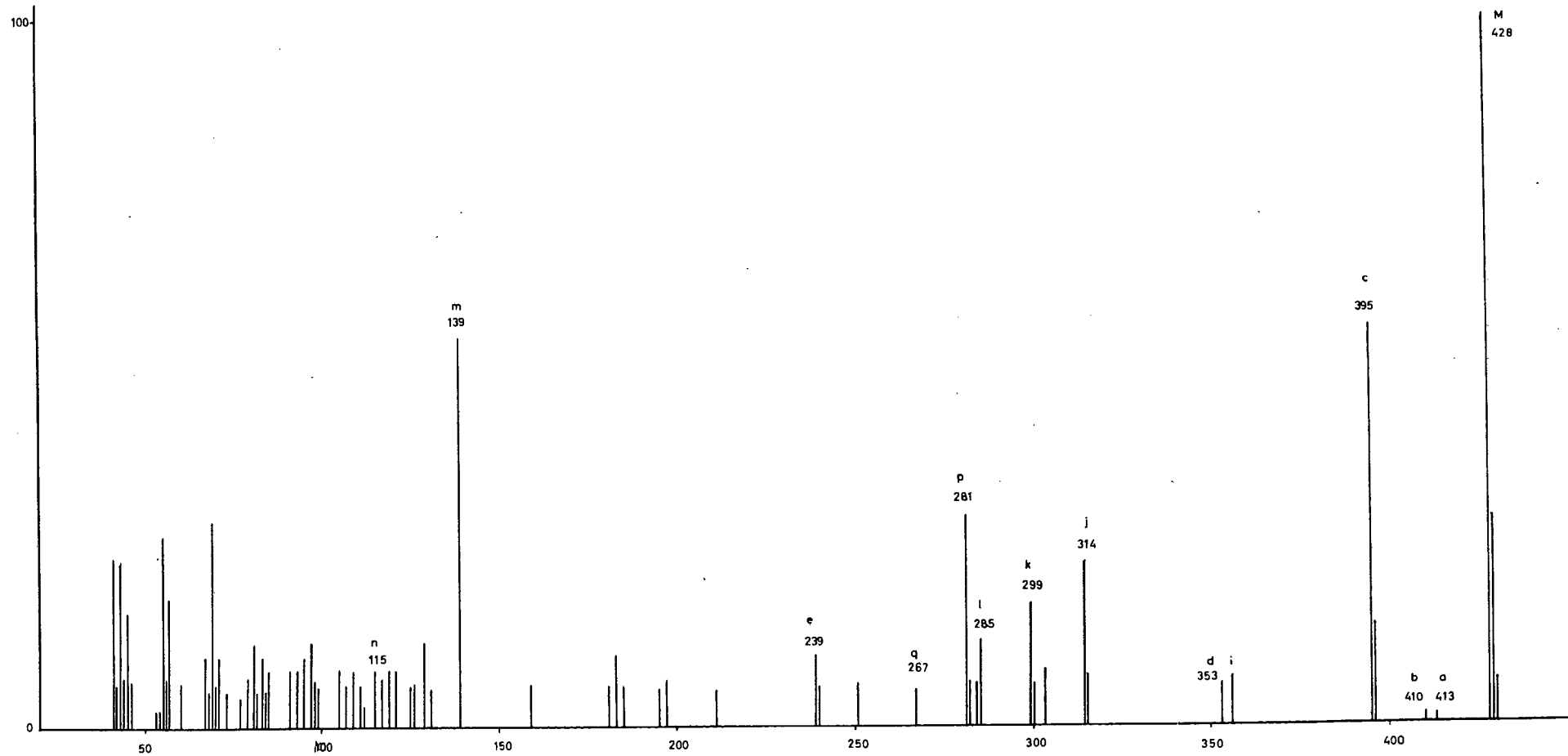


Fig. 37 The mass spectrum of praecoxigenin

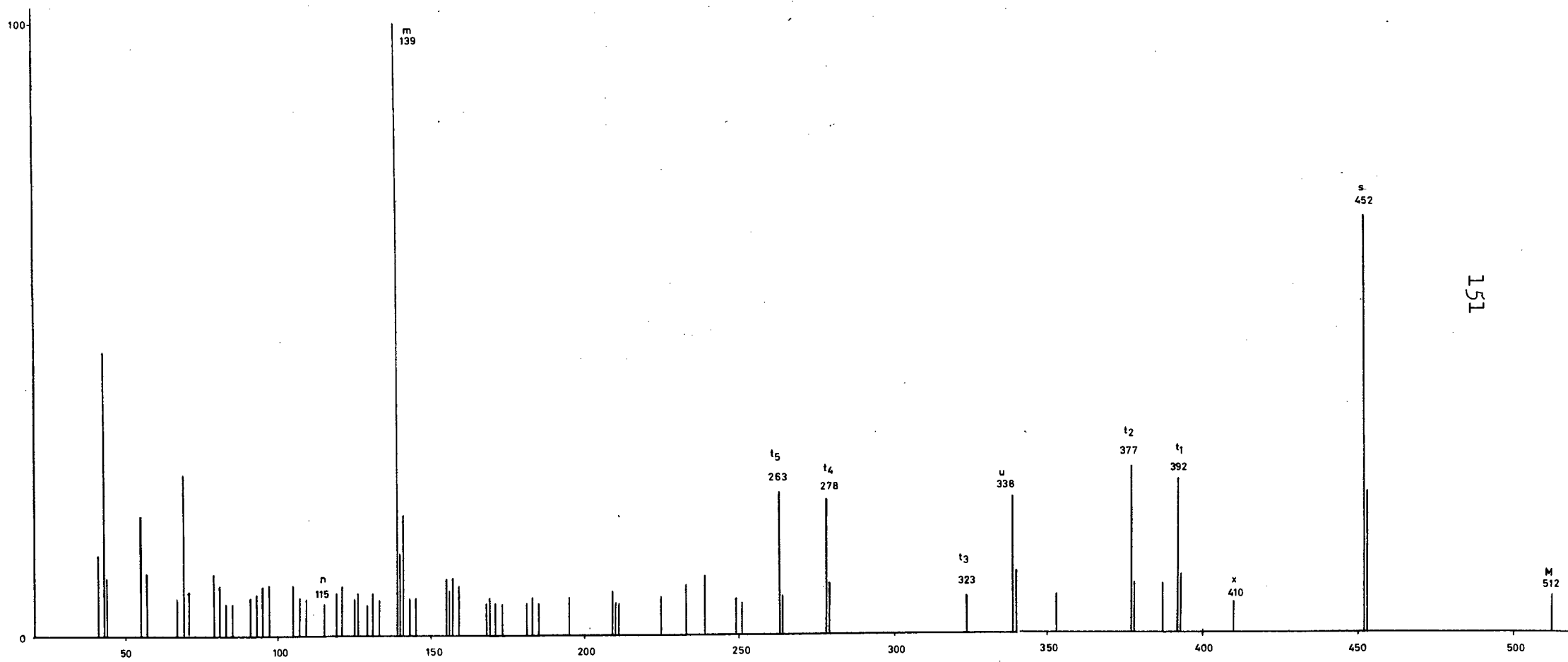


Fig.38 The mass spectrum of praecoxigenin diacetate

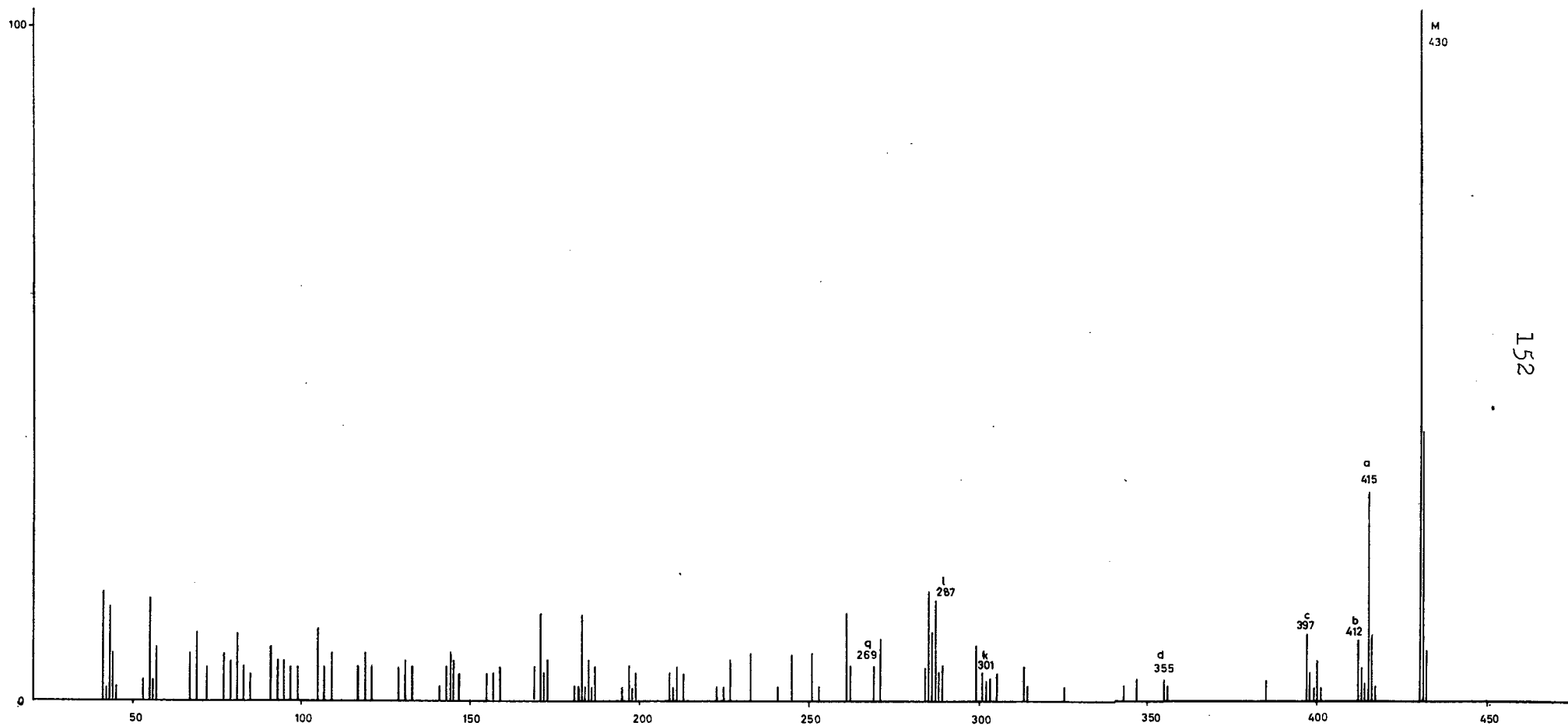


Fig.39 The mass spectrum of the product of catalytic hydrogenation of praecoxigenin

A B S T R A C T

The work of this thesis covers three major topics. The first involves the extraction, isolation and characterisation of the saponins from Agapanthus praecox cultivated vegetatively from an identified parent stock. The second deals with further confirmation of the structure of agapanthagenin, a sapogenin obtained by acid hydrolysis of the predominant saponin in this species and the third gives details of the isolation and some preliminary investigations of the structure of a new steroidal sapogenin, praecoxigenin.

The extraction of the saponins from the rhizomes presented two problems, the first involved the isolation of the mixed saponins from the crude extract and the second the separation of the saponins from the mixture in a state of purity. Chromatographic studies showed the presence of three saponins in the crude plant extract. The main constituent is a new steroidal saponin named agapanthin, $C_{51}H_{84}O_{24}$, which on acid hydrolysis yielded agapanthagenin and a sugar moiety consisting of galactose and rhamnose in the molecular ratio of 3 to 1.

Further confirmation of the structure of agapanthagenin as a 22a-spirostan-2 α :3 β 5 α triol was carried out and is summarised in Chart 17.

The glycollic nature of the two secondary hydroxyl groups was demonstrated by the fact that on oxidation with lead tetraacetate agapanthagenin (Ia) underwent fission to give XII. During oxidation with chromic anhydride a tertiary hydroxyl group should lactonise with the carbonyl group produced by oxidation of the hydroxymethylene group at position C-2. Oxidation of agapanthagenin with chromic anhydride yielded 5 α -Hydroxy-2:3-seco-22a-spirostan-2:3-dioic lactone (III) which on treatment with diazomethane gave the methyl ester (IV).

The assignment of configuration to the glycol function as a 2 α :3 β diol was based on the rate of glycol fission, which was of the same order as that of gitogenin of known configuration. This was consistent with the infrared spectrum in the three micron region due to bonded and non-bonded hydroxyl groups, and with the chemical shift and splitting pattern of the C₂ and C₃ protons in the nuclear magnetic resonance spectrum of its diacetate. The inability to

form an acetonide coupled with the fact that the dime-
sylate (V) was converted into an olefine (XIII) provided
further confirmation of the configuration of the gly-
col function as a $2\alpha:3\beta$ diol.

It has been shown that a 5-hydroxyl group can be
acetylated only if it has the α -configuration. Ace-
tylation of the tertiary hydroxyl group in agapantha-
genin diacetate has been accomplished. Partial hy-
drolysis of the triacetate (VI) has been effected yiel-
ding the 5α -monoacetate (VII). This is to be expected
since the 2α - and 3β -acetoxy groups are equatorial and
the 5α - is axial. The 5α -hydroxyl group being axial
should be readily eliminated with a coplanar hydrogen
from C_4 or C_6 giving rise to one or both of the diace-
tates (VIIIb) and (IXb). Both these compounds have been
isolated by the action of thionyl chloride on agapan-
thagenin diacetate. The Δ^5 -diacetate (IXb) with mono-
perphthalic acid gave a single epoxide formulated as
X by analogy with the major product of peroxidation
of cholesterol. Reduction of this epoxide with li-
thium aluminium hydride yielded agapanthagenin and a
trace of an unidentified compound. In conformity
with previous investigations by Roberts (53) the Δ^4 -
diacetate (VIIIb) on epoxidation yielded a single epox-

ide (XI). Reduction of this epoxide with lithium hydride again yielded agapanthagenin as the only product. Reduction of the two epoxides to agapanthagenin conclusively established the position and configuration of the tertiary hydroxyl group as 5α .

The pattern of the infrared absorption spectrum in the $800-1000\text{ cm}^{-1}$ region established agapanthagenin as an "iso" sapogenin. This assignment has been confirmed by a study of the chemical shift and splitting patterns of the C_{27} methyl and C_{26} methylene protons in the nuclear magnetic resonance spectrum.

Intimately associated with agapanthagenin in the crude extract was a second compound which was isolated in small quantities. The infrared spectrum showed the presence of a hydroxyl group and a double bond but no ketonic band. It also suggested that it is a steroidal sapogenin belonging to the 25D series. On acetylation it gave a diacetate, $C_{31}H_{44}O_6$. Analytical values were in good agreement with the formula $C_{27}H_{40}O_4$ which established it as a dihydroxy diene. The ultra violet spectrum showed no region of maximum absorption due to a conjugated double bond. The fragmentation pattern of the mass spectrum confirmed

that it has the usual spiroketal ring system with no unsaturation in either ring E or F. This sapogenin and its diacetate have melting points of 266-268°C (decomp.) and 230-231°C respectively and have not been reported in literature. The sapogenin was named praecoxigenin.

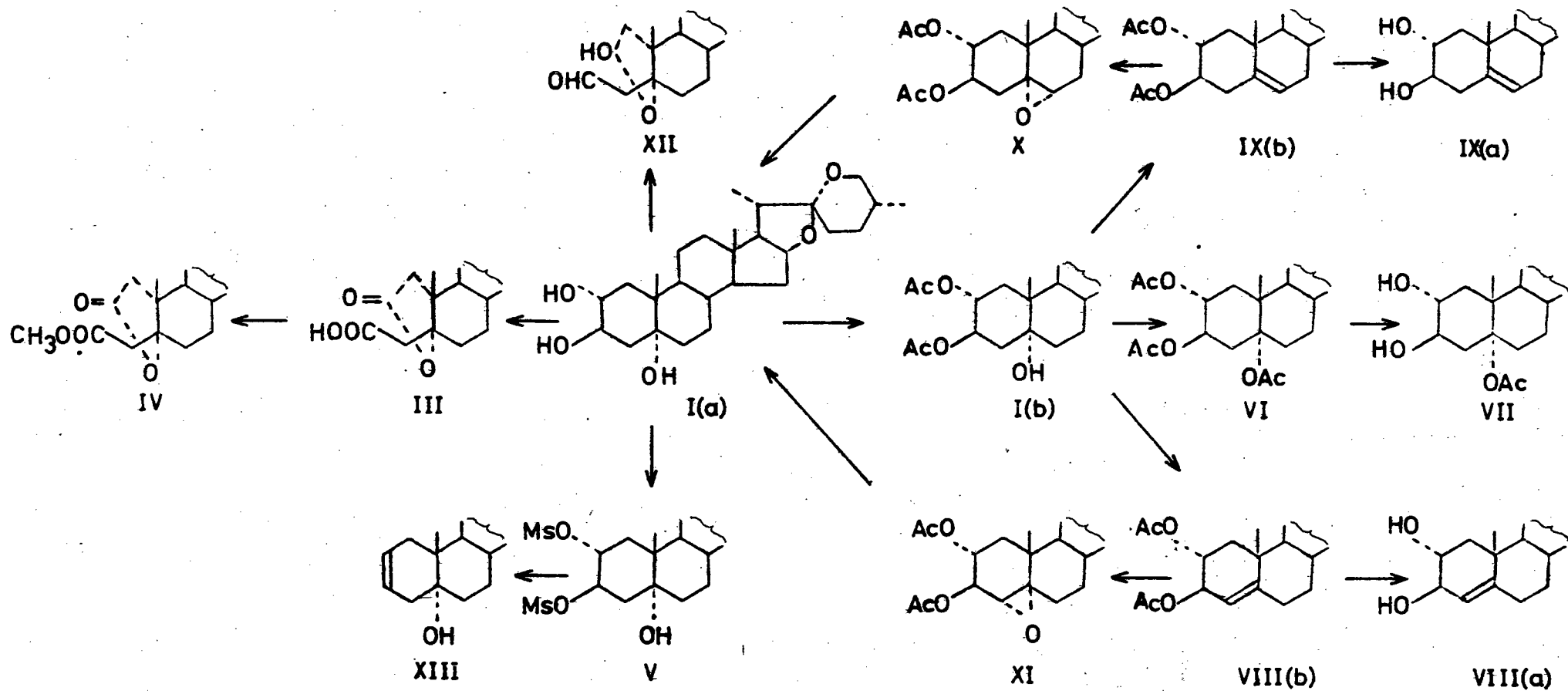


CHART 17 Some reactions of agapanthagenin.

EXPERIMENTAL

EXPERIMENTAL

All melting points were uncorrected and were determined on a Kofler hot-stage. Unless otherwise specified, all optical rotations were determined in ethanol solutions at room temperature. Infrared spectra were measured with a Perkin-Elmer model 521 spectrophotometer in KBr discs unless otherwise stated. Mass spectra were recorded on a MS-9 double focussing mass spectrometer and n.m.r. spectra on a Varian HA-100 spectrometer at the National Chemical Research Laboratory of the C.S.I.R.. Elementary analyses were performed by Weiler and Strauss of Oxford. In cases where elementary analysis could not be obtained due to lack of material, accurate masses were determined by means of mass spectrometry. All solvents used were previously distilled and the light petroleum had a boiling range of 56 - 60°.

Chromatography

Glass plates coated to a thickness of 250 u with Merck kieselgel G were used for thin layer chromatography. The plates were activated for thirty minutes

at 105° prior to use.

For routine column chromatography Merck silica gel with a particle size of between 0.05 and 0.2 mm, or Merck alumina, Brockmann activity 2, were used.

The following solvent systems were used for thin layer chromatography:

Solvent I	lower layer of chloroform - ethanol - water (65 : 37 : 8)
Solvent II	benzene - methanol (20 : 1)
Solvent III	chloroform - ethanol (9 : 1)
Solvent IV	chloroform - methanol (40 : 1)
Solvent V	cyclohexane - ethyl acetate (4 : 1)

In all cases the plates were developed to 15 cm from the origin. As a routine procedure the spray reagent used was 20% sulphuric acid, and, after spraying, the plates were heated to 100° for five minutes to reveal the spots.

Ascending paper chromatography was conducted using Whatman chromatographic paper with one of the following solvents:

Solvent VI	upper layer of butanol - acetic acid - water (4 : 1 : 5)
Solvent VII	butanol - pyridine - water (6 : 4 : 3)

The spray reagent used for sugars was aniline (0.93 g) and phthalic acid (1.66 g) dissolved in water saturated n - butyl alcohol (100 ml).

1. Tests for saponins in plant material

A solution of 0.9% sodium chloride (100 ml) was added to gelatine powder (4.5 g) and after standing for thirty minutes at room temperature the mixture was heated in a water bath, with stirring, at 80°. After cooling to 45° defibrinated blood (6 ml) was stirred in and the mixture poured on to glass plates as a thin film which was allowed to set. Spots (0.05 ml) of the mucilagenous exudate from leaves, lower stems and rhizomes of Agapanthus were carefully added to the gelatine film on the plate. At the same time a spot of a solution of digitonin (0.01%) was also run on to the plate to act as a control. The plate was examined after one hour

for areas of haemolysis which were revealed as a transparent colourless spot on an opaque red blood gelatine background. The exudate from rhizomes showed more intense haemolysis than that from leaves and lower stems.

2. Extraction of Agapanthus rhizomes

Sliced, minced rhizomes (1 Kg) were dried at 80° in an oven with fan circulation for four days. The dried material (200 g) was ground to a fine powder, placed in calico bags and extracted with ethyl alcohol (total volume 2 litres) until a portion of the alcoholic extract gave no frothing when shaken with water. Water (2 l) was added to the combined extracts to reduce the alcohol content to about 50%. On standing considerable precipitation of colloidal material occurred which was removed by filtering on a buchner funnel with kieselguhr. The filter cake was stirred with 50% alcohol and refiltered. The combined filtrates were defatted with benzene saturated with 50% alcohol in a series of liquid - liquid extractors. The defatted alco-

holic solution was concentrated to one litre to remove most of the alcohol. Sodium chloride (50 g) was added and sufficient hydrochloric acid to give the dark brown extract a pH of 4.5. The extract was then shaken four times in a separating funnel with butanol saturated with water (500 ml). The butanol layers were combined and washed with dilute salt solution (500 ml) and the washings re-extracted with butanol (250 ml). The aqueous layer was discarded. The solvent was removed under reduced pressure in a rotary evaporator leaving a dark brown residue (31 g). This residue was mixed with methanol (100 ml) filtered and added dropwise to dry acetone (2 l) with stirring. The brown crude saponin residue (15 g) was filtered off and dried under vacuum over calcium chloride.

3. Thin layer chromatography of crude saponin extract

The chromatographic pattern of the spots revealed by spraying with sulphuric acid after development with solvent 1 is given below (Table 13).

Table 13. Thin layer chromatography of crude saponin extract

Colour of spot	Rf value
Brown	0.93
Blue	0.90
Blue	0.88
Purple	0.70
Brown	0.37
Purple	0.17
Brown	0.00

In order to ascertain which of these spots were due to saponins a similar chromatogram was run and sprayed with a 2% suspension of guinea pig blood cells in normal saline. The spray reagent was prepared as follows:

Blood cells were prepared by centrifuging heparised guinea pig blood, discarding the plasma, washing the cells three times with an equal volume of saline. The centrifuged cells were then suspended in normal saline. It was found that with precautions to avoid haemolysis

the blood cell suspension could be used for at least two days without change in sensitivity.

After spraying the developed plates evenly and thoroughly with a suspension of guinea pig blood cells in normal saline they were set aside for 30 minutes. The chromatogram showed faint light spots on a brownish background at Rf values 0.17, 0.37, 0.70. It was, therefore, assumed that these three spots were due to haemolysis of the blood cells caused by the presence of saponins and that the spots at other Rf values, revealed by spraying with sulphuric acid, were due to impurities, other than saponins, in the crude saponin extract. Judging by the intensity of the spots on the plates it appeared that the saponin at Rf 0.17 was the major component in the extract and that the one at Rf 0.70 was the minor component.

4. Isolation of saponins from the crude saponin extract

After many unsuccessful attempts to isolate the saponins from the crude saponin

extract of the plant, which included crystallisation from various solvents, column chromatography, preparative layer chromatography, the following technique was used with moderate success.

The dried saponin extract (5 g) was acetylated with acetic anhydride (50 ml) and pyridine (50 ml) by boiling under reflux for two hours. After precipitation from water the acetylated product was extracted with ether. A large black ether insoluble residue was separated off from the ether extract. After washing the ether layer successively with dilute hydrochloric acid, sodium bicarbonate (5%), and drying over anhydrous magnesium sulphate, removal of the solvent gave a light brown residue (2.2 g).

Thin layer chromatography of this residue with solvent II on spraying with sulphuric acid revealed dense spots at Rf 0.67 and 0.47 with faint spots at Rf 0.87, 0.73, 0.56, 0.37 and 0.17.

This crude acetylated product (1 g) was chromatographed on neutral alumina (100 g) with benzene, benzene - chloroform (1 : 3), chloroform,

chloroform - ethanol (95 : 15) to afford three fractions of which the first was obtained as a colourless glass. Thin layer chromatography showed that it was a mixture of two compounds which could not be separated. This acetylated glass was deacetylated with methanolic potassium hydroxide (5%) and, after removal of the methanol and neutralisation with dilute hydrochloric acid, was extracted with butanol saturated with water. Removal of the butanol under vacuum gave a white amorphous residue which on thin layer chromatography with solvent I showed two spots at Rf 0.17 and 0.37. The saponin mixture was chromatographed on silica gel using solvent I. Mixed fractions were removed but by continuous elution small quantities of chromatographically pure material was isolated (Rf 0.17). All attempt to isolate the other two saponins from the plant material in a state of purity failed.

5. Relationship between the saponins and the saponinogens obtained by acid hydrolysis

In order to determine the relationship between the saponins and their saponinogens obtained

by acid hydrolysis, saponin extracts containing all three saponins, the two major saponin constituents, and the one saponin obtained pure were respectively hydrolysed with hydrochloric acid and the precipitated sapogenins filtered off. Thin layer chromatography of these residues with solvent III were run and compared with agapanthagenin, praecoxigenin and yuccagenin which had been isolated from the plant material by other methods. The results are shown in Table 14.

Table 14. Thin layer chromatography of saponins and sapogenins from Agapanthus praecox

Sample	Rf value of saponin (Solvent I)	Rf value of derived sapogenin (Solvent III)
Extract containing the three saponins	0.17	0.48
	0.37	0.57
	0.70	0.83
Extract containing two saponins	0.17	0.48
	0.37	0.57
The saponin isolated pure	0.17	0.48
Agapanthagenin	-	0.48
Praecoxigenin	-	0.57
Yuccagenin	-	0.83

From this table it can be seen that agapanthagenin was obtained by the hydrolysis of the pure saponin of Rf value 0.17. This saponin was thus named agapanthin. The two other saponins in the plant extract which could not be isolated pure but which on acid hydrolysis gave praecoxigenin and yuccagenin were named praecoxin and yucconin respectively. The latter was present in very low concentration in the plant extract.

6. Characterisation of agapanthin

The white amorphous eluate (Rf 0.17) obtained from the chromatographic separation previously outlined crystallised from methanol as oblong plates m.p. 252-253°,

$$[\alpha]_D^{23} = 52^\circ (\text{C, 0.61 in pyridine})$$

(Found : C, 56.12; H, 7.68; $\text{C}_{51}\text{H}_{84}\text{O}_{24}$ requires; C, 56.67; H, 7.78%)

V KBr : 3500-3300 broad band (O-H), 1445 (C-OH)
max 975, 915, 895, 860 cm^{-1} (spiroketal system).

7. Agapanthin peracetate

Agapanthin (100 mg) was allowed to stand with 1 ml each of pyridine and acetic anhydride at room temperature for 24 hours. The reaction mixture was poured on to ice and the deposited substance filtered and washed with water. Crystallisation from aqueous methanol gave a peracetate (100 mg) as needles m.p. 214-221°. (Found : C, 56.78; H, 6.81%). As this product was found to be a mixture it was re-acetylated as described above but was left to stand for 3 days. The product was poured into ice water and extracted with ether. The ether was washed with dilute hydrochloric acid, then with dilute sodium bicarbonate, dried over anhydrous magnesium sulphate and the solvent removed to give a white amorphous product which on repeated crystallisation from aqueous methanol gave fine needles (85 mg) m.p. 225-226°,

$$[\alpha]_{\text{D}}^{23} -47^{\circ} (\text{C}, 0.402, \text{in } \text{CHCl}_3).$$

(Found : C, 56.78; H, 6.45. $\text{C}_{77}\text{H}_{110}\text{O}_{37}$ requires C, 56.83; H, 6.77%).

ν KBr : 3510, (O-H), 1740 (ester C=O), 1220 (acetate),
max : 988, 918, 900, 865 cm^{-1} (spiroketal system).

8. Acetyl determination of agapanthin peracetate

Agapanthin peracetate, dried in vacuo over phosphorus pentoxide, was hydrolysed with sodium hydroxide in methanol for one hour in a micro acetyl apparatus. The saponification mixture acidified and distilled, and the distilled titrated with N/100 sodium hydroxide. The results are shown below (Table 15).

Table 15. Acetyl determination of agapanthin peracetate

Wt. of agapanthin used. mgs	Vol. N/100 NaOH required	% Acetyl.
8.1	6.28	33.5
10.2	7.79	32.8
8.5	6.56	33.2

Mean value = 33.4%

9. Molecular weight of agapanthin

Samples of agapanthin (\pm 100 mg) were refluxed with a mixture of 95% ethanol (4.5 ml), concentrated hydrochloric acid (4 ml) and water

(1.5 ml) for two hours, excess water was then added to the hydrolysis mixture which was filtered through a tared sintered glass crucible.

The mass of the agapanthagenin formed was determined, and from this the molecular weight of agapanthin calculated (Table 16).

Table 16. Molecular weight of agapanthin

Wt of agapanthin used for hydrolysis g	Wt of agapanthagenin formed g	Calculated molecular Wt of agapanthin
0.1075	0.0445	1082
0.1043	0.0468	1092
0.1000	0.0458	1073
0.1000	0.0466	1079

Mean molecular weight of agapanthin = 1081

10. Characterisation of the sugar moiety

10.1 Comparative paper chromatography with known sugars

Sheets of Whatman chromatographic paper 20 x 20 cm were spotted 3 cm from the bottom edge

and 2.5 cm apart with 50 ul samples of the sugar hydrolysate from 100 mg of agapanthin together with 1% solutions of known sugars in 10% isopropanol. The paper was formed into a cylinder and developed, by ascending chromatography over a distance of 18 cm, with the upper layer of solvent mixture butanol - acetic acid - water (4 : 1 : 5). After removal from the developing tank the paper was thoroughly dried, sprayed with a solution of aniline hydrogen phthalate in water saturated butanol, and heated at 105° for 5 minutes.

This chromatogram (Figure 40) indicated that the sugars present in the hydrolysate of agapanthin were rhamnose and galactose. Because of the similarity in the Rf values of galactose and glucose modifications to the chromatographic procedure were carried out to confirm the identity of the aldohexose present.

10.2 Confirmation of the identity of the aldohexose

Use was made of the fact that glucose is fermented by a yeast suspension (galactose un-

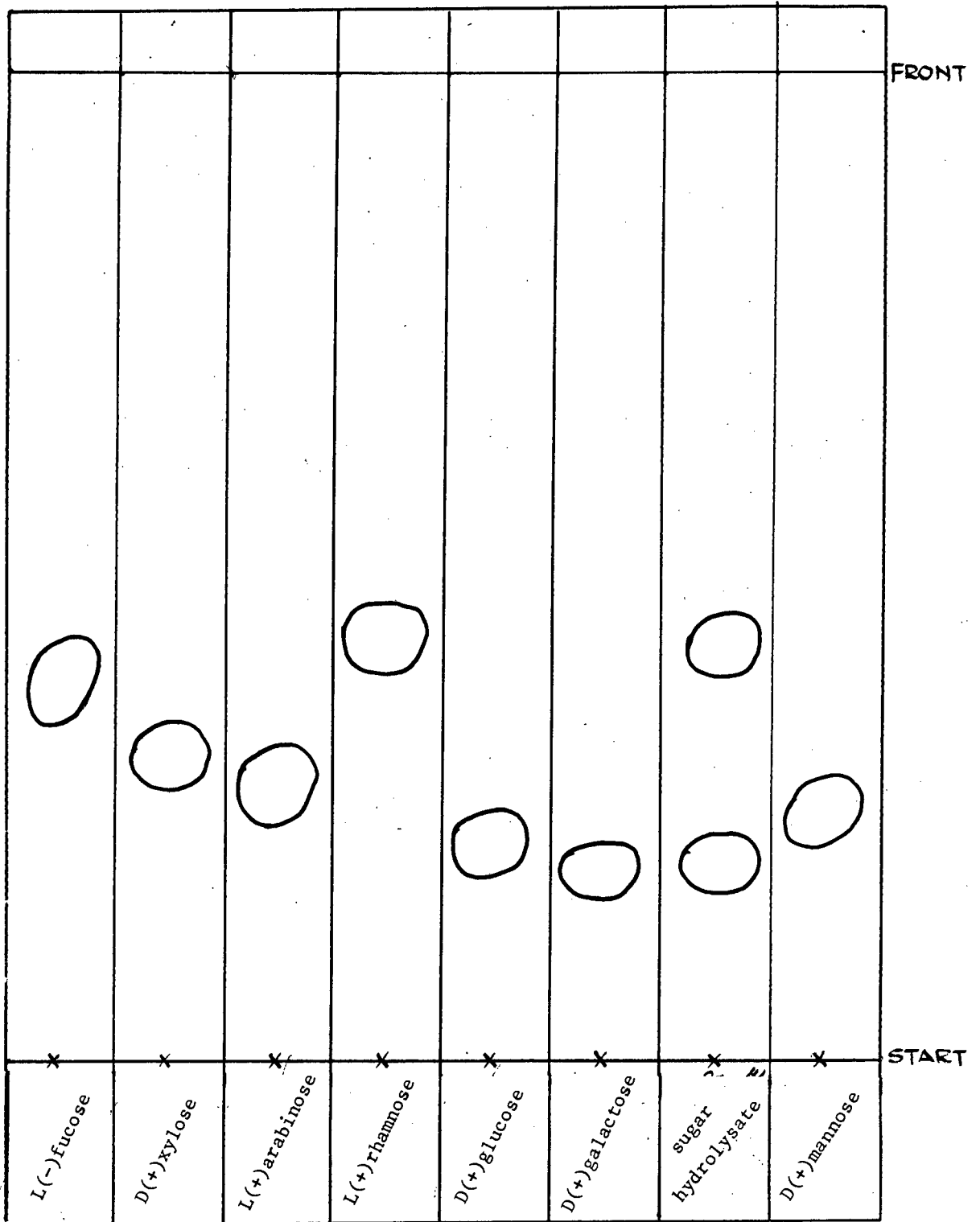


FIG. 40. Comparative paper chromatography of sugars.

der the same experimental conditions is unaffected) and would therefore not show up as a spot with the detecting agent. A 20 x 20 cm chromatographic paper was spotted 3 cm from the base and 2.5 cm apart with 50 ul of the following solutions

<u>Spot No.</u>	<u>Sugar</u>
1	rhamnose
2	galactose
3	sugar hydrolysate
4	glucose
5	rhamnose
6	galactose
7	sugar hydrolysate
8	glucose

The paper was developed by ascending chromatography for 18 hrs with the solvent system butanol - pyridine - water (6 : 4 : 3) which is considered by Hais and Macek (55) to give better resolution of glucose and galactose. After development the paper was cut vertically in half so that spots 1 - 4 were on the one half and 5 - 8 on the other. After thorough drying, the one half was sprayed with a 5% yeast suspension

and incubated in a moist atmosphere at 38° for 90 minutes. The paper was dried and both halves were sprayed with aniline hydrogen phthalate solution and heated at 105° for 5 minutes.

This chromatogram (Figure 41) indicated that the spot corresponding to the aldohexose in the agapanthin hydrolysate remained visible on the paper sprayed with the yeast suspension and thus confirmed the aldohexose in the sugar hydrolysate from agapanthin as galactose.

10.3 Empirical estimation of the molecular ratio of galactose and rhamnose in the sugar hydrolysate

Mixtures of galactose and rhamnose were made up according to Table 17.

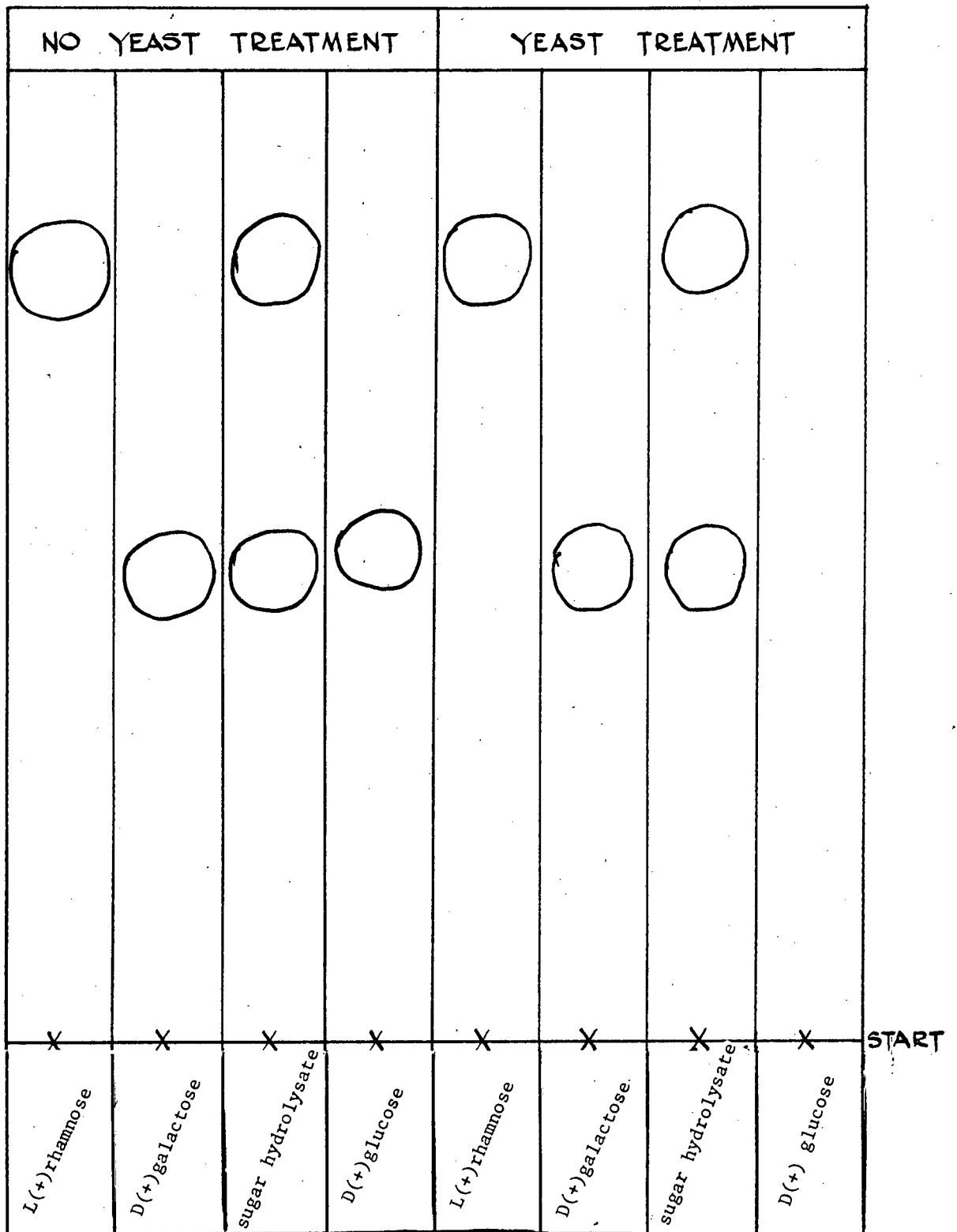


FIG. 41. Paper chromatography of sugars before and after yeast treatment.

Table 17. Molar concentrations of solutions of galactose and rhamnose

Solution	Wt in mg/10 ml		Molecular ratio	
	galactose	rhamnose	galactose	rhamnose
1	14.57	14.75	1	1
2	19.42	10.07	2	1
3	21.92	7.38	3	1
4	23.44	5.86	4	1

50 ul of these solutions were spotted on a chromatographic paper together with 50 ul of sugar hydrolysate and chromatogramed in the usual way. The ratio of the intensity of the spots obtained with mixtures 1 to 4 was compared with that obtained with the sugar hydrolysate, and showed that the sugar ratio in the agapanthin hydrolysate was nearest to 3 moles galactose to 1 mole rhamnose.

10.4 Spectrophotometric determination of the molar ratio of sugars in the hydrolysate

10.4.1 Chromatographic separation of the sugars in the hydrolysate

The hydrochloric acid hydrolysate from 100 mg of agapanthin was kept overnight in a deep freeze refrigerator and while frozen was transferred to a freeze dryer and freeze dried for 12 hours. The freeze dried sugars were made up to 2 ml in a volumetric flask. A pencil line was drawn horizontally across a piece of 20 x 20 cm Whatman No. 1 chromatographic paper 3 cm from the bottom edge. Vertical lines were then drawn at distances of 2 and 4 cm from each edge. Sugar hydrolysate (0.1 ml) was applied with a special micro pipette as an even narrow strip on the horizontal pencil line between the two inner vertical marks. The paper was spotted with 50 ul of the same solution on the horizontal line 2 cm from the right hand and left hand edges. The paper was then formed into a cylinder with the edges held just apart with plastic clips and developed by using ascending chromatography with solvent

system butanol - pyridine - water (6 : 4 : 3). After 18 hours the paper was removed and thoroughly dried. Two vertical strips 3 cm wide were cut off each edge of the paper. These were sprayed with aniline hydrogen phthalate solution and dried at 105° for five minutes. Using these two test strips showing spots due to galactose and rhamnose, horizontal strips were cut from the remainder of the paper containing these separated sugars. The strips were folded into a tight roll attached to the tip of a Wiley condenser with thin copper wire and the sugar extracted with 5 ml of water by immersing the apparatus in an oil bath, heated to 130°, for thirty minutes. The extracted sugars were then diluted to 10 ml in a volumetric flask and the sugar content of aliquot portions determined spectrophotometrically.

10.4.2 Calibration curves for galactose and rhamnose

A solution containing a mixture of galactose (50 mg) and rhamnose (50 mg) in water

(2 ml) was prepared and 0.1 ml was chromatographed as previously described. Strips containing the separated sugars were extracted with water and the extract made up to 10 ml. Aliquot portions of this extract from 0.1 to 0.5 ml, were used for the preparation of a calibration curve as follows:

A measured portion of the sugar extract was run into a 10 ml. glass ampoule, water added to make the volume up to 0.5 ml. followed by 2 ml of a 1.5% solution of p-aminobenzoic acid in glacial acetic acid and 2 ml of a 1.3% solution of phosphoric acid in glacial acetic acid.

The ampoules were then sealed and heated in a waterbath at 100° for one hour. After treatment they were cooled to room temperature and the absorbance of the solution measured against a reagent blank at 360 mμ in a Zeiss PM Q 11 spectrophotometer at a slit width of 0.05 mm.

From the relationship between sugar concentration and absorbance (Table 18) calibration curves (Figure 42) for galactose and rhamnose were prepared.

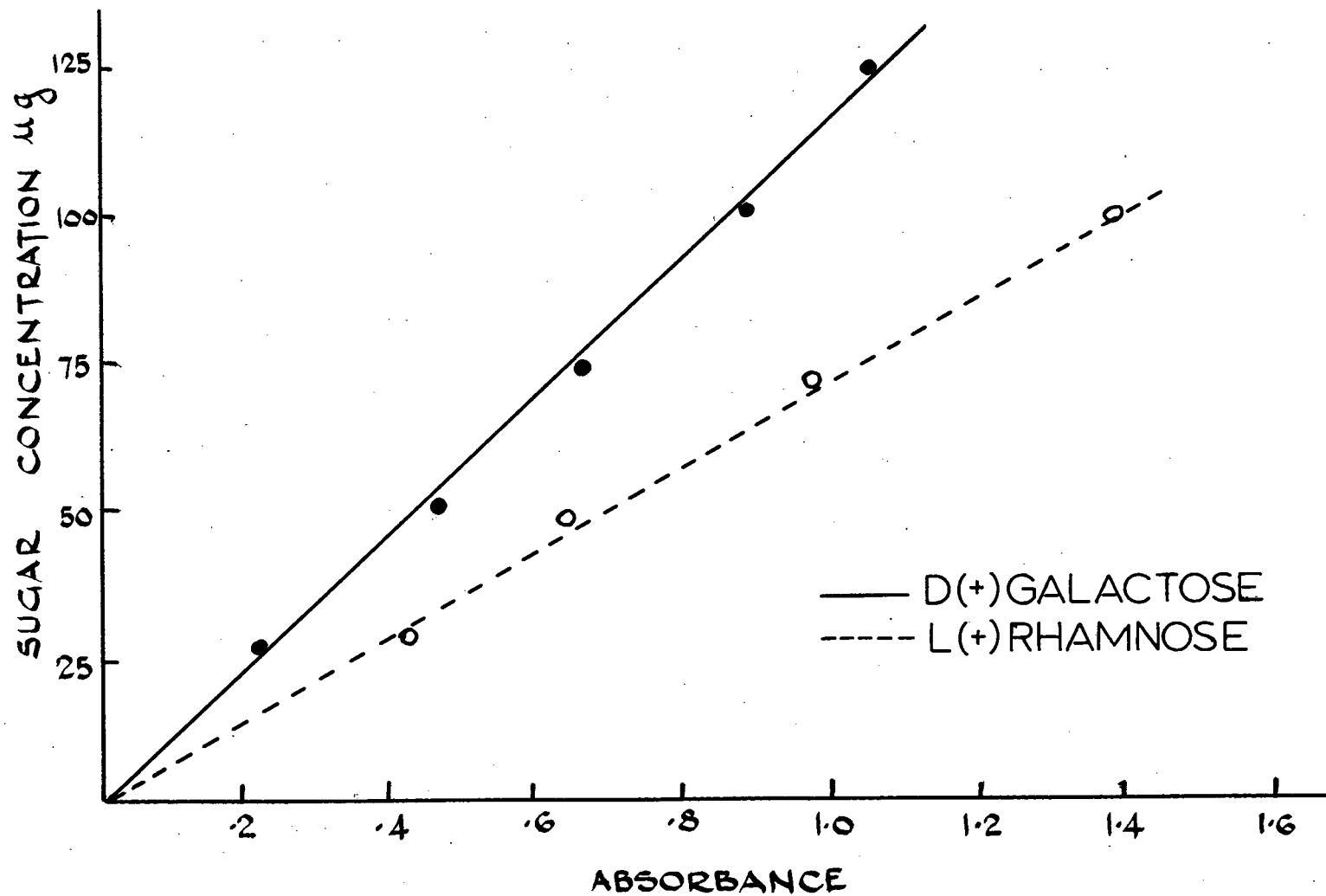


FIG. 42 SUGAR CALIBRATION CURVES

Table 18: Relationship between concentration and absorbance of solutions of galactose and rhamnose

Volume of sugar extract ml	Weight of each sugar ug	Absorbance	
		galactose	rhamnose
0.1	25	0.22	0.45
0.2	50	0.46	0.62
0.3	75	0.73	0.92
0.4	100	0.84	1.44
0.5	125	1.14	-

10.4.3 Spectrophotometric determination of galactose and rhamnose in hydrolysate

Agapanthin (100 mg) was treated according to the method described in 10.4.1 and the absorbance of aliquot portions (0.5 ml) was determined by the method described in 10.4.2. From these readings, the weight of each sugar was estimated and hence the molecular ratio. The results are reported in Table 19.

Table 19. Molecular ratio of sugars in agapanthin

Sugar	Mean absorbance	Weight of sugar ug	Molar ratio
galactose	0.72	85	3.1
rhamnose	0.38	27.5	1.0

11. Extraction of sapogenins from Agapanthus rhizomes

Freshly collected rhizomes (6 kg) were sliced and minced to give a moist mash. To this mash 2N hydrochloric acid (6 l) was added and the mixture was boiled for 4 hours making up the volume of liquid lost from time to time. The hydrolysed mash was then filtered on a large buchner funnel until most of the liquid had been removed. The mash was then removed from the funnel, water added and mixed with sodium bicarbonate until neutral and filtered again. The filtered residue was dried at 80° in an oven with a circulating fan for about 48 hours. The residue was then ground to a fine powder and re-dried until there was no further loss in weight,

to provide a powder (1.6 kg). This powder was extracted with carbon tetrachloride (1.5 l) in a modified soxhlet apparatus for 24 hours. The volume of the solvent was reduced to approximately 250 ml and the extract was filtered hot giving a light brown powder (24.2 g). Tests showed that the carbon tetrachloride contained a negligible amount of sapogenin, so that hot filtration had the advantage of separating fatty material from the crude sapogenin residue.

11.1 Thin layer chromatography of crude sapogenin extract

Thin layer chromatography of the crude sapogenin extract using solvent III and spraying with sulphuric acid showed the presence of three spots at Rf 0.48, 0.57, and 0.83. A chloroform solution of antimony pentachloride (20%) as a detecting agent also revealed the presence of three spots at the same Rf values. The colours obtained with these two detecting agents are given below (Table 20).

Table 20. Thin layer chromatography of crude sapogenin extract

Rf value of spot	Colour with antimony pentachloride in chloroform	Colour with sulphuric acid
0.48	blue	purple
0.57	red	olive green
0.83	brown	purple

The spots at Rf 0.48 and 0.57 were of equal intensity but the spot at Rf 0.83 was faint and gave a faint green fluorescence under U.V. light.

12. Seasonal variations in the sapogenin content of rhizomes

Rhizomes were collected from the same source at four different times of the year.

- (i) During the summer flowering season and for two months thereafter.
- (ii) After the summer rains in late autumn.
- (iii) During the winter period from June to the end of September.

(iv) After the spring rains in October.

Samples were sliced, minced, dried to constant weight at 80° and assayed for their sapogenin content

12.1 Assay procedure

Samples of dried rhizome (10 g) were mixed with 2N hydrochloric acid, hydrolysed for four hours, neutralized with sodium bicarbonate, and filtered. The residue was dried at 80° overnight, powdered, and the sapogenin extracted with carbon tetrachloride for 24 hours. After the evaporation of the solvent the residue was acetylated by boiling with acetic anhydride (2 ml) for 5 minutes. The acetylated crude sapogenins were transferred to a 15 ml centrifuge tube with benzene (5 ml), a saturated methanolic solution of potassium hydroxide (5 ml) added and the contents vigorously mixed. Water (5 ml) was added and the tube centrifuged. The benzene layer was withdrawn and the residual aqueous methanol extracted twice with benzene.

The benzene layers containing the crude sapogenin acetates were combined, the solvent was removed and the residue was dissolved in carbon disulphide (5 ml). The infra red spectrum relative to the pure solvent was obtained between 900 - 1000 cm^{-1} . For the determination of the quantity of sapogenin acetate in the sample the 982 - 987 cm^{-1} absorption band was used. A straight line was drawn between the two points of maximum transmittance on opposite sides of the 982 - 987 cm^{-1} band. Another straight line perpendicular to the frequency axis was drawn through the point of minimum transmittance of this band. The absorbance value of the intersection of these two straight lines was subtracted from the absorbance of the point of minimum transmittance and the corrected extinction coefficient calculated from this absorbance difference.

The infrared absorption spectra of sapogenin acetates, from dried rhizomes (10 g) collected during the four seasons of the year, in carbon disulphide using a cell of path length 0.1 cm are shown in Figure 43. From these spec-

tra the corrected absorbance and hence the absorptivities of the samples were calculated as follows:

Let the transmittance at the intercept of the two lines previously described be x , and the point of minimum transmittance be y .

Corrected absorbance of sample = $\log_{10}x - \log_{10}y$

Absorptivity = $\frac{\text{Corrected absorbance of sample}}{\text{Cell path length (cm) X concentration term.}}$

The concentration term was taken as 2000 g/l and was based on the fact that the saponin acetates from 10 g of dried plant material were dissolved in 5 ml of carbon disulphide.

To act as a standard a solution of agapanthagenin diacetate (0.090 g) in carbon disulphide (10 ml) was prepared and the infrared absorption spectrum between $900 - 1000 \text{ cm}^{-1}$ determined. The corrected absorbance of this standard calculation from the spectrum (Figure 44) was:

$$\begin{aligned}\text{Corrected absorbance of the standard} &= \log_{10} 68 - \log_{10} 30 \\ &= 0.3554\end{aligned}$$

$$\begin{aligned}\text{Absorptivity} &= \frac{0.3554}{0.1 \times 9} \\ &= 0.3950 \text{ litre, g}^{-1}, \text{ cm}^{-1}\end{aligned}$$

The seasonal variation in the sapogenin content of rhizomes collected at different times of the year is given in Table 21.

Table 21. Seasonal variation in sapogenin content of Agapanthus rhizomes

Period of year	Corrected absorbance of samples	Absorptivity litre gm ⁻¹ cm ⁻¹	Sapogenin content of dried rhizomes % w/w
Summer season	.2888	.0014	$\frac{.0014}{.3950} \times 100$ = 0.37
Late Autumn	.1920	.0096	$\frac{.0096}{.3950} \times 100$ = 0.24
Winter period	.0547	.0003	$\frac{.0003}{.3950} \times 100$ = 0.07
Early Spring	.0911	.0005	$\frac{.0005}{.3950} \times 100$ = 0.12

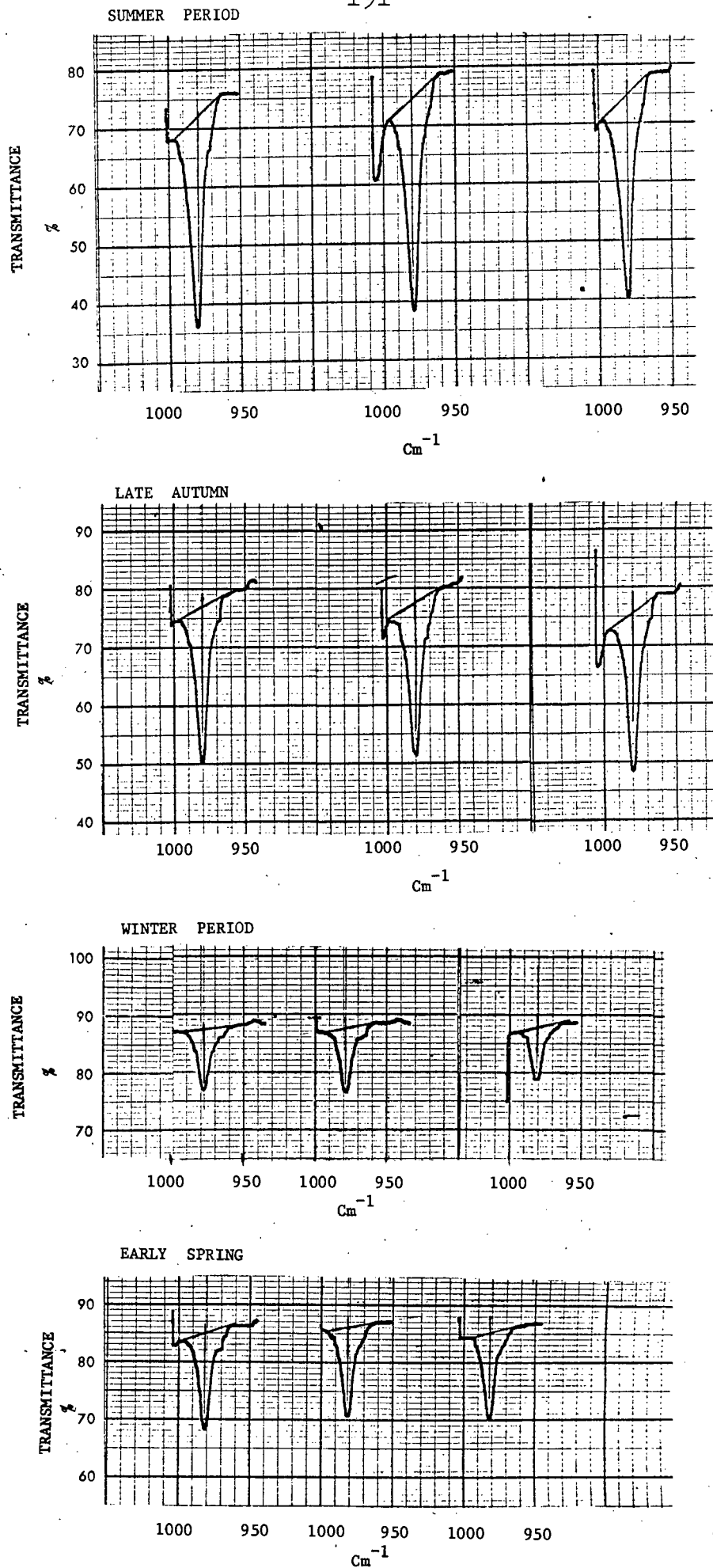


FIG. 43. IR spectra of sapogenin acetates from rhizomes (950-1000 cm^{-1} region in CS_2)

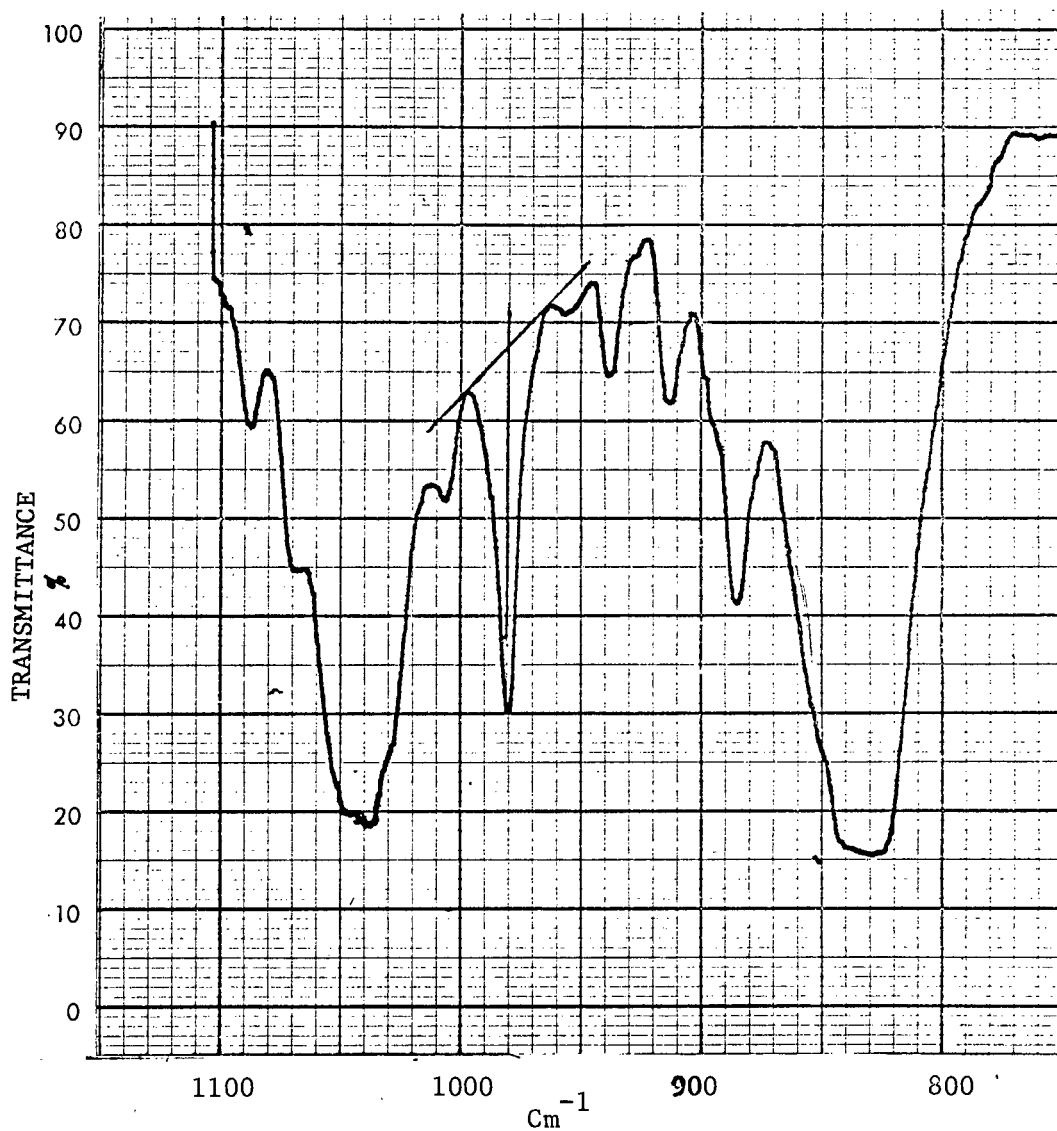


FIG. 44. IR spectrum of agapanthagenin diacetate
(800-1100 cm^{-1} region in CS_2)
concentration 9.0g/l.

13. Isolation of sapogenins from the crude sapogenin extract

13.1 Separation of agapanthagenin

The separation of agapanthagenin from praecoxigenin in crude crystalline extracts proved extremely difficult. Fractional crystallisation from solvents or solvent mixtures gave mixtures of these two sapogenins. Chromatography on columns of silica gel or florosil failed to resolve the mixture. On alumina columns, even when deactivated with 10% of 5% acetic acid, agapanthagenin was so tenaciously held that elution, even with a mixture of chloroform - methanol (1 : 1) in which agapanthagenin is readily soluble, gave negligible amounts of chromatographically pure agapanthagenin. Preparative layer chromatography using glass plates coated with kieselgel G was tried without success. Progress with this work was thus hampered by the difficulty in obtaining workable amounts of pure agapanthagenin from the mixture. After prolonged elution of the mixture (10 g) on alumina (1 kg) with chloroform - methanol (1 : 1),

the absorbent was extracted deactivated by the addition of water, dried and extracted with the same solvent mixture to give traces of a white amorphous powder which on crystallisation from chloroform yielded colourless square plates of agapanthagenin (Ia) Stephen reports m.p. 285° (19) (Found : C, 71.90; H, 10.00. Calculated for $C_{27}H_{44}O_5$: C, 72.3; H, 9.8%).

ν_{max} KBr : 3440, 3380, 3155, (O-H), 980, 918, 900, 860 cm^{-1}
(spiroketal system).

13.2 Isolation of praecoxigenin (IIa)

The crude sapogenin mixture (10 g) was chromatographed on silica gel (1 kg) and eluted with 50 ml fractions of chloroform - methanol in the proportion of 95 : 5. Fifty fractions were removed and each fraction was subjected to thin layer chromatography on kieselgel G. The elution pattern is shown on Table 22.

Table 22. Thin layer chromatography of crude saponin mixture

Fraction number	Rf value of spots
1-5	0.83
	0.57
6-50	0.57
	0.48

Fractions 6 - 50 were combined and re-chromatographed on silica gel as before, each time using the first 20 fractions eluted. By this process after 6 chromatograms the first ten fractions of the final chromatogram was shown to be chromatographically pure and after removal of the solvent yielded pure praecoxigenin (23 mg). This crystallised from ethanol to give flat needles m.p. 267-268° with decomposition,

$$[\alpha]_D^{23.6} -56.0^\circ (c, 0.88)$$

(Accurate mass (M.S.) : 428.291845, $C_{27}H_{40}O_4$ requires : 428.292643).

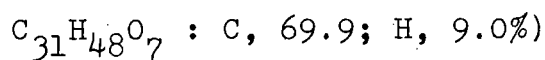
ν KBr : 3580, 3400, (O-H), 3050, 1620, (C = CH₂)
max : 980, 920, 900, 860 cm^{-1} (spiroketal system)

14 Derivatives of agapanthagenin14.1 Agapanthagenin diacetate (2 α :3 β -diacetoxy-22a-spirostan-5 α -ol) (Ib)

To agapanthagenin (500 mg) pyridine (3 ml) and acetic anhydride (1 ml) was added and the mixture left standing at room temperature for 24 hours. The mixture was poured on to ice, extracted with ether, washed successively with hydrochloric acid (5%) sodium bicarbonate solution (5%) water, and dried over anhydrous magnesium sulphate. Removal of the solvent yielded a residue (500 mg) which crystallised from ethanol in needles, Stephen reports m.p. 298-299°

$$\left[\alpha \right]_{\text{D}}^{24} -101^{\circ} \text{ (c, 2.1) (19).}$$

(Found : C, 70.1 : H, 9.1 Calculated for



ν KBr : 3500, (O-H), 1730, (ester C = O), 1265, 1235,
max (acetate), 980, 920, 900, 860 cm^{-1} (spiroketal system)

n.m.r.:

CDCl_3 : 0.72 (m, 6 protons, C-18 Me with C-27 Me superimposed)

1.05 (s, 3 protons, C-19 Me)

1.98 (s, 6 protons, acetate Me groups)

3.35 (m, 1 proton, C-17 proton)

4.35 (d, 2 protons, $J = 4\text{Hz}$ C-26 protons)

5.20 (m, 2 protons, C-2 and C-3 protons)

Pyridine:

0.65 (d, 3 protons, C-27 Me)

0.80 (s, 3 protons, C-18 Me)

1.00 (s, 3 protons, C-19 Me)

2.00 (s, 6 protons, acetate Me groups)

3.49 (s, 1 proton, C-17 proton)

4.50 (m, 2 protons, C-26 protons)

5.45 (six line pattern multiplet, 1 proton
C-2 or C-3H, A_2BX system $J_{AX} = 10\text{Hz}$,
 $J_{AB} = 4\text{Hz}$)

5.80 (six line pattern multiplet, 1 proton
C-2 or C-3H, A_2BX system $J_{AX} = 10\text{Hz}$,
 $J_{AB} = 4\text{Hz}$)

14.2 Treatment of agapanthagenin (22a-spiro-
stan-2 α :3 β :5 α -triol) with lead tetra-
acetate

Agapanthagenin (110 mg) was dissolved in a solution of lead tetraacetate (3%) in purified acetic acid (25 ml). After standing at room temperature for 24 hours, an aliquot portion (5 ml) was removed for the estimation of amount of reagent used up and the remainder was diluted with water, extracted with chloroform, washed with sodium thiosulphate solution (5%), followed by sodium carbonate solution (2N) and then water. After drying over anhydrous magnesium sulphate the solvent was removed to yield an amorphous product which crystallised from methanol in flat needles, m.p. 182-183°,

$$[\alpha]_{\text{D}}^{23.5} -30.0^{\circ} (c, 0.61).$$

$$\nu_{\text{max}}^{\text{CHCl}_3} : 3,400 (\text{O-H}), 1720, 1441 \text{cm}^{-1} (\text{aldehyde})$$

Lead tetraacetate reagent (5 ml) required 32.0 ml of 0.0196N sodium thiosulphate as compared with 26.7 ml of sodium thiosulphate required for the reaction mixture (5 ml), showing

that one equivalent of the reagent was consumed. The mass spectrum gave a peak at m/e 446 due to the molecular ion.

14.3 Preparation of 2 α :3 β :5 α -Triacetoxy-22a-spirostan (VI)

Agapanthagenin diacetate (1.8 g) was refluxed in chloroform (20 cc) with acetyl chloride (4 g) and dimethylaniline (6.4 g) for 20 hours. Water was added, the chloroform removed under reduced pressure, the excess of acid neutralized with sodium hydrogen carbonate and the product extracted with ether. The residue after removal of ether was chromatographed on alumina (30 g) with light petroleum - benzene (3 : 2) and crystallised from methanol in plates m.p. 202^o,

$$[\alpha]_D^{23.5} = -26^{\circ} \quad (c; 4.1).$$

(Found : C, 69.3; H, 8.7; C₃₃H₅₀O₈ requires : C, 69.0; H, 8.7%)

$$\nu_{\max}^{\text{CCl}_4} : 1725, \text{ (ester C=O)}, 1220 \text{ (C-O acetate)}$$

14.4 Preparation of 22a-spirostan-2 α :3 β :5 α triol-5 α monoacetate (VII)

To a solution of agapanthagenin triacetate (VI) (1 g) in methanol (45 ml) finely powdered potassium hydroxide (2.5 g) was added with shaking until a clear solution was obtained. After 30 minutes at 20° the product separated out and was crystallised from methanol as needles m.p. 230°,

$$\left[\alpha \right]_D^{23.5} -16^\circ \text{ (c, 2.1)}$$

(Found : C, 71.0; H, 9.3 C₂₉H₄₆O₆ requires :
C, 71.0; H, 9.4%)

ν KBr : 3475, (O-H), 1710, (ester C=O), 1230, 1245, acetate)
max : 980, 920, 900, 860 cm⁻¹ (spiroketal system)

14.5 Dehydration of agapanthagenin diacetate with thionyl chloride - pyridine

To a solution of agapanthagenin diacetate (Ib) (4 g) in pyridine (10 ml) at 0°, thionyl chloride (1 ml) in pyridine (5 ml) was added dropwise with shaking; the mixture was held at 0° for one hour, diluted with ice water and ex-

tracted with ether. The ether layer was shaken with hydrochloric acid (5%), sodium bicarbonate solution (5%), water and dried over anhydrous magnesium sulphate. Removal of the solvent yielded a white solid (2.6 g) which was chromatographed on alumina (250 g). Elution with light petroleum - benzene (4 : 1) yielded prismatic needles (800 mg) which crystallised from methanol in needles m.p. 178° . This product gave no depression in melting point when a mixed melting point was carried out with pure yuccagenin diacetate (IXb).

Further elution with light petroleum - benzene (1 : 4) yielded an eluate (1.2 g) which crystallised from methanol in plates of $2\alpha:3\beta$ -Diacetoxy-22a-spirost-4-en (VIIIb) m.p. $212-214^{\circ}$.

$$\left[\alpha \right]_{\text{D}}^{23.5} = -42^{\circ} \quad (c, 3.2)$$

(Found : C, 72.3; H, 8.8; Ac, 16.8. $\text{C}_{31}\text{H}_{46}\text{O}_5$ requires : C, 72.4; H, 8.9; Ac, 16.7%).

ν_{max} KBr : 1730, (ester C=O), 1225cm^{-1} (acetate)

The previous experiment was repeated using thionyl chloride (2.2 ml) at 10°. The product was isolated in the usual way and chromatographed on alumina to yield yuccagenin diacetate (IX) (950 mg), Δ^4 isomer of yuccagenin diacetate (VIIIb) (410 mg), and a dark brown oil (600 mg).

14.6 Deacetylation of 2 α :3 β -Diacetoxy-22a-spirost-4-en

2 α :3 β -Diacetoxy-22a-spirost-4-en (VIIIb) (90 mg) was heated under reflux for one hour with methanolic potassium hydroxide (5%). The methanol was removed under reduced pressure and the residue extracted with ether. After drying with anhydrous magnesium sulphate and removal of the solvent, the product was crystallised from ethanol to yield needles of 22a-spirost-4-en-2 α :3 β -diol (VIIIa), m.p. 205-206°,

$$\left[\alpha \right]_D^{23.6} -75.2^\circ \quad (c, 1.33)$$

(Found : C, 75.4; H, 9.9; C₂₇H₄₂O₄ requires: C, 75.35; H, 9.8%).

14.7 Deacetylation of 2 α :3 β -Diacetoxy-22a-spirost-5-en (IXb)

This product was deacetylated by the same method as used for the deacetylation of VIIIb to yield yuccagenin (IXa) m.p. 248°

14.8 Treatment of agapanthagenin (Ia) with methanolic potassium hydroxide

To a solution of agapanthagenin in methanol (150 ml) finely powdered potassium hydroxide (25 g) was added with stirring until a clear solution was obtained. After 48 hours at 20° excess water was added and the reaction product was filtered off. Thin layer chromatography using solvent III showed two spots at Rf 0.48 and 0.80 which would be consistent with a mixture of agapanthagenin (Ia) and yuccagenin (IXa).

14.9 Treatment of yuccagenin diacetate (IXb) and its isomer (VIIIb) with tetranitromethane

Tetranitromethane (1 drop) was added to

solutions of yuccagenin diacetate (IXa) and its Δ^4 isomer (VIIIb) in chloroform. Yuccagenin diacetate gave a dark brown colour whereas the colour of the Δ^4 isomer was pale yellow.

14.10 Epoxidation of yuccagenin diacetate (IXa)
and its isomer (VIIIb)

14.10.1 Preparation and standardisation
of monoperphthalic acid

A solution of monoperphthalic acid was prepared according to the method outlined in Organic Syntheses (56).

Into a round bottomed flask (250 ml) equipped with a mechanical stirrer and cooled in an ice bath, sodium hydroxide (50 ml of 15%) was added. The solution was cooled to -10° and hydrogen peroxide (21 ml of 30%), similarly cooled, was added in one portion. When the temperature had again dropped to -10° , pulverised phthalic anhydride (15 g) was added with stirring. When all the phthalic anhydride had dissolved, a solution of sulphuric acid (50 ml

of 20%), cooled to -10° , was added. After filtration without suction through glass wool the acid solution was extracted four times with ether (first extraction 100 ml remainder 50 ml). The combined ether extract was shaken three times with a solution of ammonium sulphate (30 ml of 40%), the ether solution dried with anhydrous sodium sulphate and stored in a fridge. The peracid content of this solution was determined by adding to aliquot portions a solution of potassium iodide (30 ml of 20%) and titrating after 10 minutes with sodium thiosulphate.

Aliquot portions of this solution (2 ml) required 12.86 ml of 0.0854N sodium thiosulphate giving a concentration equivalent to 0.05 g of monopero-phthalic acid per ml.

14.10.2 Preparation of $2\alpha:3\beta$ -Diacetoxy-
 $4\alpha:5\alpha$ -epoxyspirostan (IX)

To $2\alpha:3\beta$ Diacetoxy- $22a$ -spirost- 4 -en (VIIIb) (1.14 g) ethereal monopero-phthalic acid (21 cc; 0.05 g per ml) was added and the

mixture heated under reflux for 7 hrs. The ether was removed under reduced pressure, the residue digested with dry chloroform (25 cc) and after filtration the chloroform was removed. The residue chromatographed on alumina (100 g) gave with light petroleum ether - benzene (3 : 2) an eluate which crystallised from methanol in needles of (XI) m.p. 222-224^o,

$$\left[\alpha \right]_D^{23.5} -22^{\circ} \text{ (c, 1.8)}$$

(Found : C, 70.5; H, 8.8; C₃₁H₄₆O₇ requires : C, 70.2; H, 8.6%)

14.10.3 Preparation of 2 α :3 β -Diacetoxy-5 α :6 α -epoxyspirostan (X)

Epoxidation of yuccagenin diacetate (IXb) (160 mg) was carried out as in experiment 14.10.2 with ethereal monopero-phthalic acid (30 ml, 0.05 g per ml). The product was chromatographed on alumina (100 g) with light petroleum - benzene (4 : 1) to give a product which crystallised from methanol as needles (X) m.p. 204-205^o,

$[\alpha]_D^{23.5} 47^\circ$ (c, 3.65)

(Found : C, 70.6; H, 8.9; $C_{31}H_{46}O_7$ requires :
C, 70.2; H, 8.6%)

14.11 Reduction of the two isomeric epoxides (X)
and (XI) with lithium aluminium hydride

14.11.1 Reduction of 2 α :3 β -Diacetoxy-
4 α :5 α -epoxyspirostan (XI)

Reduction was affected by heating 2 α :3 β -Diacetoxy-4 α :5 α -epoxyspirostan (XI) (100 mg) dissolved in dry ether (20 ml) with lithium aluminium hydride (0.08 g) under reflux for two hours. Dilute hydrochloric acid was added and the product after filtering and washing was dried over calcium chloride under vacuum. Thin layer chromatography of the product using solvent III gave a dense spot at Rf 0.48 and a faint spot at Rf 0.22. Fractional crystallisation of the mixture from methyl ethyl ketone gave agapanthagenin (Ia) m.p. and mixed m.p. 284°. The second product (Rf 0.22) which was in trace amounts was not characterised.

14.11.2 Reduction of 2 α :3 β :-Diacetoxy-
5 α :6 α -epoxyspirostan (X)

Reduction of this epoxide (X) (120 mg) was carried out as in experiment 14.11.1 using lithium aluminium hydride (90 mg) to give a single reaction product which gave a m.p. and mixed m.p. with agapanthagenin of 284°.

14.12 Oxidation of agapanthagenin (Ia) with chromic anhydride

To agapanthagenin (3 g) in acetic acid (100 ml) a solution of chromic anhydride (2 g in acetic acid) was added dropwise with stirring. The mixture was kept at 20° for 45 minutes. After the addition of methanol (10 ml) most of the solvent was distilled off under vacuum. The mixture was made alkaline with ammonia, then just acid with hydrochloric acid and extracted with ether. The ether extract was well washed with water and after removal of the ether the product, after repeated crystallisation from methanol, gave needles of 5 α -Hydroxy-2:3-seco-22 α -spirostan-2:3-dioiclactone

(III) m.p. 250°

$[\alpha]_D^{23.5} -16^\circ$ (c, 4.05)

(Found : C, 70.7; H, 8.9; $C_{27}H_{40}O_6$ requires
C, 70.5; H, 8.8%)

14.12.1 Equivalent weight of the lactone -
acid (III)

The crystalline oxidation product (III) (0.1222g) dissolved in neutral methanol on titration with 0.05N sodium hydroxide required 5.35 ml, giving an equivalent weight for the lactone-acid (III) of 456.

14.12.2 Methylation of the lactone -
acid (III)

N-methyl-N-nitrosotoluene-p-sulphona-
mide (400 mg) in ether (20 ml) was added drop-
wise to 3M methanolic potassium hydroxide (10 ml)
in a distillation flask heated to 65°. Diazo-
methane which distilled over was collected in
ether (100 ml) cooled in ice. Ethereal diazo-

methane (10 ml) was added to the lactone-acid (III) (40 mg) and the reaction mixture left at room temperature for 24 hours. Removal of the solvent gave a glass m.p. 165°

ν_{max} CS_2 : 1786, (C=O lactone), 1739 cm^{-1} (C=O methyl ester)

14.13 Mesylation of agapanthagenin (Ia)

To agapanthagenin (105 mg) in 2 ml of pure dry pyridine at 0° methanesulphonylchloride (115 mg) was added and the reaction mixture was kept at 0° for 48 hours. The product was added to ice cold water, taken up in ether and worked up in the usual way to give 69 mg of a product which crystallised from methanol as needles of the dimesylate (V) m.p. 218° with decomposition

$[\alpha]_{\text{D}}^{23.5}$ -52.0° (c, 0.70)

ν_{max} CHCl_3 : 3528 (O-H), 1323, 1167 cm^{-1} (-O-SO₂-)

14.13.1 Solvolysis of the dimesylate (V)

To the dimesylate (V) (51 mg), dissolved in pure dry acetone (2 ml) in a glass vial, sodium iodide (120 mg) was added. The vial was sealed and heated at 100° for 24 hours. After cooling, the vial was opened, the solvent was removed and the residue dissolved in chloroform, which was successively washed with sodium sulphite solution (5%), water and dried with anhydrous magnesium sulphate. Removal of the chloroform yielded a product which crystallised from ether as flat needles (XIII) m.p. 205°

$$[\alpha]_{\text{D}}^{23.5} -54.0^{\circ} \quad (c, 0.84)$$

14.14 Attempted preparation of an acetonide of agapanthagenin

Agapanthagenin (25 mg) was extracted in a soxhlet apparatus for six hours with acetone (20 ml) containing p-toluenesulphonic acid. After the addition of sodium carbonate solution (5%) and evaporation to dryness the residue was

extracted with chloroform-methanol (1 : 1).

Thin layer chromatography on the residue, after the removal of solvent, using solvent system III, gave a single spot at Rf 0.48 which was indicative of unchanged agapanthagenin.

15. Kinetics of the oxidation of agapanthagenin and gitogenin with lead tetraacetate

15.1 Purification of acetic acid

Acetic acid (analar) was refluxed with chromic anhydride for thirty minutes, to render it aldehyde free, and then fractionally distilled. The water content of the distillate, as determined by the Karl Fischer method, was 1.81% w/w. This water was removed by adding acetic anhydride (198 ml) and boiling under reflux for three hours. This dried acetic acid was stored in dark bottles under anhydrous conditions.

15.2 Experimental procedure

The method as described by Hockett et al. (57)

was used. 0.2 m mole of agapanthagenin and gitogenin were weighed into separate 100 ml standard volumetric flasks, dissolved in 20 ml of acetic acid, and transferred to a constant temperature water bath controlled to 25° ($\pm .05^{\circ}$). A lead tetraacetate solution was made up in acetic acid, standardised with 0.02N sodium thiosulphate, and allowed to come to temperature by immersing the flask in the water bath. A volume of standard lead tetraacetate solution was added to the solution of these sapogenins so that the mole ratio of oxidant to sapogenin was 15.2 to 1. The volume was immediately made up to the mark with acetic acid. The time from the first contact of the oxidising agent with the sapogenin solution was noted. The reaction mixture was immediately returned to the water bath. Samples of 10 ml were removed at pre-determined times and added to 25 ml of a stopping solution containing a mixture of 0.5 g of sodium iodide and 5 g of sodium acetate.

The liberated iodine was titrated with 0.02N sodium thiosulphate using a metrohm piston

pipette. A sharp end point was obtained by adding a few drops of a 5% solution of sodium starch glycollate. In all cases the end point was taken to be the first disappearance of the blue colour. It was found that the precipitated oxidation product did not interfere with the sharpness of the end point. Experiments showed that the lead tetraacetate solutions were stable under the experimental conditions used.

Results

Initial sapogenin concentration = .002 M (Red)

Initial lead tetraacetate concentration = .0307 M (Ox)

Volume of 0.02N thiosulphate required for lead tetraacetate in a 10 ml aliquot of a blank determination = 30.7 ml (b)

Volume of 0.02N thiosulphate equivalent to the amount of sapogenin in a 10 ml aliquot of reaction mixture = 2.00 ml (a)

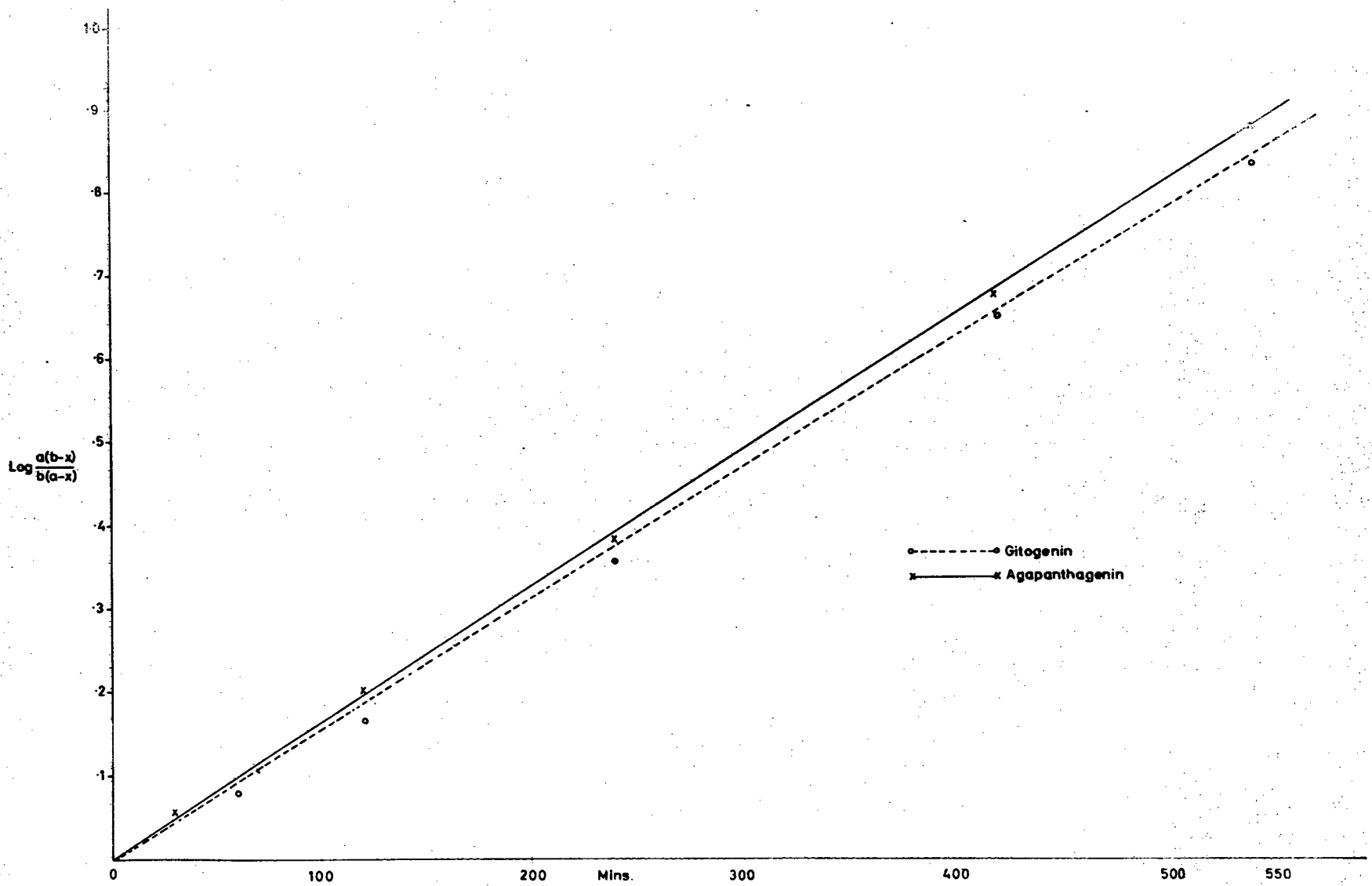


Fig.45 Glycolic oxidation of sapogenins with lead tetraacetate

The results of the kinetic oxidation of gitogenin and agapanthagenin are given in Table 23.

Table 23. Results of oxidation of sapogenins with lead tetraacetate

Reaction time (mins)	Volume of titre of 0.02N sodium thiosulphate (ml)	
	gitogenin	agapanthagenin
30	30.49	30.50
60	30.35	30.30
120	30.20	29.93
240	29.54	29.50
420	29.13	29.10
540	28.98	28.95

A graph (Figure 45) was drawn of

$\log_{10} \frac{a(b-x)}{b(a-x)}$ against time for each sapogenin.

$x = b$ - volume of the 0.02N sodium thiosulphate titre.

The slope of the line for each sapogenin was calculated by the method of least squares

and the reaction constant k calculated from the following equation

$$k = \frac{2.303 \times \text{slope}}{\text{time (secs)} \times (\text{Ox-Red})} \text{lit-mole}^{-1} \text{sec}^{-1}$$

The results are shown in Table 24 below.

Table 24. Rate constant for oxidation of sapogenins with lead tetraacetate

Sapogenin	k lit-mole ⁻¹ sec ⁻¹
gitogenin	2.07×10^{-3}
agapanthagenin	2.17×10^{-3}

16. Derivatives of praecoxigenin (IIa)

16.1 Acetylation of praecoxigenin (IIa)

To praecoxigenin (70 mg) acetylating mixture (8 ml) (pyridine - acetic anhydride 3 : 1) was added and the mixture left at room temperature for 18 hours. The product was poured on to ice, extracted with ether which was washed

successively with hydrochloric acid (5%), sodium bicarbonate solution (5%) and water. After drying with anhydrous magnesium sulphate the ether was removed to yield a residue which crystallised from ethanol as flat needles (IIb) m.p. 230-231°,

$$\left[\alpha \right]_D^{23.5} -69.0^\circ \text{ (c, 1.33)}$$

(accurate mass (M.S.); 512.312728; $C_{31}H_{44}O_6$ requires : 512.313766)

ν KBr 1732 (C=O acetate), 1242 (C-O acetate)
max : 982, 928, 900, 866 cm^{-1} (spiroketal system)

n.m.r.

$CDCl_3$ 0.67 (s, 3 protons, C-18 Me)
1.02 (s, 3 protons, C-19 Me)
2.00 (s, 6 protons, acetate Me groups)
3.49 (s, 1 proton, C-17 proton)
4.50 (m, 2 protons, C-26 protons)
5.2 (m, 3 protons, C-2 and C-3 protons
superimposing a proton of a tri-
substituted olefine)

16.2 Catalytic hydrogenation of praecoxigenin (IIa)

Praecoxigenin (8.55 mg) dissolved in glacial acetic acid (10 ml) was hydrogenated using Adams catalyst (8 mg). The uptake of hydrogen is shown in Figure 46 as ml of hydrogen against time. (Volume of hydrogen taken up, 0.46 ml, $C_{27}H_{40}O_4$ requires for one double bond, 0.45)

After hydrogenation the catalyst was removed by filtration through celite and the product was poured into excess water and left overnight. The precipitated dihydropraecoxigenin (XIV) (4 mg) was collected. The mass spectrum of this product showed a peak at m/e 430 which would confirm that one molecule of hydrogen had been taken up.

ν KBr : 3400 (O-H), 1670 cm^{-1} (C=C alkene)
max

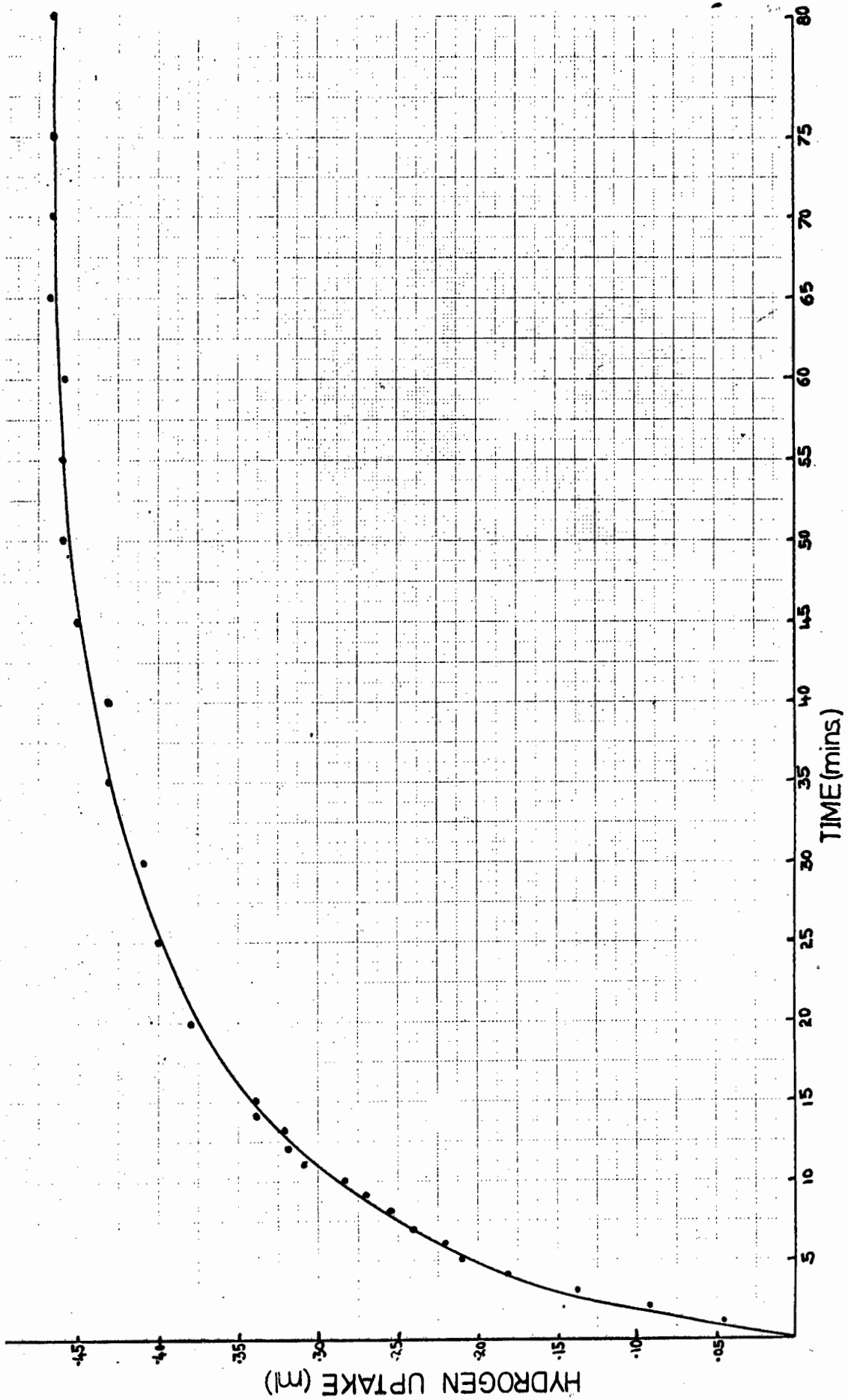


FIG. 46 HYDROGENATION OF PRAECOXIGENIN

A P P E N D I X

APPENDIXDescription and geographical distribution of Agapanthus
praecox

The plant worked on in this investigation is A. praecox Willd. subsp. orientalis (Leighton) Leighton.

The genus Agapanthus was established by L'Heritier in his Sertum Anglicum 17 (1788). The genus was placed in Liliaceae Tribus Allieae by Bentham and Hooker f. in their Genera Plantarum III : 756 (1883) and this position was generally accepted until Hutchinson in his Families of Flowering Plants Monocotyledons (1934) removed Agapantheae from Liliaceae and placed it in Amaryllidaceae. Hutchinson considered the umbellate inflorescence to be of greater taxonomic significance than the superior ovary in distinguishing the families Liliaceae and Amaryllidaceae. Phillips in his Genera of South African Flowering Plants (1951) adhered to the older classification and maintained Agapanthus in the family Liliaceae, a view still largely upheld in South Africa.

This plant was initially erroneously referred to as

A.umbellatus L'Herit. (Sert. Angl. 17 : 1788) and it is figured under this name in the first plate of the Flowering Plants of South Africa (1921). However, Miss F.M. Leighton (18) pointed out that A.umbellatus L'Herit. is in fact a synonym of A.africanus (L.) Hoffman-segg. Subsequently, in her revision of the genus Agapanthus, Miss Leighton (17) identified the plant figured as A.umbellatus in Fl. Pl. S. Afr. t. 1 (1921) as being A.praecox Willd. subsp. orientalis (Leighton) Leighton. More recently the plant has been figured in Fl. pl. Afr. t. 1476, 1477 (1966) under this latter name.

The genus Agapanthus occurs from the Cape Peninsula to the mountain ranges just south of the Limpopo River. The altitudinal range of tolerance of A.praecox subsp. orientalis is from sea level to + 1500 metres. A.praecox subsp. orientalis occurs in the Uitenhage district of the Cape Province and is spread mainly eastwards to Natal.

The plant may be described as follows:

Plant tufted from the base with robust rhizomes.
Leaves: firm, dull green and slightly glaucous, up to about 30 cm long and 3-4 cm broad on the

the flowering stems, slightly folded upwards and somewhat canaliculate, shortly tapered to the apex, with central rib down lower half of back.

Peduncles: 75 cm to 1 m tall, stout and rigid; spathe valves ovate, about 6 cm long.

Umbel: very dense with a hundred or more flowers on stiff pedicels 4-10 cm long radiating in all directions.

Perianth: mainly mauve with cobalt-blue veining, about 4 cm long and united into a tube for about $\frac{1}{3}$ of its length; lobes with a maximum spread of about 3 cm, somewhat undulate, outer oblong-lanceolate, about 7.5 mm broad, inner oblanceolate-oblong, 1-1.1 cm broad.

Stamens: attached in the mouth of the perianth tube, declinate, more or less equal in length.

Capsule: about 2.5 x 1 cm; style about 1.5 cm long.

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