APPLICATIONS OF GAS CHROMATOGRAPHY - MASS SPECTROMETRY TO ORGANIC CHEMISTRY

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CONTENTS

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B. S. Middleditch

Summary*

The work described in this thesis was based on applications of the combined technique of gas chromatography - mass spectrometry (GC-MS)[1] to a variety of topics in organic chemistry and biochemistry. The research included studies of the scope of the technique (based on model compounds) and applications to actual analytical problems.

Following the introduction, a section of the thesis is devoted to work on steroids. Results obtained with progesterone and testosterone analogues confirm the value of GC-MS in distinguishing isomers. The use of trimethylsilyl (TMS) ether derivatives in GC-MS is well established, but the advantages of (chloromethyl)dimethylsilyl ethers as derivatives for GC-MS have been little explored. The utility of these derivatives is illustrated and discussed for the example of 1/α-alkyl-17β-hydroxy steroids. The mass spectral fragmentations of TMS ether derivatives of androst-5-en-3β-ol analogues and of other unsaturated 3β-hydroxy steroids have been investigated. The results of this survey have been applied to the characterisation of yeast sterols, sterols from a bacterium (Methylococcus capsulatus) grown on methane,[2] steroidal drug metabolites,[3] and a steroidal enzyme-reduction product.[4]

Corticosteroids cannot be examined directly by GC-MS because of

*References cited in this summary are restricted to publications incorporating work described in the thesis.

the low thermal stability of the side chain. Earlier work has shown that their boronate derivatives are quite stable. The mass spectra (recorded by GC-MS) of representative corticosteroid boronates are discussed in respect of their use in structural assignments.

Similar difficulties are encountered in GC-MS of β-hydroxy amines because of their relatively high polarity and low thermal stability. The use of boronate derivatives in the characterisation of catecholamines and related β-hydroxy amines by GC-MS is discussed,[5] and a more detailed investigation of the mass spectral fragmentations of the derived 1,3,2-oxazaborolidines has been carried out.[6]

O-methyloxime (MO) derivatives are of value in the analysis by GC-MS of aldehydes and ketones. Salient features of the spectra of MO derivatives of aliphatic aldehydes and ketones are enumerated. Aldehydes from the cuticular leaf waxes of Chenopodium album L. and Lolium perenne L. have been identified by GC-MS of their MO derivatives.[8] Unsaturated aliphatic hydrocarbons from the green form of the freshwater alga Botryococcus braunii have been ozonised and cleaved to form aldehydes which have been identified as their MO derivatives. The structures of the hydrocarbons have thus been inferred.[9]

An exploratory study of the use of GC-MS in the analysis of air pollutants has been carried out. The gas chromatographic and mass spectrometric properties of some polycyclic aromatic hydrocarbons have been surveyed and a number of these compounds have been tentatively identified in dust collected from air conditioner filters.

Perfluorodecalin has been found to be a convenient mass calibration standard for low resolution mass spectra.[10] The need for, and problems associated with, computer-assisted data handling in GC-MS are discussed. The development of an on-line real-time data acquisition system is described.

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I
INTRODUCTION

INTRODUCTION

The objective of analytical chemistry is to identify and determine quantitatively the chemical constituents of sample materials. A wide variety of methods is available, but there is a constant demand for techniques which afford higher sensitivity or greater selectivity. It is generally accepted, at the present time, that combined gas chromatography-mass spectrometry (GC-MS) provides the most powerful procedure for the analysis of many organic samples. Its suitability for organic chemical analysis depends on its dual mode of operation: it first of all separates the components of a mixture by gas chromatography (GC) and then provides additional structural information by mass spectrometry (MS).

The classical analytical chemist relied on such methods as fractional distillation and crystallisation to isolate components of mixtures. Identification was possible by comparison of physical properties, such as melting and boiling points and optical activity. Characterisation of hitherto unknown compounds was carried out by correlation of chemical properties. Methods were also available for the estimation of various elements. Positive identification was carried out by synthesis of possible structures and comparison with the "unknown".

Classical procedures, both in analysis and characterisation, have been largely supplanted by more powerful methods based on

physical techniques. Infrared (IR) spectra can be used as "fingerprints" for comparison of samples,[1] and additional information is provided by the presence of absorption bands at frequencies typical of certain functional groups.[2] Nuclear magnetic resonance (NMR) spectra can be interpreted to give evidence of the environment of certain atoms in a molecule.[3] Mass spectrometry - to be discussed in more detail below - can also be used to provide spectra as fingerprints or as sources of more specific structural information.[4] Ultraviolet (UV)[5] spectra are effectively limited in application to conjugated systems, but within such a field they are often capable of giving detailed information about the chromophoric part of the molecule. Each of these methods has advantages and disadvantages and is more suited to some applications than others. To the organic chemist, however, the most important consideration is often that of sensitivity. In this respect, mass spectrometry has a great advantage over IR and NMR spectroscopy. McFadden has listed[6] typical limits of detection and identification (Table 1). It should be emphasised that the quoted limits depend to a large extent on the sample, and also that they are continually being improved upon.[7] In practice, measurements are usually carried out well above the limits of sensitivity whenever sufficient sample is available. One effect of the increase in sensitivity of nonselective analytical methods is

Table 1. Ultimate sensitivities (g.) of various analytical techniques.[6]

	Detection	Identification
Chemical analysis		
selective reagents	10^{-5}	$10^{-5} - 10^{-4}$
microreactor	10^{-6}	$10^{-6} - 10^{-5}$
IR	$10^{-6} - 10^{-7}$	$10^{-6} - 10^{-5}$
NMR	10^{-4}	3.10^{-3}
computer averaging (24 hr)		10^{-5}
MS		
standard inlet	$10^{-7} - 10^{-6}$	$10^{-5} - 10^{-4}$
direct probe	$10^{-11} - 10^{-10}$	$10^{-9} - 10^{-8}$
GC-MS	$10^{-12} - 10^{-11}$	$10^{-10} - 10^{-9}$

that samples must be isolated in a high degree of purity: impurities and artefacts often mask or modify the spectra obtained. The difficulties of handling samples in very small amounts should not be overlooked. It is, for example, impossible to isolate microgram quantities of material by fractional crystallisation or fractional distillation. Fortunately, it has been found possible to exploit the properties of solubility and adsorptivity of substances in order to effect their separation. This approach was first employed in 1906 by Tswett[8] who succeeded in separating components of plant pigments using a column of solid adsorbent though which a solvent was flowing, hence the term "chromatography". A modern definition of chromatography[9] covers the technique in its various guises: "Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of the phases constituting a stationary bed of large surface area, the other being a fluid that percolates through or along the stationary bed". The stationary phase can be a solid* ("adsorption chromatography") or a liquid* ("partition chromatography") and the mobile phase a liquid or gas. Brenner and Olson[10] have summarised the development of the various forms of chromatography as in Table 2, which has been amended to include gel chromatography. Of

*The division into adsorption and partition chromatography as between solid and liquid phases is a simplification, since silica gel has a surface gradually merging from tightly bound water to water in equilibrium with liquid water, or with atmospheric water vapour.

Table 2. Summary of the different chromatographic methods and the first significant contributors.[10]

	adsorption chromatography		partition chromatography	
stationary phase	solid		liquid	
mobile phase	liquid (LSC)	gas (GSC)	liquid (LLC)	gas (GLC)
form of development:				
elution	Tswett (1906) Kuhn, Winterstein, and Lederer(1931)	Damköhler and Thiele (1943) Cremer (1947-1951) Janák (1953-1954)	paper chromatog. Martin and Synge (1941) liquid-gel chromatog. Giddings and Mallik (1966)	James and Martin (1952) Ray (1954)
frontal analysis.	Tiselius (1940) Claesson (1949)	Phillips (1953-1954)	Phillips (1952)	Phillips (1954)
displacement	Tiselius (1943) Claesson (1949)	Schuftan (1931) Turner (1943) Claesson (1946) Turkel'taub (1950)	Levi (1949)	

particular interest in the present work are the gas chromatographic methods: gas-solid and gas-liquid chromatography.

GAS CHROMATOGRAPHY

In the gas chromatograph (Fig. 1) the sample is swept through a column by a stream of carrier gas. In gas-liquid chromatography the column is packed with a finely divided, inert solid ("support material") upon which is coated a non-volatile liquid ("stationary phase"). Alternately, the column inner wall is coated directly with stationary phase. Individual components of the mixture, which emerge from the column in an order dependent on their vapour pressures and on their affinity for the stationary phase, are detected and a "chromatogram" is produced - giving a record of their retention times and an indication of relative concentrations (which may be determined if the detector response is known). Characterisation of samples is often effected by comparison of standardised retention times, but more positive identification can be obtained by supplementary techniques such as mass spectrometry. It is possible to collect some or all of the material emerging from the column for mass spectrometric analysis. However, since the sample emerges from the column in the vapour phase, gas chromatography is amenable to direct instrumental coupling with mass spectrometry.

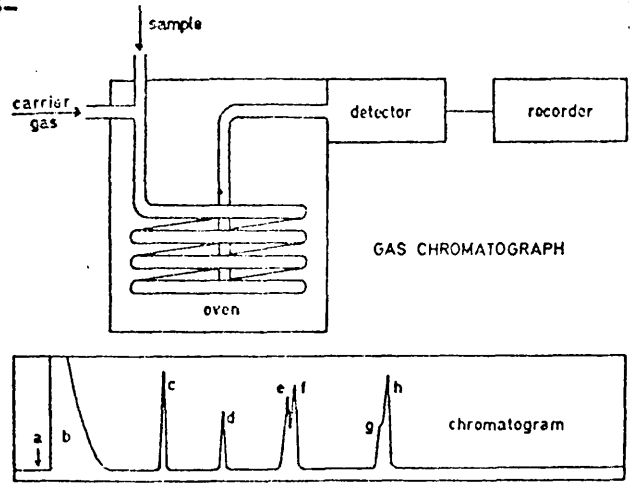


Fig. 1. Gas Chromatography (schematic).

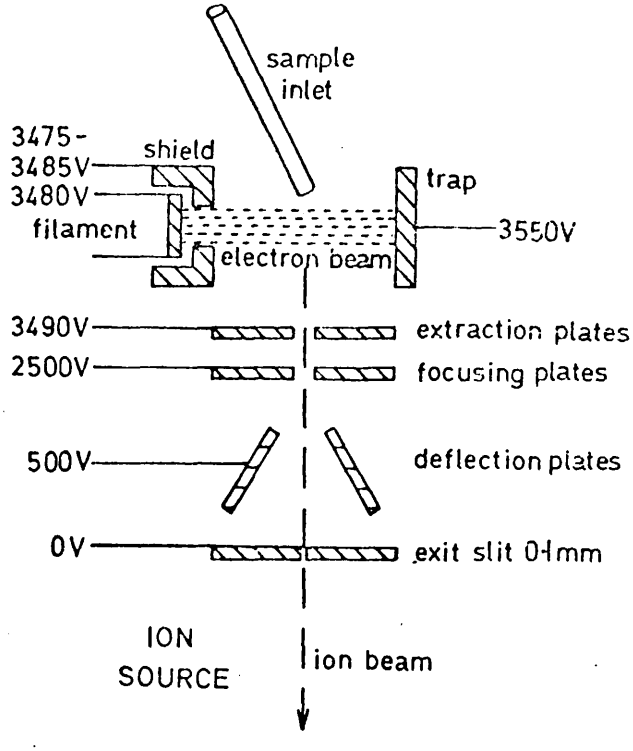


Fig. 2. The Electron-impact Ion Source (schematic).

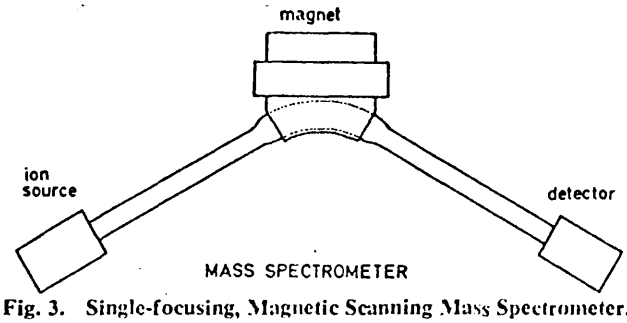


Fig. 3. Single-focusing, Magnetic Scanning Mass Spectrometer.

MASS SPECTROMETRY

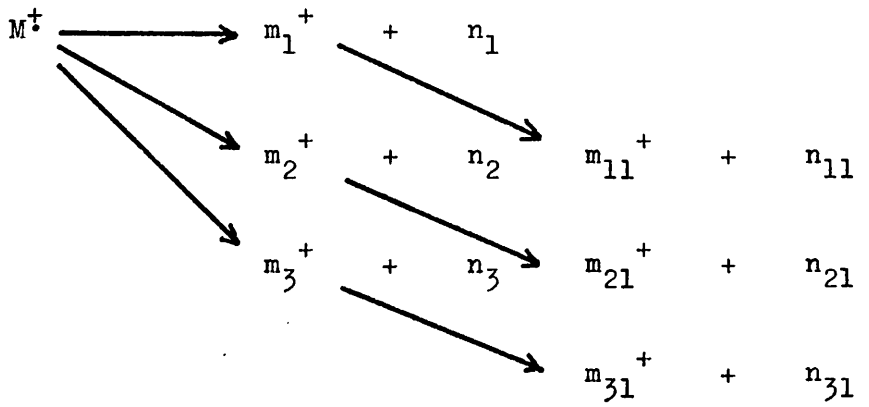
Mass spectrometers have been in use for more than half a century and have been subjected to major changes in both complexity and diversity of design and application. The principle of their operation, however, remains relatively simple: the sample molecules are ionised in a vacuum, and the energised molecular ions which are produced decompose to give characteristic fragment ions. The relative abundances of the various ions are measured, and this information is used in the identification of the sample. In the instruments most commonly used for gas chromatography-mass spectrometry, an electron-impact ion source produces a beam of positive ions, which is focused electrostatically and separated magnetically: the individual ionic abundances are recorded electrically.

In the electron-impact source (Fig. 2), the sample molecules (M) are directed into a high energy (usually 5-100 eV) electron beam. The ions produced are withdrawn from the source and directed along a flight tube through a series of slits of successively decreasing potential. If the electron energy is greater than the ionisation potential of the sample molecules, molecular ions ($M^{+\cdot}$) are produced by the loss of electrons:

$$M \longrightarrow M^{+\cdot}$$

The energised molecular ions may then decompose to give

primary fragment ions (m_1^+, etc.) which, in turn, may break down to produce secondary fragment ions (m_{11}^+, etc.), and so on until sufficient energy has been carried away by neutral fragments (n) to leave stable fragment ions:



Most of the positive ions thus produced are singly-charged, although a few may become multiply-charged by further electron-loss. In practice, singly-charged ions are assigned mass-to-charge ratio (m/e) values corresponding to the sums of the atomic weights (C=12.0000) of the constituent atoms, the charge being taken as unity. Multiply charged ions may be observed at m/e values corresponding to the appropriate fraction of their masses.

The flight tube leads into a magnetic analyser (Fig. 3) which spreads the ion beam into a "spectrum": the lighter ions are deflected more than the heavier ones. The spectrum can be "swept" over a detector by varying the magnetic field strength.

Ions emerging from the ion source have a potential

energy $\underline{e}V$, where V is the accelerating voltage. This is equal to its kinetic energy, $\frac{1}{2}\underline{m}v^2$, where v is its velocity, so:

$$\underline{e}V = \tfrac{1}{2}\underline{m}v^2$$

The magnetic field, of flux density B, exerts a centripetal force $B\underline{e}v$ on this ion. This is balanced by the centrifugal force $\underline{m}v^2/R$, where R is the radius of the ion path, so:

$$B\underline{e}v = \underline{m}v^2/R$$

$$\text{i.e.} \quad \underline{m/e} = B^2R^2/2V$$

In a magnetic-scanning instrument, $\underline{m/e}$ is directly proportional to B^2.

Occasionally, ions of mass m_1 decompose between the ion source and electromagnet to produce fragment ions of mass m_2 and neutral fragments. The neutral fragments carry away a fraction of the kinetic energy, so ions of mass m_2 formed in this region of the analyser tube possess less kinetic energy than ions of the same mass produced in the ion source. Consequently, they have lower momentum, are deflected to a greater extent, and appear at a lower apparent $\underline{m/e}$ value than the equivalent ions formed in the ion source. This apparent mass, m*, is approximately related to m_1 and m_2 by the expression:

$$m^* = m_2^2/m_1$$

The neutral particles may carry away varying amounts of energy from ions of mass m_1. Therefore, the $\underline{m/e}$ values of ions of apparent mass m* are somewhat dispersed and appear in the

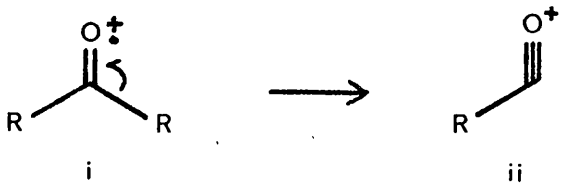
spectrum as diffuse peaks. They are erroneously referred to as "metastable" peaks. They can, of course, be used to determine fragmentation routes. Also, it is possible to ascertain that ions "linked" by a "metastable" ion are produced from the same component of a mixture.

This type of mass spectrometer, on its own, could be used to identify pure samples introduced to the ion source in the vapour phase. It is, however, usually extremely difficult to identify individual components of mixtures by direct mass spectrometry. Various methods could be used for the separation of mixtures before their identification by mass spectrometry, but gas chromatography is the most convenient because it deals with the sample already in the vapour phase.

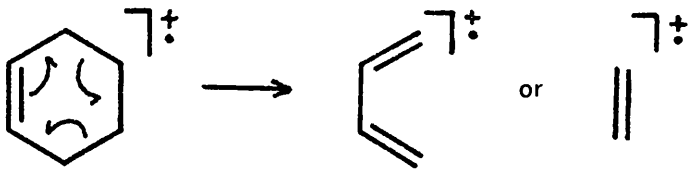
The information obtainable from a mass spectrum depends largely on the type of sample and the instrumental conditions. Most samples give a molecular ion, which is often of greater relative intensity with an ion beam of moderate energy (12-15 eV as opposed to the conventional 70eV). Examples of sample types for which the molecular ion is often absent are alcohols and acetates, for which the ions of highest mass often appear to be $[M-18]^{+}$ or $[M-60]^{+\cdot}$, respectively, corresponding to elimination of water or acetic acid. It is nearly always possible to convert an alcohol to a derivative which is more stable to electron impact, such as a trimethylsilyl (TMS) ether. In fact,

derivatives must be formed from many compounds because they are involatile.

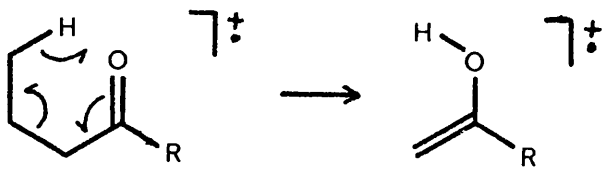
Electron impact-induced fragmentation is rarely a random process: for all but the simplest molecules some bonds are more labile than others. The preferred fragmentations may be simple, as in the α-cleavage of ketones (i→ii). They may involve more extensive electron-rearrangement as in the "retro-Diels-Alder" fragmentation of cyclohexene-type systems (iii→iv or v). Frequently, fragmentation is accompanied by atomic rearrangement, as in the McLafferty fragmentation of ketones (vi→vii). These preferred fragmentations, which are also more prevalent than others at lower electron energy, are accompanied by many more fragmentations: the complete spectrum comprising a "fingerprint" of the sample molecule. The spectra of more than 17,000 organic compounds have been published[11] and much progress has been made on the correlation of fragmentation mode with structural features.[4] It is often easy to distinguish structural isomers by mass spectrometry. For example, nordrimenone (I) gives a base peak at m/e 82 whereas isonordrimenone (II) undergoes rearrangement to give a base peak at m/e 83. These ions are the base peaks in both the 70eV and 15eV spectra and it can be seen (Fig. 4) that these ions and the molecular ions are of greater relative abundance at 15eV. The spectra of stereoisomers are usually rather similar, any differences in



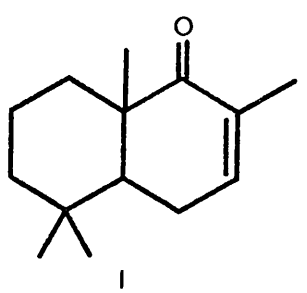
i ii



iii iv v



vi vii



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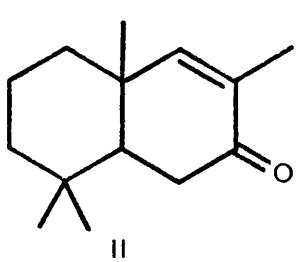

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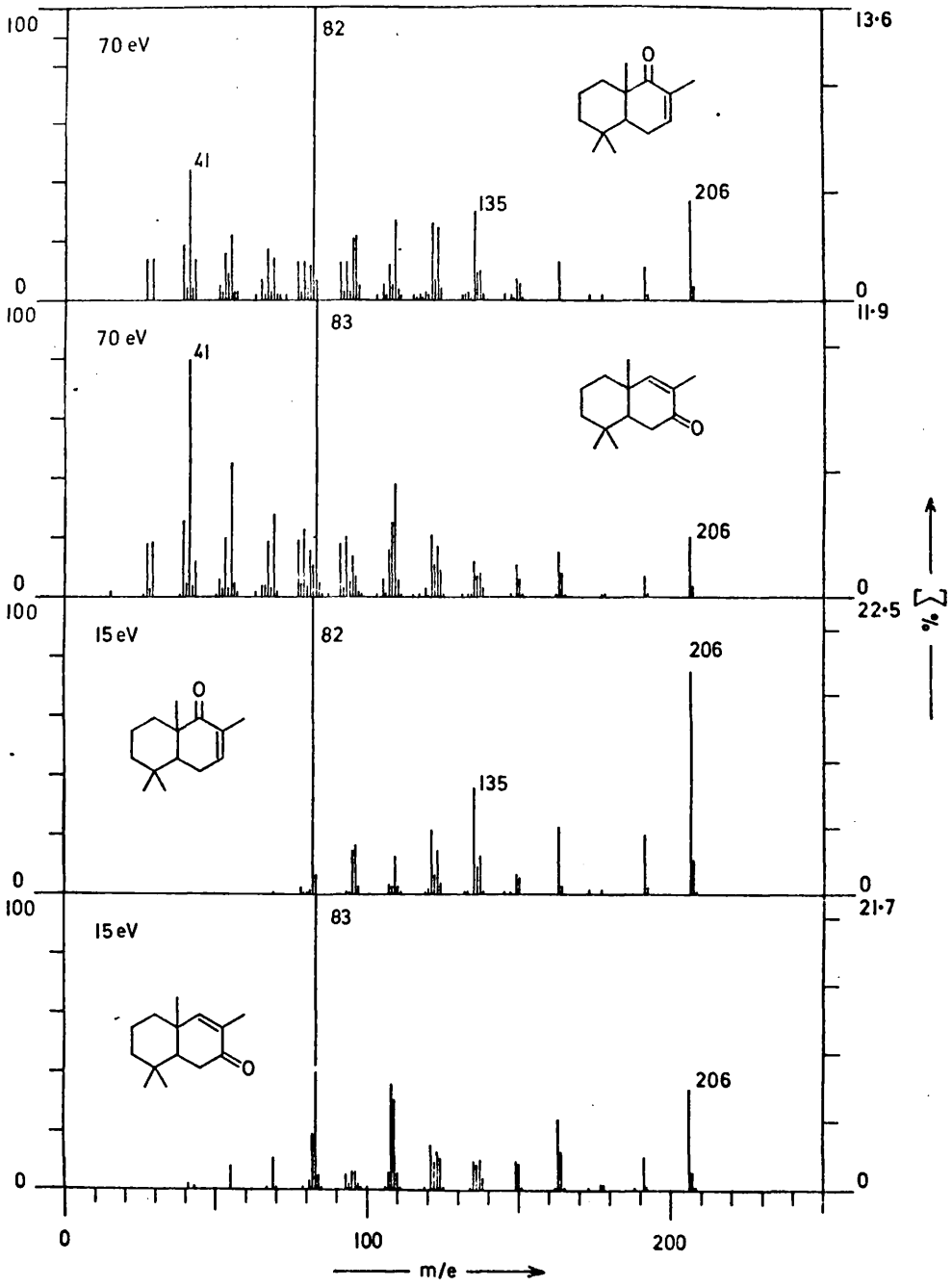

100
70 eV
82
13·6
41
135
206
0
100
70 eV
83
11·9
41
206
0
100
15 eV
82
206
135
0
22·5
100
15 eV
83
21·7
206
0
0
100
200
m/e
%

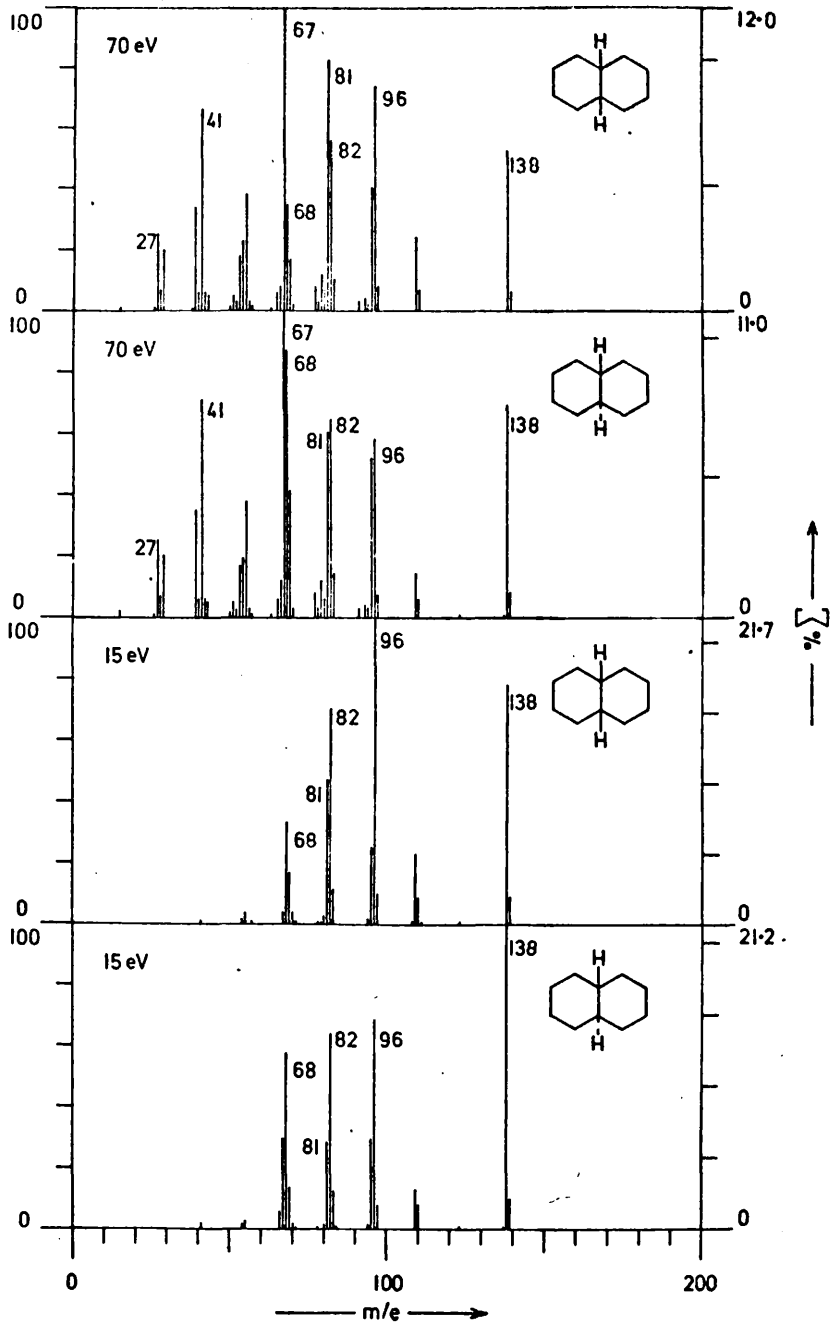
Fig. 4

relative abundances of certain ions being statistically insignificant. Such differences may, however, be accentuated in the low voltage spectra (cf. spectra of cis- and trans-decalin at 70eV and 15eV in Fig. 5). In many instances, stereoisomers are more easily differentiated by gas chromatography.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Before GC and MS can be operated in combination with each other, one major problem must be overcome: the sample emerges from the chromatograph in a stream of carrier gas, whereas the mass spectrometer must operate under high vacuum (Fig. 6). The carrier gas flow rate through the gas chromatograph is, typically, about 30 ml/min. A 1 μg sample emerging during a period of 10 sec. would, therefore, be present in much less than 1% concentration in the gas stream. A suitable ion source pressure could be maintained if only a small proportion of the total effluent from the gas chromatograph were introduced to the mass spectrometer, but this would lead to an extremely low net sensitivity of the combination. Selective removal of all or the majority of the carrier gas would be preferable. Several methods are, in fact, available for carrying this out, each based on the different physical properties of the carrier gas and sample.

Various types of porous tube separator (Fig. 7) have


100
70 eV
67
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41
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12·0
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70 eV
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68
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138
96
27
0
11·0
0
100
15 eV
96
138
82
81
68
0
21·7
0
100
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138
82
96
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81
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21·2
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100
200
m/e
% ∑

Fig. 5

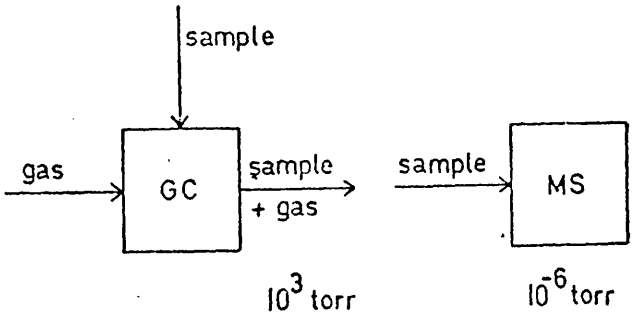


Fig. 6 Gas and sample flow, and typical operating pressures in GC-MS.

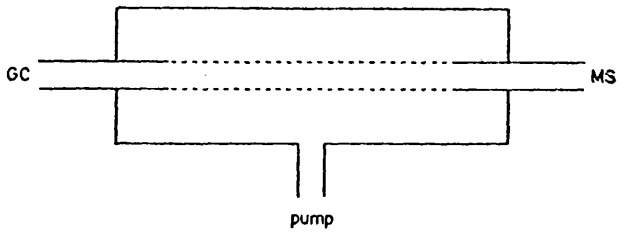


Fig. 7 Porous Tube-type Molecule Separator.

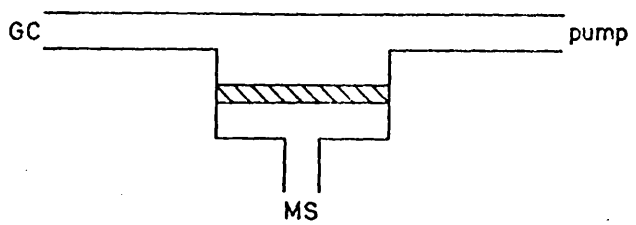


Fig. 8 Membrane-type Molecule Separator.

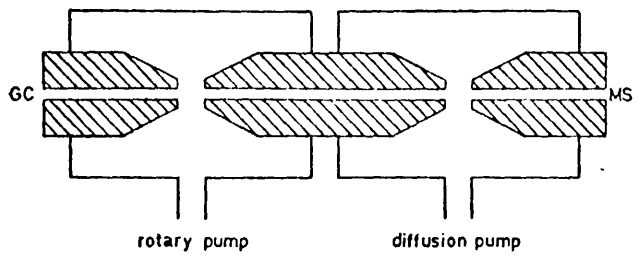


Fig. 9 Jet-type Molecule Separator.

been described. They make use of the preferential diffusion of carrier gas (helium) through the porous tube, which is surrounded by a chamber maintained at a low pressure by means of a rotary pump. The separator tube can be made of porous glass,[12-14] Teflon[15,16] or porous stainless steel.[17] The porous glass separator is the most commonly used, but suffers from the disadvantage that polar samples can become adsorbed onto the glass surface. This leads to "tailing" of the sample and often imposes a lower limit on the sample size.[18] This problem can be overcome to some extent by "silanization" of the glass.[19] Teflon is apparently more susceptible to "memory" effects.[20]

The membrane-type molecule separator (Fig. 8) makes use of the selective permeability of the sample molecules through a silicone membrane.[21,22] The carrier gas (which need not be helium) is removed by a rotary pump. An interesting feature of this type of separator is that there is a time delay of several seconds between the sample's leaving the column and entering the ion source: an ancillary detector at the exit of the column can alert the operator before the sample actually enters the mass spectrometer.

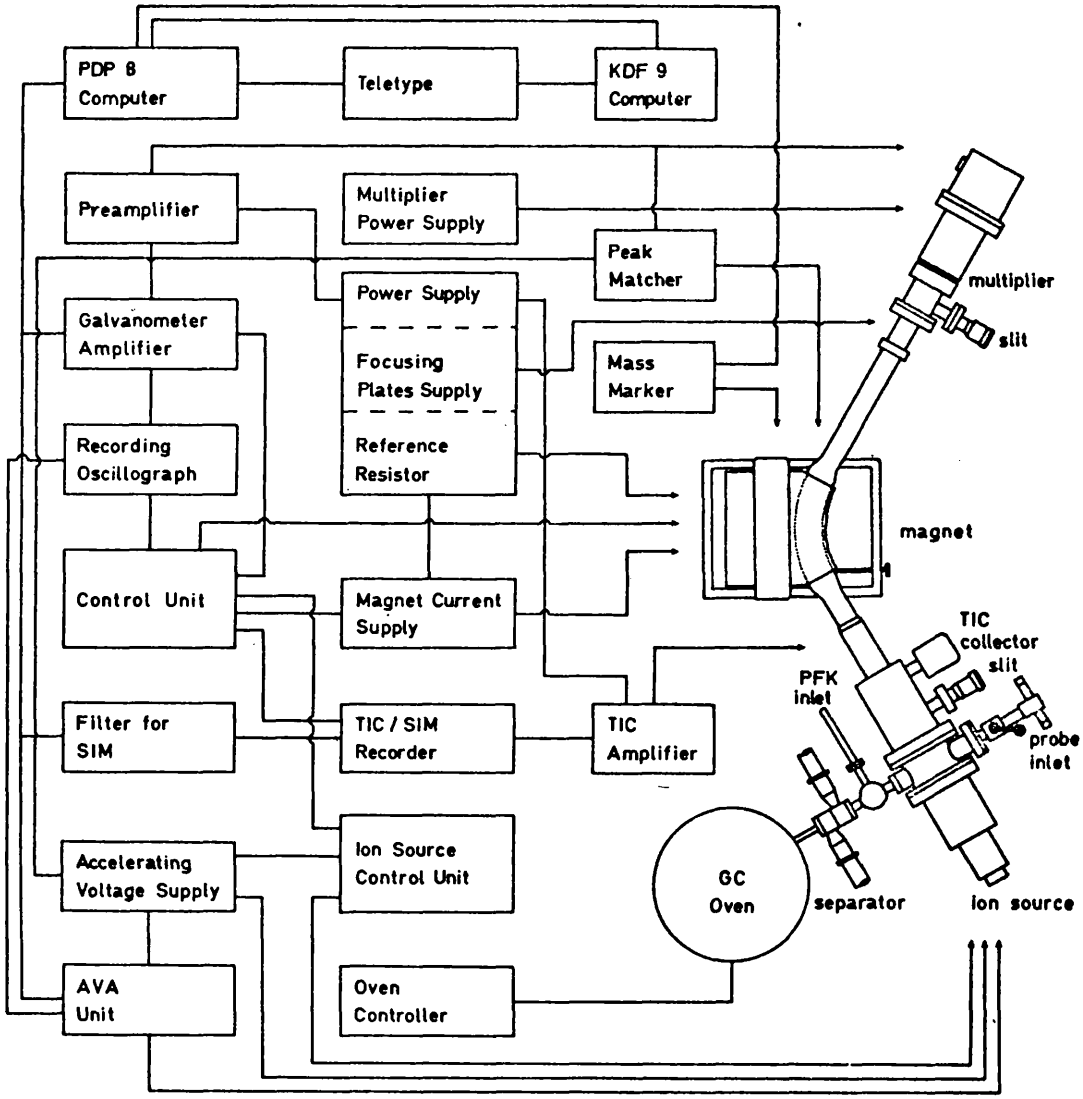
The jet-type separator (Fig. 9) comprises two pairs of stainless steel jets, each pair surrounded by an evacuated chamber: the second chamber is at a lower pressure than the first.[23, 24, 25] The carrier gas, again helium, diffuses away

from the supersonic stream more rapidly than the sample and is selectively removed. Since the sample makes little or no contact with the separator, there is not the same problem of decomposition as is encountered with the other separators.

Each separator has its advantages and disadvantages but the choice depends largely on the problem of commercial availability unless the laboratory has appropriate workshop facilities. The geometry of the interface, in limiting remixing of sample, is apparently more critical than "dead" volume.

Sample enrichment is not the only problem to be overcome in the direct coupling of gas chromatography with mass spectrometry. The sample may be emerging from the gas chromatograph for only a short period of time. The mass spectrometer, then, has to be modified for rapid scanning. In one commercially available combined instrument, the magnetic field can be increased from 0 to 14 kgauss (corresponding to m/e 0-1000) in under 5 sec: the ions are detected by an electron multiplier connected to a wide-band D.C. amplifier, and the mass spectra are produced on a recording oscillograph. A chromatogram is obtained by monitoring the total ion current.[26] (Fig. 10).

It has been mentioned that helium is often used as a carrier gas, particularly with porous tube and jet-type separators. The value of such an inert carrier gas in preserving labile



GAS CHROMATOGRAPHY - MASS SPECTROMETRY INSTALLATION

Fig. 10

compounds up to the point of electron impact should not be underestimated. Precise retention data are of prime importance in many structural assignments, and care should be taken to ensure that retention indices at the positions of actual MS scans are noted. This can usually only be effected by determining retention times from runs carried out with mixtures of sample and suitable standards. Obviously, the sample must be well resolved from the standards.

GC-MS is not, by any means, limited to the instrumentation described above: many other types of mass spectrometer have been used, including time-of-flight,[27] quadrupole, and double-focusing spectrometers. If capillary columns are used in the gas chromatograph, the carrier gas flow rate may be so low as to obviate the need for a molecule separator.

ANCILLARY TECHNIQUES OF GC-MS

The combination GC-MS instrument is capable of performing operations other than conventional GC-MS, but the flexibility of any particular model depends on its design.

Single Ion Monitoring. The magnetic field or accelerating voltage may be adjusted so that any ion in the spectrum is brought into focus on the detector. If the abundance of ions of this mass is monitored during a GC run, a "single ion" chromatogram is produced. Substances giving rise to character-

istic ions in their spectra may be selectively detected. For example, Henneberg and Schomburg have used this method to detect lead alkyls in petroleum (m/e 207 = Pb^+).[28] We have found[29] that single ion monitoring can be successfully employed to detect steroids containing distinctive structural features (Fig. 11) and have applied the technique to the detection of possible drug metabolites in urine extracts (see below). Using a two-channel potentiometric recorder, we have been able to determine the position of the possible metabolites in the total ion current chromatograms so that a full spectrum could be obtained during a subsequent run.

Multiple Ion Detection. Using a similar method, but with two adjacent detectors, it is possible to monitor simultaneously the intensities of two classes of ion. This has been carried out by Gorshkov et al.[30] who claim that the ratios of abundances of common fragment ions, such as m/e 39 and 41 or 41 and 43, are of diagnostic significance in the analysis of petroleum naphthas. However, this technique can only be used if appropriate instrumentation is available [eg. MC-1307 ("CHROMASS-2"), MS-1, M2-2M, MI-1305] and is rarely employed outside the Soviet Union.

A more widely applicable method was developed by Sweeley et al.[31] for use with conventional mass spectrometers.

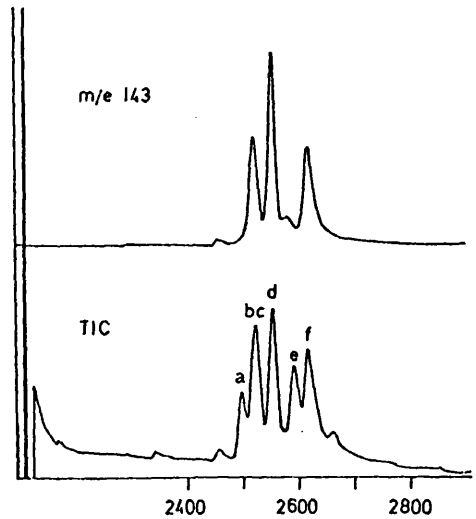


Figure 11 Simultaneous single ion (m/e 143) and total ion current (TIC) chromatograms of TMS derivatives of 17β-hydroxy-5β-androstan-3-one (a, I = 2495), 17β-hydroxy-17α-methyl-5β-estran-3-one (b, 2520), 5β-androstane-3α, 17β-diol (c, 2525), 17α-methyl-5β-estrane-3α, 17β-diol (d,2560), Δ^4-17β-hydroxyandrosten-3-one (e, 2590) and Δ^4-17β-hydroxy-17α-methylestren-3-one (f, 2615). Conditions: 6 ft OV-1,190-240° at 3°/min.

The two or more ions of interest are sequentially brought into focus onto the detector by varying the accelerating voltage. The detector signal is reproduced by an oscillographic recorder with a slow chart speed. In this manner, several single ion chromatograms may be produced simultaneously. This is of particular value if partially resolved GC eluates give different characteristic ions or if the compounds of interest each give rise to more than one characteristic ion. The method has been used with success in the search for metabolites of Chlorpromazine.[32]

Isotope Labelling. Hydroxylic, enolic and other readily exchangeable hydrogen atoms can be selectively replaced by deuterium atoms by GLC with a suitable stationary phase.[33-36] The presence of strongly acidic or basic catalysts, *e.g.* phosphoric acid[33] or potassium hydroxide,[34] is required for effective replacement of enolic hydrogen atoms by deuterium atoms. Deuterium labelling is used extensively in the investigation of mass spectral fragmentation modes.[4] Conversely, the mass shifts of parent and fragment ions upon deuteriation are a valuable indication of their structure and hence of the environment of functional groups in the molecule. GC-MS affords a useful method of labelling and identifying small quantities of material, and exploratory investigations have

been carried out on steroidal ketones and their derivatives.[37] Apiezon L was selected as the stationary phase because it has low polarity and good thermal stability. It had, moreover, also been shown to be compatible with potassium hydroxide in the GLC of free amines.[38,39] Barium hydroxide was found to be a suitable catalyst since, although it is a weaker base then potassium hydroxide, it gave satisfactory results and caused less column "bleed" - a critical consideration for GC-MS. The columns used were packed with OV-1 (1%), and Apiezon L (1%) incorporating barium hydroxide (1%) on Gas-Chrom Q (100-120 mesh). Before use, the column was "saturated" with deuterium by injection of D_2O or MeOD.

There have been various reports of isotopic exchange of radiolabelled ions by GLC[40-43], but it should be pointed out that, at the isotope dilutions employed, mass spectrometry is of insufficient sensitivity to differentiate the labelled molecules.

Reaction Gas Chromatography. Many systems have been devised for modification of sample before, in, or after the GC column.[44-46] However, little use has been made of the potential of combined GC-MS in this field, identification of products resting mainly on retention times. Examples of reaction gas chromatography are:

(i) Hydrogenation. Hydrogen is usually used as carrier

gas, with a precolumn containing catalyst. The technique was first employed by Rowan for the analysis of hydrocarbons.[47] The method has since been extended to the study of a wide variety of compounds.[48,49] Practical applications have included the identification of insect attractants,[50] the queen bee substance,[51] alkaloids,[52] and fatty acids from wool wax.[53]

(ii) Dehydrogenation. There has been less practical application of dehydrogenation, but studies have been made on alicyclic and heterocyclic compounds[54] and monoterpenes.[55,56]

(iii) Carbon Skeleton Chromatography. In an extension of the work of Thompson et al.,[57] Beroza et al. have developed a technique for the catalytic saturation of multiple bonds and stripping of functional groups containing oxygen, nitrogen, sulphur and halogen from the molecule. This work has recently been reviewed by Beroza.[58]

(iv) Pyrolysis. Pyrolysis of samples often gives rise to products which yield characteristic chromatograms (pyrograms). This is particularly useful for the identification of polymers: Groten has found that different pyrograms were obtained for each of more than 150 polymers.[59] The technique is of limited use for samples of low molecular weight, although it has been shown that cis- and trans-isomers of some hydrocarbons give different pyrograms.[60]

(v) Other Techniques. Many more applications of reaction-GC have been described, but few have been used in conjunction with GC-MS.

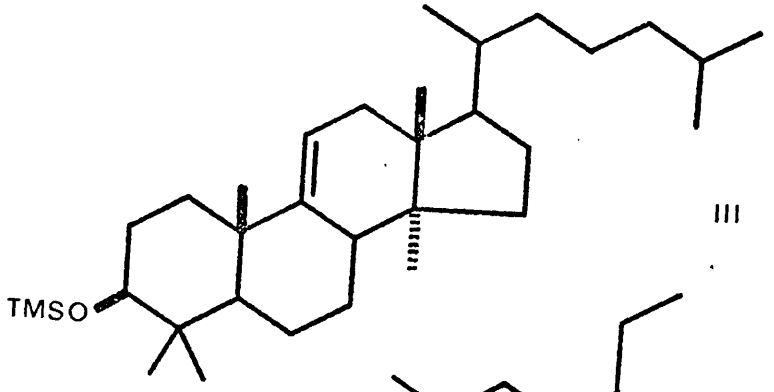
Other Techniques. Various instrumental techniques have been used in direct combination with GC,[61] but only infrared spectroscopy[62] has been used to any great extent in tripartite combination with GC and MS.[63] It is usually difficult to obtain infrared spectra at the rate at which samples emerge from a GC column, but a technique has been developed for interrupting the carrier gas flow during IR and MS scans.[64]

A more recent development, pioneered by Lovelock et al.,[65,66] is carrier gas transmodulation. If hydrogen is used as the carrier gas, it can be selectively removed by passage through a heated palladium-silver tube. It may be replaced, if required, by another gas. A logical development of this technique is the inclusion of hydrogenation or dehydrogenation processes between the gas chromatograph and the mass spectrometer, thus providing further useful information on the structures of the samples.

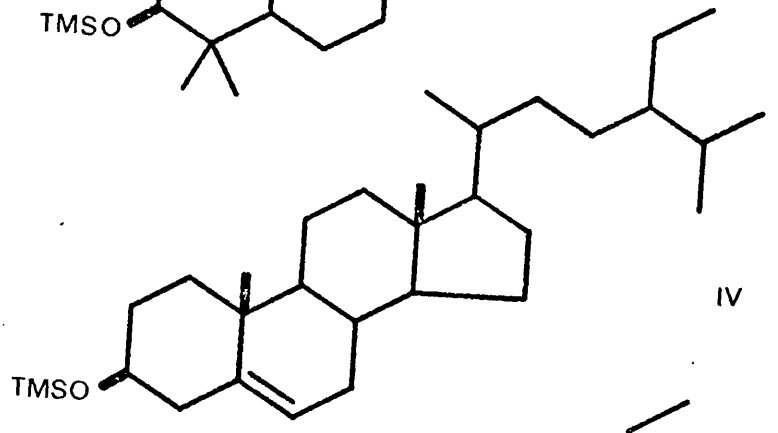
Precise Mass Measurement. While unlikely to be of use with combined GC-MS,[67] precise mass measurement facilities are available for a number of low resolution mass spectrometers to be used in conjunction with conventional sample introduction systems. If the mass of any ion can be determined at sufficiently

high accuracy (normally within 5-10 p.p.m.) it is possible to calculate the empirical formula of the ion.[68] Tables have been compiled to facilitate this task.[69,70] Direct measurement of mass[71] is only feasible with a suitable high-resolution instrument. It is also possible to calculate ratios of masses to a high degree of accuracy by measurement of the ratio of the accelerating voltages required to focus each of a pair of ions onto a detector.[72] This is known as "peak matching" because, in its practical form,[73] the method involves superposition of MS peaks on an oscilloscope screen. This latter method is also suitable for use with low-resolution instruments, although it cannot be used for measurements of peaks comprising unresolved isobaric ions (i.e. ions of the same nominal m/e). In practice, low-resolution peak matching is limited to molecular ions and simple fragment ions, such as $[M-15]^+$ and $[M-18]^{+\cdot}$.

We have applied the technique to the investigation of plant sterols.[74] A 3-component mixture was found to give TMS ethers with molecular ions at m/e 486 (major component), 484 and 472 (minor component). The authentic TMS ether III was used as a mass standard because it was of similar volatility and molecular weight. The precise mass of the molecular ion of III was 500·4413. 2 μg each of the standard and the unknown mixture were introduced to the ion source via the



III



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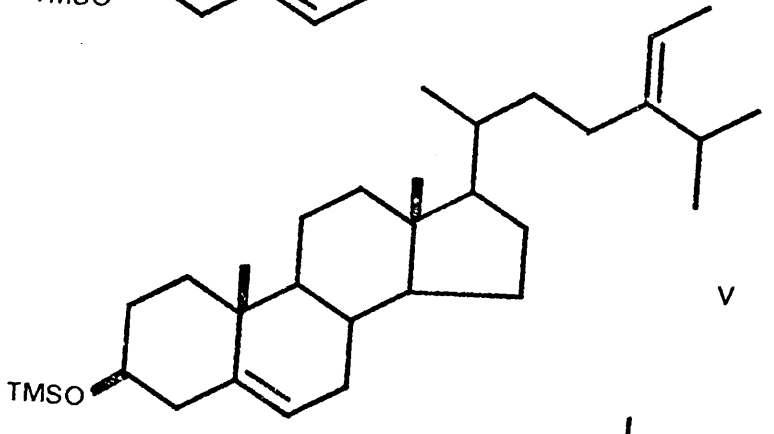

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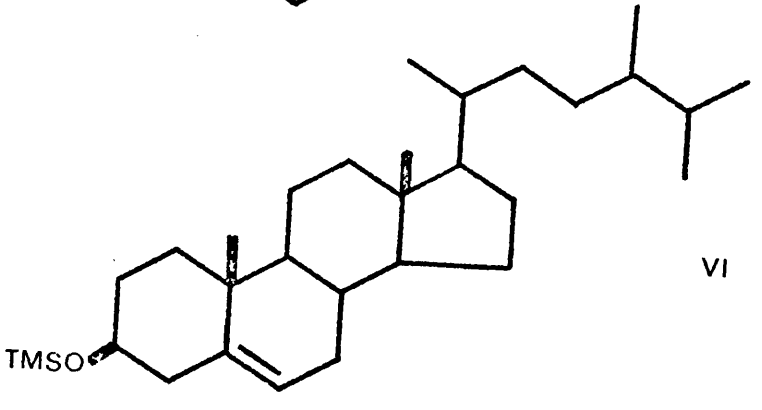


VI

direct probe and the molecular ions of the standard and the major component of the mixture were located by adjustment of the accelerating voltage. Final adjustment of the peak matcher controls was carried out using a further 5 μg sample of each. The measured ratio of the masses of the molecular ions was 1·028806, giving a molecular weight for the major component of 486·4292. This is within 7 p.p.m. of $C_{32}H_{58}OSi$ (486·4256). Subsequent examination of the mass spectrum showed that the major component was β-sitosterol TMS ether (IV). Similar measurements were carried out on the other two components of the mixture, and they were identified (with the aid of GLC) as fucosterol TMS ether ($C_{32}H_{56}OSi$)(V) and campesterol TMS ether ($C_{31}H_{56}OSi$)(VI).

APPLICATIONS OF GC-MS

The immense value of GC-MS as an analytical tool can be gauged by the number of papers which have been published on the subject: more than 300 in the past five years alone. Several reviews of the literature of GC-MS have appeared, including those contributed by Leemans and McCloskey,[75] Ryhage and Wikström,[26] and McFadden.[6] A more detailed review has recently been presented by Watson,[76] and more recent work has been summarised by Brooks.[77] Geochemical applications

have been described by Calvin,[78] and Eglinton and Murphy.[79] Useful bibliographies are compiled periodically by LKB-Produkter,[80] and GC-MS abstracts by Science Technology Agency.[81]

The main advantages of GC-MS over other methods of chemical analysis are that only very small quantities of materials are required (typically, 1 μg) and mixtures are separated and characterised in the same instrument. GC-MS has, therefore, an enormous potential in the field of natural product analysis. Examples of applications which have already been exploited are given in Table 3. The power of the technique is well illustrated by its application to the study of lipids from diseased human arteries: sufficient material for analysis by modern chromatographic techniques can be obtained from single aortas, and mass spectrometry is the only generally-applicable technique suitable for characterisation of the small quantities of components so separated.[82]

GC-MS is also of great utility even when relatively large quantities of material are available. This is particularly so for the study of flavours and aromas although here, too, it is advantageous to work on a small scale.[83] Some examples of aromas which have been investigated are given in Table 4.

It is rather difficult to define the sensitivity of GC-MS: it depends on the sample. High sensitivities could

GC-MS APPLICATIONS: NATURAL PRODUCTS

STEROIDS IN:	HYDROCARBONS IN:
Amniotic fluid	Algae
Bile	Bacteria
Blood	Bituminous coal
Brain	Cattle manure
Faeces	Foodstuffs
Insects	Fungal spores
Plants	Petroleum
Urine	Plant waxes

AMAZONIAN HALLUCINOGENIC DRUGS
DRUG METABOLITES
EMULSIFIERS IN GASTRIC JUICE OF CRABS
HOP CONSTITUENTS
INSECT JUVENILE HORMONES
LIPIDS OF DISEASED HUMAN ARTERIES
LIPIDS OF QUEEN OF ORIENTAL HORNET

Table 3

GC-MS APPLICATIONS: AROMAS

Apple	Hop oil
Banana	Human breath
Bee	Locust
Beef	Maple syrup
Black currant	Milk
Bone glue	Orange
Cheese	Pea
Clover	Peanut
Coffee	Strawberry
Cranberry	Tobacco

Catty odours in food
Weed taint in milk

Table 4

be quoted for samples which give simple but highly characteristic spectra, such as benzene, toluene, methyl chloride and carbon disulphide. For samples which give more complex spectra, larger quantities are required. Some samples are detectable, by virtue of characteristic ions, at much lower concentrations than are needed to obtain a full enough spectrum for identification purposes. For example, we have applied the single ion monitoring technique to detect TMS derivatives of 17α-methyl 17β-hydroxy steroids (see below) in quantities of less than 10 ng. The recording of full spectra of good quality, however, requires about 1 μg of sample. Another limitation on sensitivity is due to loss of sample on the GC column: cortisone t-butyl boronate gives fairly intense spectra with 3 μg of sample, but only very weak spectra with 1 μg. In general, we have found that satisfactory spectra are usually obtained for samples of 1 - 3 μg if the GC conditions are adjusted to give retention times of 15 - 20 minutes.

Additional problems arise from GC. Column "bleed", if it is too high, can rapidly contaminate the ion source of the mass spectrometer. For this reason, low concentrations of stationary phase are employed, typically 1%. Column temperatures are maintained at 250° or less: samples of high retention index are best run on shorter columns. The homogeneity of GC

peaks can be checked by multiple scanning[84] and useful data can be extracted from unresolved GC peaks by the multiple ion detection technique.[31]

The following sections of this thesis relate to work carried out by the author since October 1967. His previous use of GC and MS was confined to analysis of vapour phase photolysis and reaction products: in this work, MS analysis was carried out on collected GC fractions.[85]

The GC-MS facilities available for the present work in the Chemistry Department of the University of Glasgow were based on an LKB 9000 gas chromatograph-mass spectrometer (LKB-Produkter AB, Stockholm). This comprises a gas chromatograph with temperature programmer, a Ryhage-type jet molecule separator and a single focusing mass spectrometer equipped with a 60^{o} sector, 20 cm radius magnetic analyser and sweep generator for fast scanning of spectra. A rhenium filament is used to provide an ion source of the electron bombardment type. The measuring system comprises a 14-stage electron multiplier, electrometer and a wide-band amplifier feeding a direct-writing UV oscillograph. A direct probe inlet was available and a heated inlet system for the introduction of marker substances (see below) was constructed by the author. During the three-year period, several more accessories were installed: accelerating

voltage alternator (for multiple ion detection) (April 1969), mass marker (June 1969) and peak matcher (March - April 1968 and July 1970). Various gas chromatographs and other equipment were available, as referred to in the text.

The sections on GC-MS of steroids and boronate derivatives comprise part of a progressive programme of work on the development of methods and techniques of GC-MS analysis. The section on aliphatic compounds describes work carried out primarily in collaboration with workers in other laboratories. The work on particulate air pollutant analysis was a trial of the suitability of GC-MS for studies in this area, and the section on data handling details our attempts at automation of this phase of GC-MS.

II
STEROLS

STEROIDS

Steroid analysis poses a particularly challenging problem for the analytical chemist. Firstly, there is (within the group of similar tetracyclic structures) a multiplicity of skeletal types, with both nuclear and side chain variants. There is also the possibility of multiple bonds and substituent functional groups, such as hydroxylic, ketonic, acidic and aldehydic. Finally, the question of stereochemistry must be decided. Perhaps no single technique, other than X-ray crystallography, is capable of solving all of these problems. Much structural information can, however, be gleaned from gas chromatographic and mass spectrometric data of steroids and their derivatives. Moreover, only small quantities of material are required for gas chromatographic-mass spectrometric investigation.

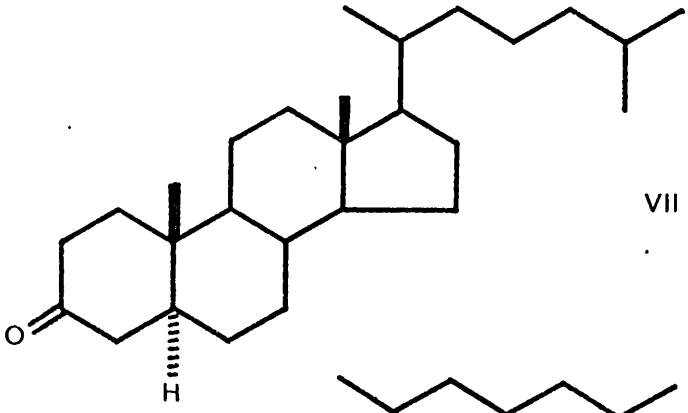
Rapid progress has been made in the field of gas chromatographic analysis of steroids since the first report[86] in 1959. Reviews have been compiled at frequent intervals.[87-103] Mass spectrometry has made similar progress since 1956,[104] although fewer general reviews[105-107] have been compiled: mainly because of the fact that GC has attained wider acceptance as a practical tool, particularly in the biomedical field. A recent review[77] of GC-MS gives good coverage of applications to steroids. The approaches used in gas chromatographic-mass spectrometric investigation of steroids are best discussed in three sections, dealing

respectively with steroid ketones, sterols and multifunctional steroids (including corticosteroids).

(i) Ketones. More work has been carried out on the mass spectrometry of steroidal ketones than on any other steroid type. Consequently, more is known of the fragmentations associated with the various ketones. Many of these processes have been clarified by deuterium labelling studies, particularly by Djerassi's group.[108] It is found that, in general, the ketone function has little fragmentation-directing influence. In the absence of other functional groups, there may be ions at $[M-18]^{+\cdot}$ and $[M-33]^{+}$ corresponding to successive elimination of water and a methyl group, but the spectra are normally dominated by peaks due to "erosion" products of the steroid nucleus, chiefly eliminations of fragments from rings A and D of the nucleus. Transfer of hydrogen atoms is widespread and often random. Certain structural configurations may, however, give rise to favoured fragmentation modes. For example, the McLafferty rearrangement of 20-isopropyl-5α-pregnan-16-one produces an ion (m/e 259) accounting for more than 80% of the total ion current.[109] The presence of a double bond in a suitable position may have a pronounced effect on the fragmentation of steroidal ketones. The spectra of Δ^4-3-ketosteroids have been the subject of comprehensive studies: they are discussed further in the following section. It should be noted that, in general, the molecular ion is stabilised by the presence of a conjugated

enone moiety. Vinylic fission is generally a very unfavourable process and "typical" fragmentations of steroidal ketones[110] and their derivatives[111] are often suppressed in the spectra of these compounds. The apparently anomalous formation of ions at m/e 43 and $[M-43]^+$ from Δ^{16}-20-ketones has been rationalised by postulation of loss of the 17-acetyl group accompanied by concerted migration of the angular C-18 methyl group.[110] It is often advantageous to examine the spectra of fragmentation-directing derivatives which enhance the structural differences of the parent molecules.

Ethylene ketal, ethylene thioketal and dimethylamine derivatives are particularly specific in their fragmentation-directing behaviour.[112] This high specificity is often somewhat of a disadvantage since little general structural information is obtained from the spectra. For example, loss of ring A is negligible in the spectrum of cholestan-3-one (VII) but gives rise to a peak of medium intensity in the spectrum of coprostan-3-one (VIII).[113] The spectra of the derived ethylene ketals IX and X are virtually identical.[114] On the other hand, Δ^4- and Δ^5-3-keto-19-methylsteroids[115] and Δ^4-, Δ^5, and $\Delta^{5(10)}$-3-keto-19-norsteroids[116] can be well distinguished by mass spectrometry of their ethylene ketals. Also, Whalley and co-workers have shown[117,118] that 19-nor- and 19-methyl-3-ketosteroids can be distinguished by mass spectrometry of the derived 2-spiro-2'-(1,3-dithian) analogues: highly characteristic ions are observed



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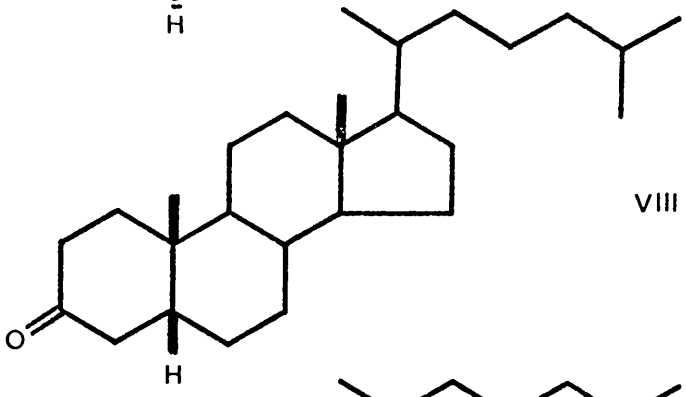

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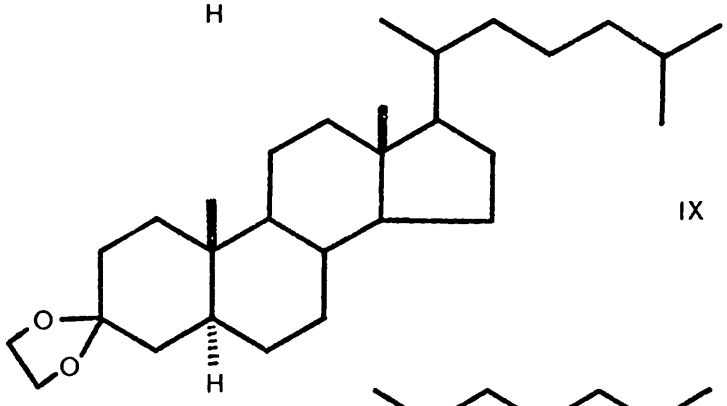

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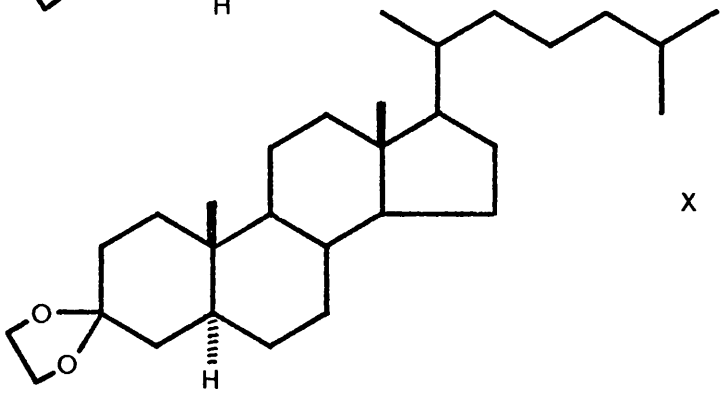


X

at m/e 145 and m/e 159, respectively. The results are not so sharply defined in the spectra of derivatives of steroids having a double bond in ring A or B. The gas chromatographic separation of ketosteroids as ethylene thioketal derivatives has been described,[119] but the most widely used derivatives for gas-phase analytical characterisation of steroidal ketones are the oximes, particularly the O-methyloximes.[120] Syn- and anti-oxime derivatives may be formed from steroidal ketones, and in certain instances can be separated by GC[121-125] or TLC.[122,123,126] The relatively high polarity of simple oximes essentially limits their use to mono-ketones which have no other functional groups. This range of application can, however, be extended by conversion to the much less polar O-trimethylsilyloximes.[111,127-130] A further type of derivative suitable for GC-MS of steroidal ketones is the enol-TMS ether.[131,132] As mentioned in the Introduction, ketones can be readily deuteriated in transitu in GC-MS, and considerable information can thereby be obtained about the environment of the ketonic function. Another approach to the characterisation of steroidal ketones is via their reduction to sterols, for which a different range of methods is available.

(ii) Sterols. Spiteller-Friedmann and Spiteller have reviewed[107] the mass spectral fragmentations of sterols. Other than the ubiquitous elimination of water from the molecular ions and from certain fragment ions of sterols (a process which is also observed in the spectra of

many ketones), the majority of the fragment ions arise from fragmentation of the unsaturated parent steroid. Fissions directed by the hydroxyl group are often greatly accentuated in the spectra of the TMS ether derivatives.[133] These derivatives are of lower polarity than the corresponding sterols and thus exhibit excellent gas chromatographic properties.[134] Many papers have been published on the GC-MS of steroid TMS ether derivatives, both with reference compounds and natural products. Many of the early observations are reviewed by Brooks *et al.* in a comprehensive paper [135] on sterol trimethylsilyl ethers and the recent literature is cited in the first of the Chemical Society Specialist Periodical Reports on Mass Spectrometry.[77] It should be noted that rearrangement ions may be produced from sterols *via* migration of trimethylsilyloxy groups[136-138] analogous to those observed in the spectra of other TMS derivatives.[139-151] Comparison of the spectra of TMS derivatives with those of the corresponding d_9-TMS derivatives[152] gives a clear indication of the TMS-containing ions. Similarly, mass shifts are observed in the spectra of (chloromethyl)dimethylsilyloxysteroids, which also have higher retention indices.[153] The retention indices of the bromo- and iodo-analogues[154] are even higher, but the iodo compounds have been used as derivatives of estradiol and estrone for electron-capture GC.[155] Acetates have long been used as derivatives for mass spectrometry of sterols,[156] but they are of limited use for GC-MS

since they have relatively high retention indices. The acetate function is readily eliminated, as acetic acid, under electron impact and has little fragmentation directing influence. Trifluoroacetates were used[157] in much of the early work on GC and GC-MS of bile acids and sterols. Heptafluorobutyrates,[158] which have retention times similar to trifluoroacetates, are useful in electron-capture GC and are suitable for routine gas chromatographic analysis of estrogens.[159] They have also recently been used as derivatives for GC-MS,[160] but because of the high molecular weights (Oestriol tris-heptafluorobutyrate = 876) obtained they are of limited applicability. Methyl ethers also appear to be useful derivatives for GC-MS.[161]

(iii) Multifunctional steroids (including corticosteroids)

The most widely used derivatives are the TMS ethers (of sterols) and O-methyloximes (of steroidal ketones) and, accordingly, if a steroid contains both ketonic and hydroxyl functions it may be advantageous to use the joint derivative (O-methyloxime trimethylsilyl ether: MO-TMS) for gas chromatographic-mass spectrometric analysis. In practice, it is relatively easy to prepare the MO-TMS derivatives by sequential reaction of the functional groups. The gas chromatographic and mass spectrometric properties of these derivatives are found to be such as would be expected of steroids containing MO and TMS moieties.[121]

A major problem of corticosteroid analysis by vapour phase

methods is that many of the characteristic C-17 side chains are thermally labile. For example, it has been shown that corticosteroids with a 17α,21-dihydroxy-20-oxo side-chain (cortisol, cortisone, and 11-deoxycortisol) are partially degraded to 17-ketosteroids and changed also in other ways, giving complex peaks in the expected C_{21}-region.[162] The various methods which have been applied to gas chromatographic analysis of thermal degradation products and oxidation products of corticosteroids have been reviewed by Bailey.[163] It is obviously advantageous to carry out analytical work with a derivative which retains the side chain - particularly for GC-MS. TMS ether derivatives are useful for polyhydroxy compounds,[164] and TMS-enol TMS derivatives have recently been shown to stabilise effectively the side chains of various 20-keto steroids.[165] The MO-TMS derivatives of Gardiner and Horning[121] are more widely used. Cyclic dimethylsilyl derivatives have been found to be useful derivatives for diols.[166] We have investigated the use of cyclic boronate derivatives for GC and GC-MS analysis of diols and ketols: these results are discussed below.

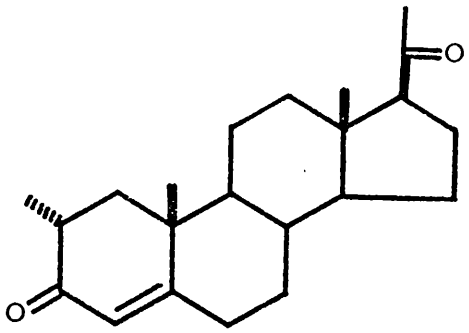
As an aid to the identification of steroids by MS, Spiteller and co-workers have recently compiled lists of "key fragments" of steroids and their derivatives (with references). It is claimed that it may be possible to use these in an automated system (computer or punched card) to characterise "unknown" steroids.[167-170] The amount of information required for a claim of "identification"

depends to some extent on what is known of the source and history of the sample, but it seems likely that we are nearing a situation where steroid identification can be carried out with confidence even if no authentic sample is available for comparison.[171]

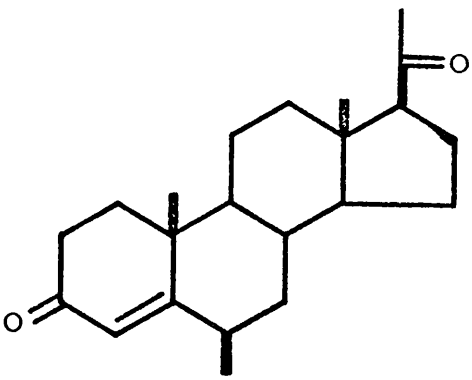
GC-MS of steroids and derivatives is exemplified, below, by accounts of investigations carried out on both authentic steroids (and their derivatives) and those isolated from natural sources. The mass spectrometry of boronate derivatives of steroids will be discussed in a later section of this thesis.

DIFFERENTIATION BETWEEN STEROIDS OF SIMILAR STRUCTURE BY GC-MS

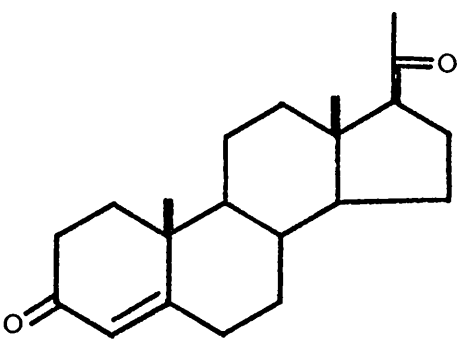
Molecular weights of the majority of steroids may be ascertained by GC-MS. For those steroids which give molecular ions in low abundance, eg. some sterols, the molecular weights can usually be inferred from those of suitable derivatives. A major problem of analysis of steroids by GC-MS is, then, that of differentiating between isomers. Fortunately, as described in some detail in the previous section, different functional groups, or their derivatives, give rise to characteristic fragmentation modes. Positional isomers can often be readily distinguished if substituent shifts in major fragment ions can be demonstrated. For example, 2α-methylprogesterone (XI) can be distinguished from 6β-methylprogesterone (XII) by comparison of ions formed by fragmentation of ring A (Figs.12 and 13). Elimination of C-2 and C-3, with substituents, gives rise to an ion at $[M-56]^{+\cdot}$



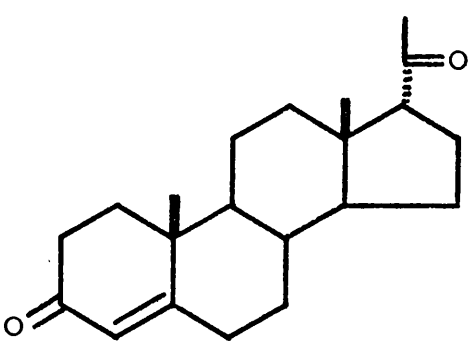
XI



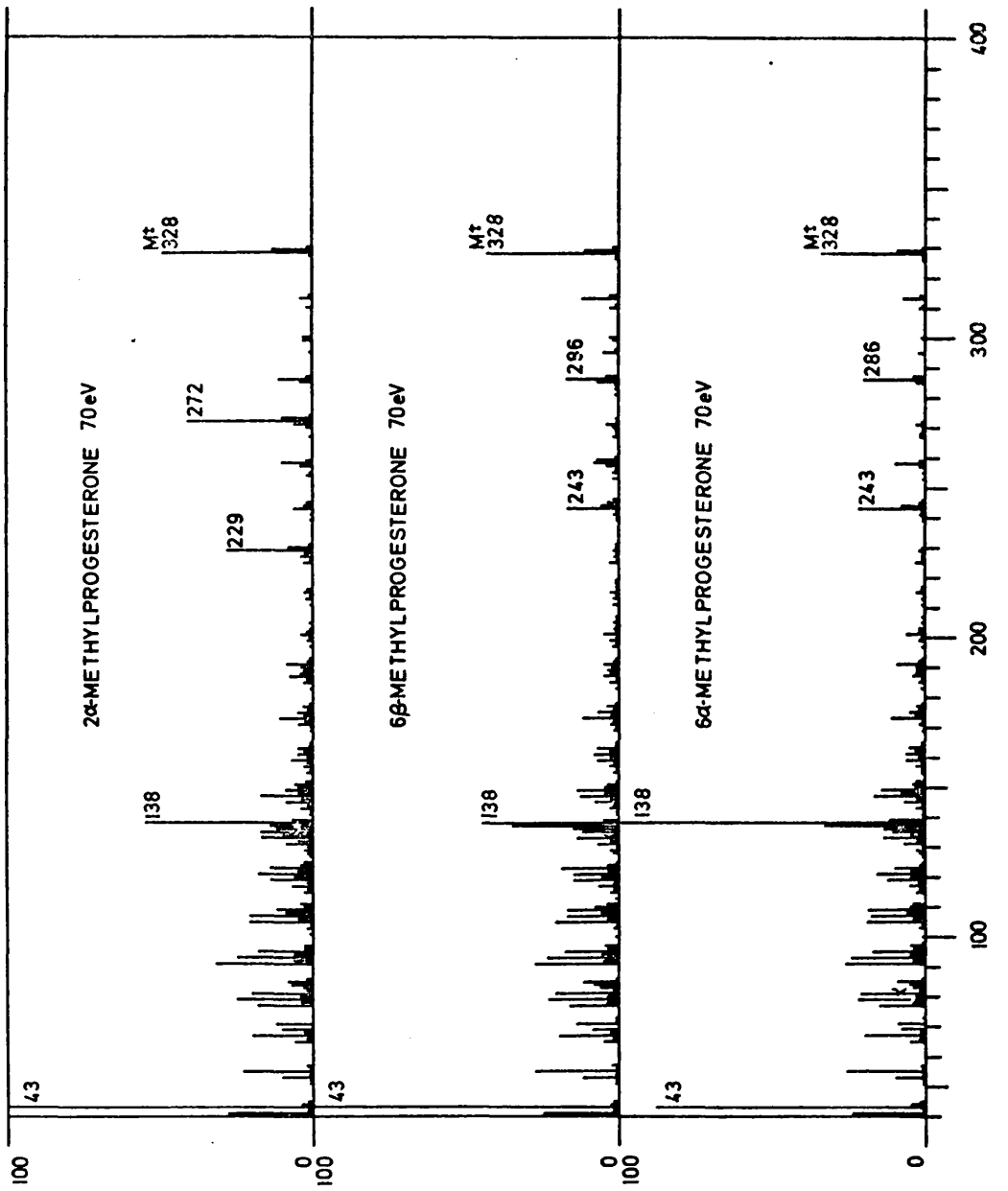
XII



XIII



XIV


2α-METHYLPROGESTERONE 70eV
6β-METHYLPROGESTERONE 70eV
6α-METHYLPROGESTERONE 70eV
43
138
229
243
272
286
296
328
M+
100
0
100
200
300
400

Figs 12-14

(m/e 272, 41%) in the spectrum of XI, and an ion at $[M-42]^{+}$ (m/e 286, 17%) in the spectrum of XII. This mass shift is not immediately apparent, since there is a fairly abundant ion at m/e 286 (11%) in the spectrum of XI. A further indication of the structural difference in ring A is given by ions due to further loss of 43 m.u. (the 17-acetyl group). These ions appear at m/e 229 (28%) and m/e 243 (17%) in the spectra of XI and XII, respectively. These differences are even more striking in the spectra obtained at an electron energy of 15eV. A large number of other cases of differentiation between positional isomers could be cited.

Characterisation of double bond isomers of steroid TMS ethers is discussed in a subsequent section (p. 107). Double bonds in certain positions in the steroid nucleus or side chain give rise to characteristic fragmentation modes, such as the retro-Diels-Alder and McLafferty rearrangements.

Mass spectra of stereoisomers are, in general, qualitatively similar; even quantitatively, they usually differ but little.[172] Such differences as exist may be enhanced by employing lower electron energies,[173] reduced source temperature,[174] or a photoionisation source.[175] These methods apparently minimise thermal reactions and reduce the initial energy content of molecular ions, thereby producing fragment ions via shorter decomposition sequences. Ions arising from

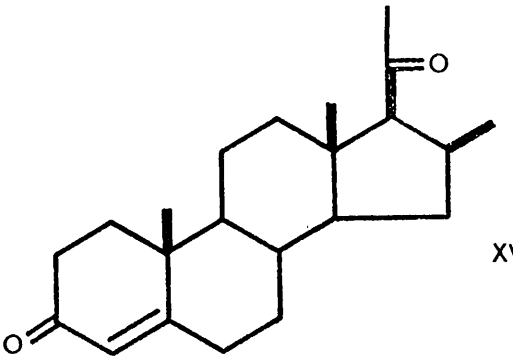
primary and secondary processes are produced in greater abundance than those formed by subsequent fragmentations and are more likely to reflect the initial molecular structure.[172]

A wealth of mass spectral data on epimeric steroids has been produced by Zaretskii and co-workers. They have discussed the spectra of epimeric steroidal secondary[176] and tertiary[177-179] alcohols as well as ring-junction stereoisomers of steroids[180-182] and related model compounds.[183] The initial work[177] indicated that differences between spectra of epimers were due to steric "crowding". It was also noted that water was eliminated more readily from epimers with axial hydroxyl groups than from those with equatorial hydroxyl groups. Following criticism from Pandit _et al._[184] that the heated metal sample-introduction system employed could give rise to inadvertent thermal dehydration, the work was repeated using a glass inlet system and a lower source temperature. It was found that differences between spectra of epimers were even more pronounced.[178] Even so, deuterium labelling experiments showed that some dehydration preceeded _via_ a 1,2-elimination, indicating that thermal decomposition was taking place.[185] There is therefore some doubt as to the authenticity of these results but, under controlled conditions, it is apparently possible to distinguish ring junction epimeric steroids and terpenoids by examination of "ionisation efficiency" curves (_i.e._ plots of ion currents _vs._ electron energy).[186]

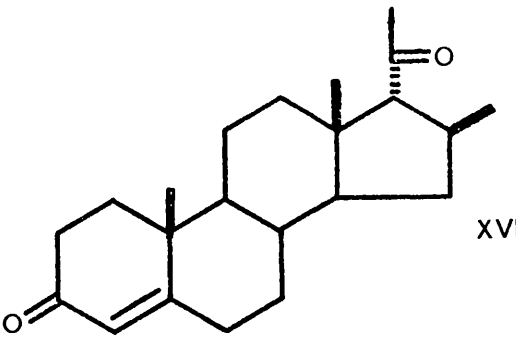
Zaretskii _et al._ have also found significant differences between

the spectra of progesterone (XIII) and 17-isoprogesterone (XIV) and between those of 16β-methylprogesterone (XV) and 16β-methyl--17- isoprogesterone (XVI).[187] This is somewhat surprising in view of the report by Djerassi's group that the mass spectra of 5α-pregnan-20-one (XVII) and its 17α-epimer (XVIII) are very nearly identical.[188]

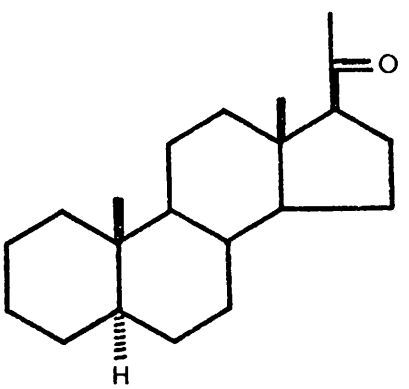
It is now reported that significant differences exist between the spectra of XV and XVI when these are obtained by GC-MS. These differences are more notable in the 15eV spectra [Fig. 15 (XV) and Fig. 16 (XVI)] than in the 70eV spectra. The $[M-84]^{+\cdot}$ ion (m/e 244, 100%) accounts for 68% of the total ion current in the spectrum of XVI. This ion is presumably formed by elimination of C-16, C-17 and substituents from the molecular ion. Analogous ions are observed in the spectra of 5α-pregnan-20-one (XVII), 5α-pregnane,[188] 16,16--dimethylprogesterone, and its 17α-epimer.[187] Fragmentation of XV occurs to a much lesser extent ($M^{+\cdot}$: m/e 328, 100%, 16.6%Σ) and is more random in nature. The ion of m/e 244 is present in relatively low abundance (38%, 6.5%Σ). The major fragment ion (m/e 124, 77%), formed by cleavage of the C-6/7 and C-9/10 bonds and concomitant transfer of two hydrogen atoms, is typical of certain steroidal Δ^4-3-ketones. There are also ions at $[M-124]^{+\cdot}$ (m/e 204, 19%) and m/e 205 (37%), presumably formed by related processes. A furtner ion, characteristic of the Δ^1- and Δ^4-3-ketone moieties, arises at



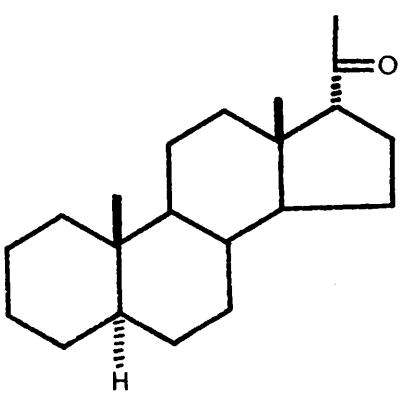
XV



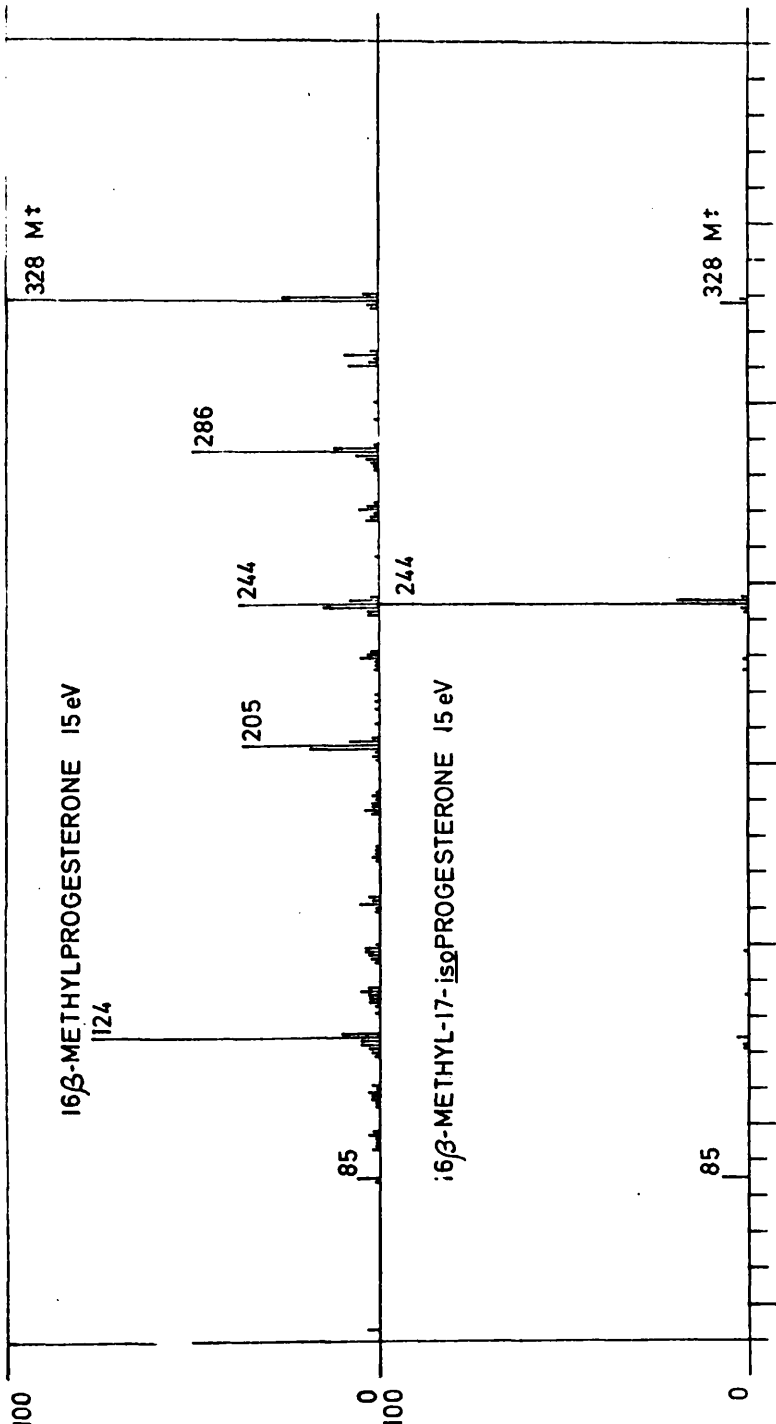
XVI



XVII



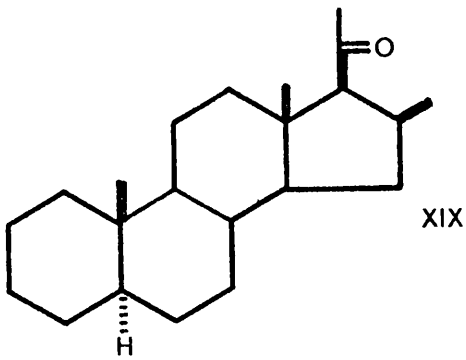
XVIII


16β-METHYLPROGESTERONE 15 eV
328 M+
286
244
205
124
85
100
0
16β-METHYL-17-isoPROGESTERONE 15 eV
328 M+
244
85
100
0

Figs. 15,16

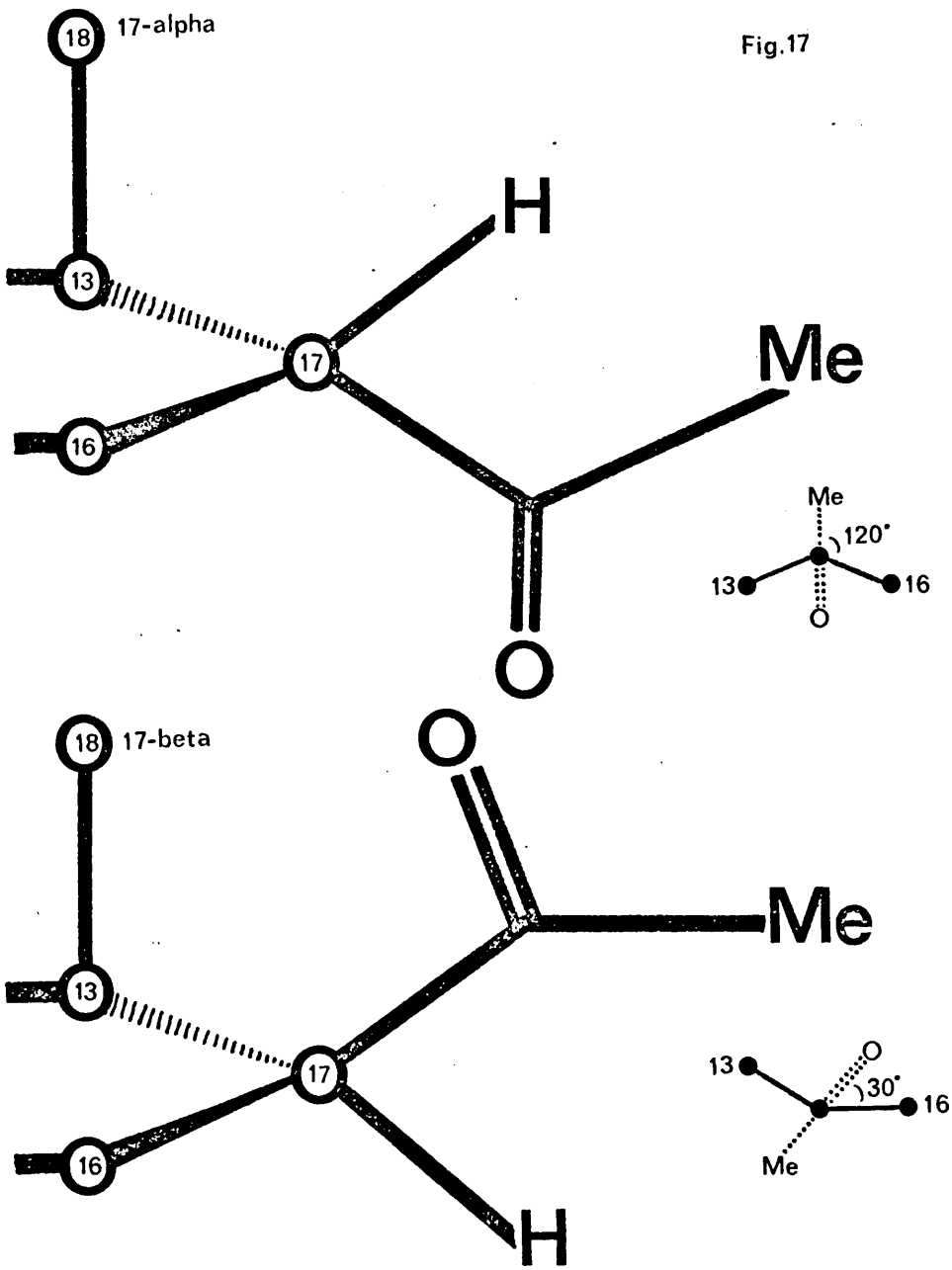
$[M-42]^{+\cdot}$ (m/e 286, 50%). The most notable additional features of the 70eV spectra are the differences in intensity of ions at m/e 43 (XV:100%, XVI:59%) and at m/e 85 (XV: 19%, XVI: 43%). The ion of m/e 43 arises mainly from C-20 and C-21 by α-cleavage, and the ion of m/e 85 comprises C-16 and C-17 with substituents and an additional hydrogen atom. The rationale for the differences between the spectra of XV and XVI is rather obscure: the controlling factor appears to be the ease of partial fission of ring D in XVI, as compared with XV. It has been noted that fission of the C-15/16 bond is enhanced by 16-methyl substitution,[188] but this factor, of course, applies to both XV and XVI. The corresponding fragmentations of both 6α,16α-dimethylprogesterone and 6α,16β-dimethylprogesterone give rise to ions at m/e 260 in very low abundance,[189] so the relative stereochemistry at C-16 and C-17 appears to have no effect on this fragmentation mode. It should, however, be borne in mind that these data[189] were obtained using a conventional heated inlet system. In an attempt at explaining this partial fragmentation, Zaretskii et al. could only suggest that the 17-acetyl group results in increased ring strain in ring D.

Before the steric effects on the fragmentation of XV and XVI can be determined, the favoured conformations of ring D and the 17-acetyl groups in these compounds must be considered. Allinger et al. have reviewed the literature on the conformations of 17-acetyl groups and have investigated the conformations of these groups in XVII, XVIII, and 16β-methyl-5α-pregnan-20-one (XIX).[190] They found


O
H

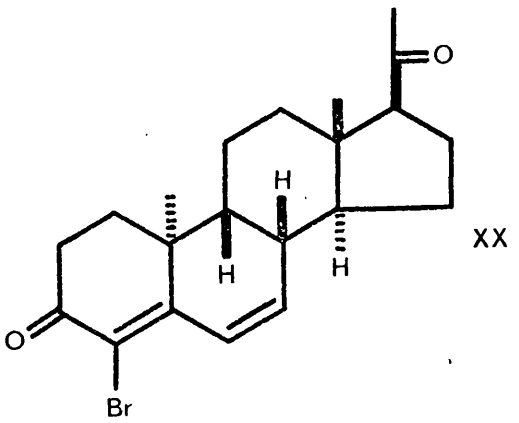
XIX

Fig.17


18
17-alpha
H
13
17
Me
16
O
Me
120°
13
16
O
18
17-beta
O
Me
13
17
16
H
13
O
30°
16
Me

that the preferred conformations of the 17-substituents were as shown in Fig. 17. It should be noted that the 20-keto and 21-methyl groups are well clear of C-13, C-16, and C-18 in 5α-17-*iso*pregnan-20-one (XVIII), but that the 20-keto group is relatively close to the 16β--methyl group in XIX and C-18 in XVII and XIX. Allinger *et al.* also found that the presence of a 16β-methyl group, as in XIX, had little effect on the conformation of the 17-acetyl group. The conformation of ring D is more difficult to ascertain. The results of X-ray crystallographic analyses are usually published in the form of bond lengths and bond angles, from which it is not easy to extract details of conformation. However, Altona *et al.* have determined the conformation of ring D for a number of steroids, including 4β-bromo-9β,10α--pregna-4,6-diene-3,20-one (XX).[191] The conformation of ring D of XX is shown in Fig. 18. It is reasonable to assume that the conformations of ring D of XV and XVI are similar to that of XVIII and that the conformations of the 17-acetyl groups in XV and XVI are similar to those in XVI (or XIX) and XVIII, respectively, although the structure of ring A can influence the preferred conformation of ring D.[191]

When these conformational factors are taken into consideration in a discussion of mass spectral fragmentations of XV and XVI, it can be seen that the explanation proposed by Zaretskii *et al.* is unsound. They suggested that the 17α-acetyl group imparted more strain to ring D than the 17β-acetyl group, whereas there is more "crowding"



XX

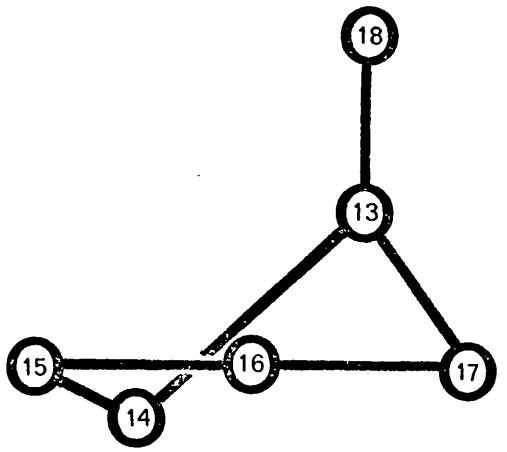
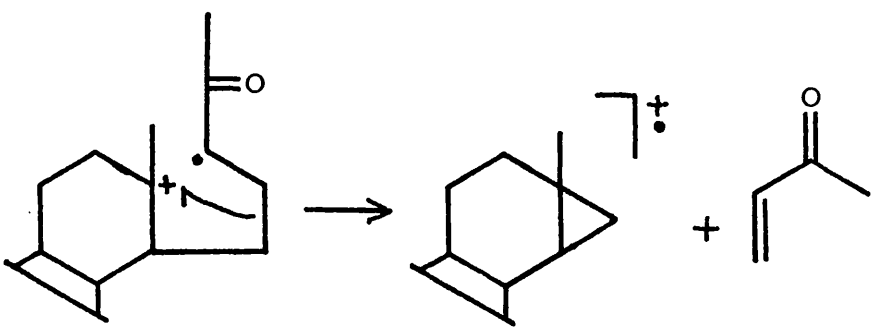


Fig. 18



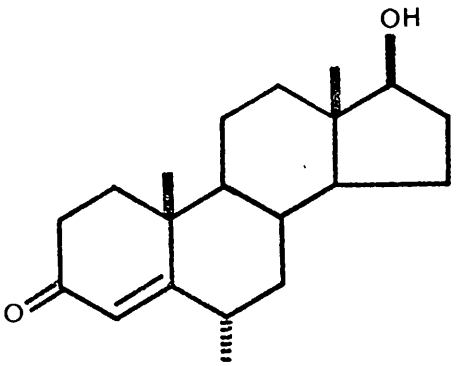
Scheme 1

of substituents in ring D of XV than XVI and therefore, presumably, more D-ring strain in XV than XVI.

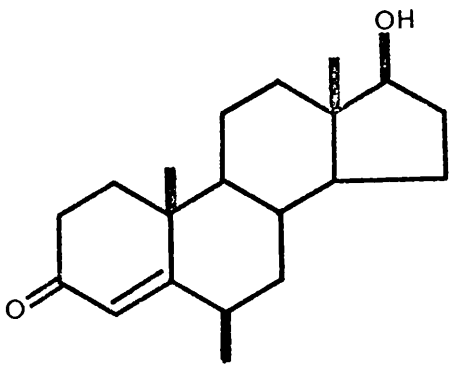
In order to determine the fragmentation mechanisms of XV and XVI, the site of charge localisation in the molecular ion must first be considered. It may be postulated that formation of the $[M-84]^{+}$ ion arises *via* initial cleavage of, and charge localisation at, the C-13/17 bond.[188,192] In the analogous fragmentation of 5α-pregnan--20-one, Djerassi and co-workers postulated that the next stage is fission of the C-15/16 bond and concomitant bond-formation between C-13 and C-15, producing a cyclopropane ring (Scheme 1). If this mechanism were applied to XV, there would be considerable steric hindrance between the C-18 methyl group and the 17β-acetyl group as they approach during the bond-formation step. There is no such steric hindrance in the fragmentation of XVI. This can be clearly seen if Dreiding molecular models[193] are examined. Consequently, formation of the $[M-84]^{+}$ ion from XVI is preferred. This fragmentation route is apparently capable of dissipating much of the energy content of the molecular ion of XVI. The equivalent energy content of the molecular ions of XV must, therefore, be expended *via* alternative fragmentations, hence the differences between the spectra of XV and XVI. It would be interesting to examine the spectra of other analogues, *eg.* the 18α,17β- and 18α,17α-isomers. It is, however, doubtful whether consideration of spectra of simpler molecules such as

substituted indanes would be informative.[105] Moreover, the structure of ring A appears also to influence the fragmentations of ring D of these steroids:[188] elimination of C-16 and C-17, with substituents, from 5α-pregnan-20-one and its 17α-epimer accounts for much less than 0.5% of the total ion current in each case. The preferred primary fragmentation of these compounds involves loss of C-15 to C-17, with substituents, and an additional hydrogen atom (probably from C-8). The stereochemistry at C-17 apparently has no effect on this fragmentation mode, hence the close similarity between the spectra of these isomers.

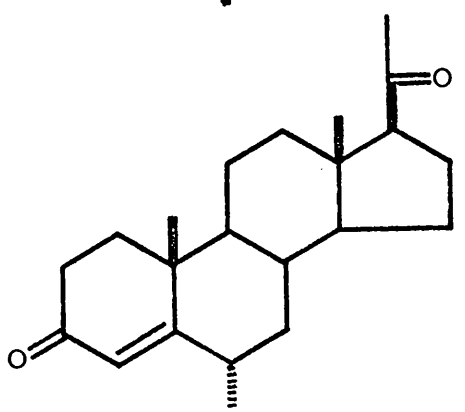
A further possibility of the influence of steric effects on mass spectra, viz. selective blocking of hydrogen transfer, becomes apparent in the spectra of 6α-methyltestosterone (XXI) and 6β-methyltestosterone (XXII) and in the spectra of 6α-methylprogesterone (XXIII) and 6β-methylprogesterone (XII). As noted above, steroidal Δ^4-3-ones (unsubstituted in rings A and B and at C-11) undergo fragmentation of the C-6/7 and C-9/10 bonds with transfer of hydrogen atoms from C-8 and C-11 resulting in the formation of an ion of m/e 124 containing ring A with C-6 and C-19. Shapiro and Djerassi have postulated two alternative mechanisms for this process.[194] The first (Scheme 2a) involves hydrogen transfer from C-11 to C-10 and from C-8 to C-4, while the second involves hydrogen transfer from C-8 to C-10 and from C-11 to C-6 (Scheme 2b). The second mechanism is supported by the observation that this ion, at m/e 138, appears as the base peak in the spectra of



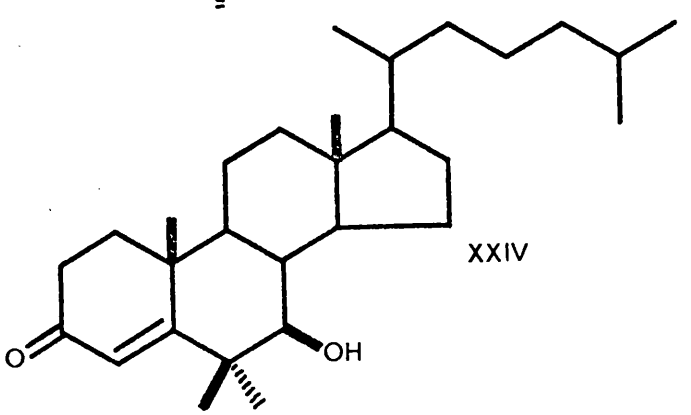
XXI



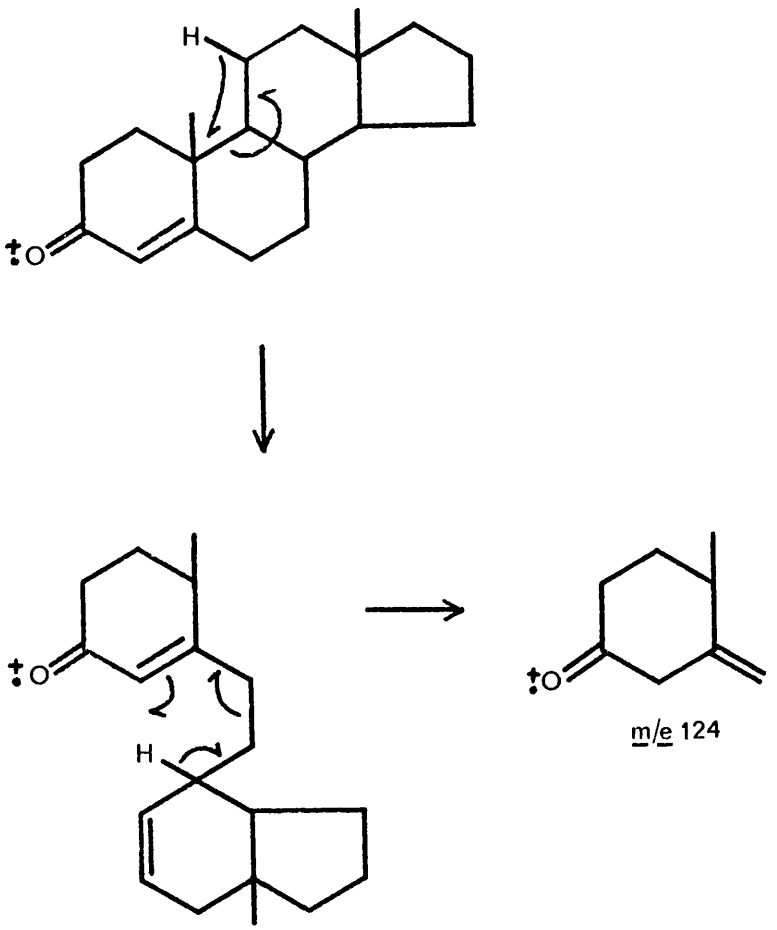
XXII



XXIII

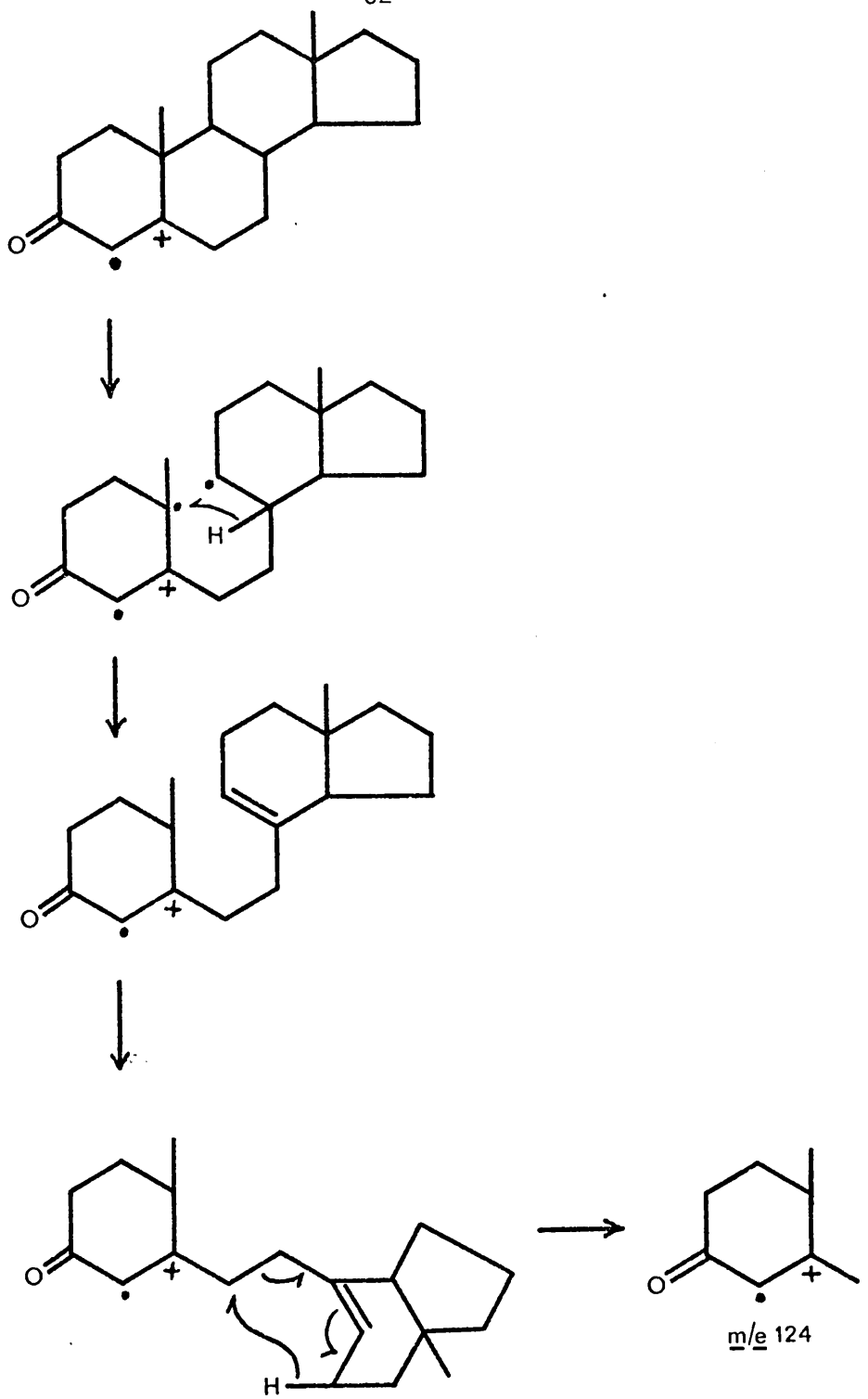


XXIV



m/e 124

Scheme 2a



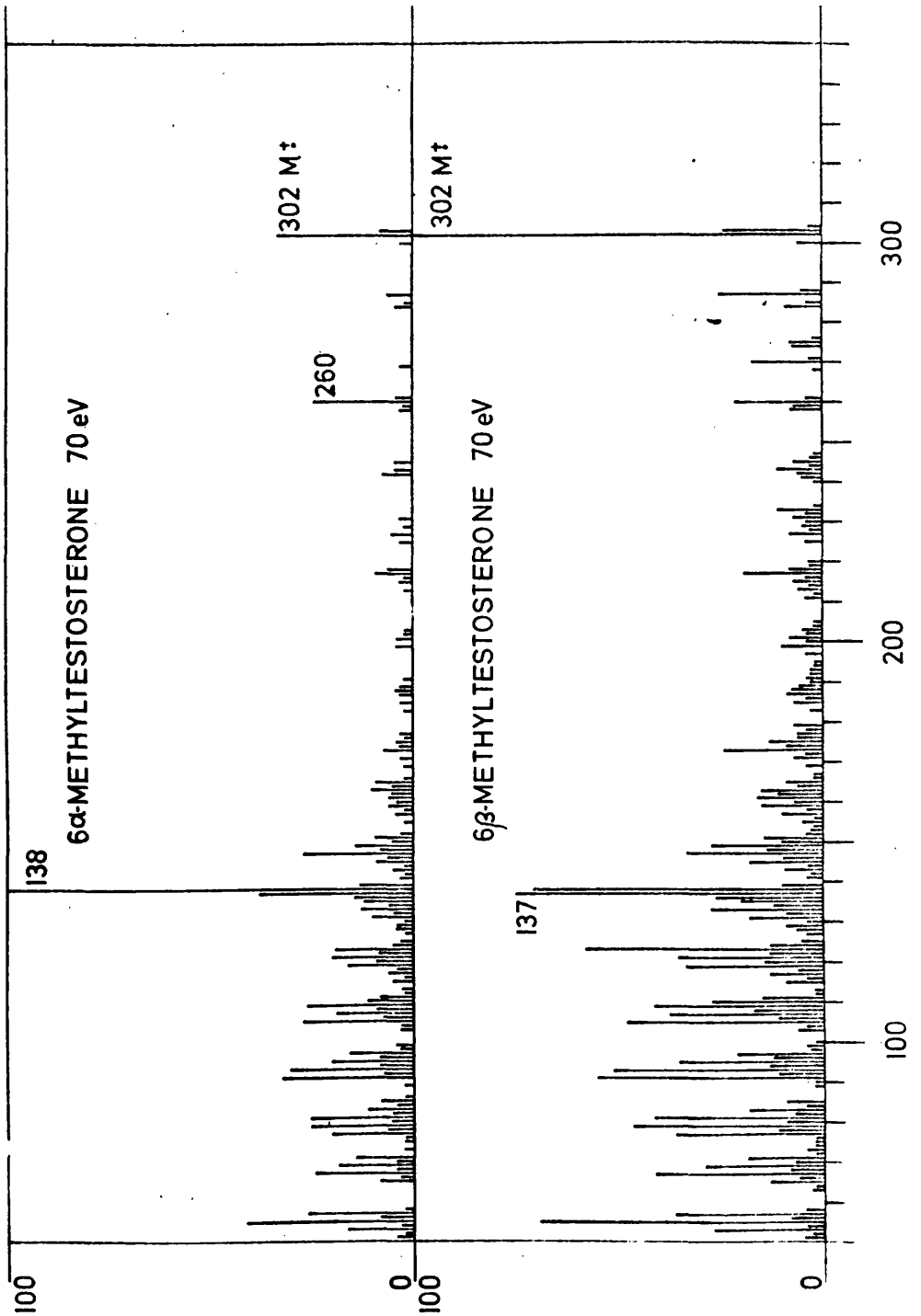
Scheme 2b

the 6α-isomers XXI (Fig. 19) and XXIII (Fig. 14) and that the ions at m/e 138 and m/e 137 (presumably formed by a similar route, but involving transfer of only one hydrogen atom) are of approximately equal abundance in the spectra of the 6β-isomers XXII (Fig. 20) and XII (Fig. 13). It should also be noted that ions of m/e 138 constitute the base peaks in the spectra of 6α,16α-dimethylprogesterone and 6α,16β-dimethylprogesterone.[189] The first alternatve mechanism apparently applies in the fragmentation of 7β-hydroxy-6,6-dimethyl-cholest-4-en-3-one (XXIV) with the formation of ions at m/e 152 (100%) and m/e 151 (70%).[195]

THE USE OF (CHLOROMETHYL)DIMETHYLSILYL ETHER DERIVATIVES IN GC-MS

The work of Eaborn and co-workers on the gas chromatographic properties of (chloromethyl)dimethylsilyl (CMDMS) ethers has already been mentioned.[153,154,196,197] In view of the excellent chromatographic properties of TMS ethers, and the useful fragmentation directing influence of the TMS group in mass spectrometry, the advantages of CMDMS ethers as derivatives for use in GC-MS are not immediately apparent. In fact, with one exception,[198] CMDMS ethershave not been employed for gas chromatographic - mass spectrometric analysis.

The identity of an "unknown" steroid can often be ascertained by GC (preferably on more than one stationary phase) and GC-MS of both the steroid and a suitable derivative. Such an identification can be carried out with a high degree of confidence if an authentic

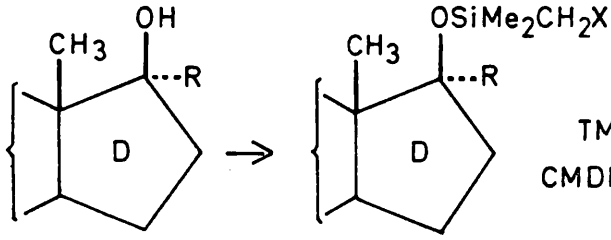

6α-METHYLTESTOSTERONE 70 eV
138
302 M+
260
100
0
6β-METHYLTESTOSTERONE 70 eV
137
302 M+
100
0
100
200
300

Figs 19,20

sample of the steroid is available for comparison, or even if the relevant gas chromatographic and mass spectrometric data are found in the literature. It is, nevertheless, often desirable to employ other physical and chemical techniques as an aid to identification, and to synthesise new compounds for comparison of chemical and physical properties. When this is not possible, eg. if only small quantities of the "unknown" steroid are available or if reference samples cannot be readily synthesised, it is important to extract the maximum amount of information by GC-MS. Comparative examination of TMS ethers and d_9-TMS ethers, which have similar retention times, provides a simple method of strengthening such information in respect of hydroxylic steroids.[152] We considered that further structural evidence could be obtained by using CMDMS ethers; accordingly, we selected, for an initial survey by GC-MS, a series of CMDMS ethers of steroids.

Fig. 21 lists retention indices and retention index increments for eight free sterols, TMS ethers, and CMDMS ethers. It can be seen that CMDMS ether formation gives rise, fairly consistently, to a retention index increment of about +400 (1% OV-1, 220-235°). [It should also be noted that the increment (TMS→CMDMS) is +270 - ie. the added 34 m.u. behaves almost exactly like an added CH_2, with only marginal extra polarity.] This relatively large increment (+400) suggests that CMDMS ethers may effectively be employed for gas chromatographic separation of sterols, diols, and triols from each other and from other components of complex natural product mixtures. This has been

RETENTION INDEX INCREMENTS



TMS: X=H
CMDMS: X=Cl

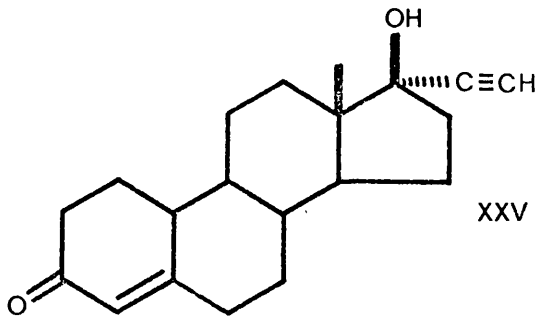
Substituents in androstane	Retention data (1% OV-1, 220-235°)				
	Free	TMS		CMDMS	
	I	I	ΔI	I	ΔI
(R: CH_3)					
Δ^3 5β-H	2230	2360	130	2630	400
3β-TMSO 5α-H	2600	2715	115	-	-
Δ^4- 3-one 19-nor	2550	2680	130	2950	400
Δ^4- 3-one	2605	2730	125	3005	400
Δ^4- 3-one 9α-F 11β-HO	2810	2935	125	-	-
$\Delta^{1,4}$- 3-one	2640	2770	130	3050	410
(R: C ≡ CH)					
Δ^4 - 3-one	2625	2730	105	3035	410
Δ^4 - 3-one 19-nor	2590	2700	110	2970	380

Fig. 21

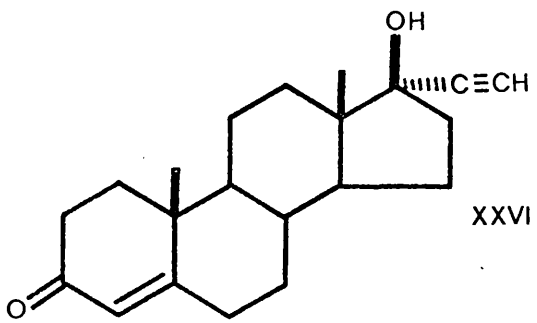
achieved with some degree of success by Madani[199] for hydroxylated metabolites of Nilevar (see below, pp. 160-70). This approach is particularly useful if molecular ions of the free sterols and TMS ethers (and CMDMS ethers) are present in low abundance, since it is difficult in these cases to determine the number of hydroxyl groups in the molecule. Similarly, increased retention index increments have been found for O-benzyloximes (as compared with O-methyloximes), yielding information on the number of ketonic groups in the molecule.

The mass spectra of CMDMS ethers are comparable with those of TMS ethers, with appropriate mass shifts. Mass spectra of these derivatives of norethisterone (XXV), ethisterone (XXVI), and secrosterone (XXVII) are represented in Figs. 22 and 23. Notable features of the CMDMS ether spectra are the presence of ions containing both isotopes of chlorine (^{35}Cl and ^{37}Cl), and the presence of ions at $[M-15]^+$ and $[M-49]^+$, due to loss of methyl and chloromethyl radicals, respectively.

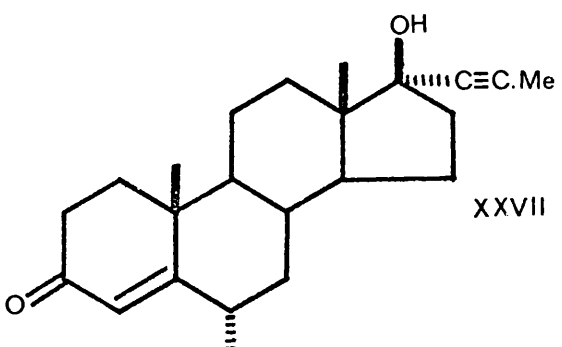
Spectra of CMDMS ether derivatives of 17β-hydroxy-17α-methyl steroids have been examined in more detail. Base peaks in all of the 70 eV spectra of TMS and CMDMS ethers of this class of steroid which we have studied appear at m/e 143 and 177, respectively, corresponding to C-15/16/17 and substituents, less one hydrogen atom. At lower electron energies, abundant fragment ions are observed due to the corresponding nuclear fragments (Fig. 24). Mass spectra of 17α-methyl-19-nortestosterone (XXVIII) and its TMS and CMDMS ether



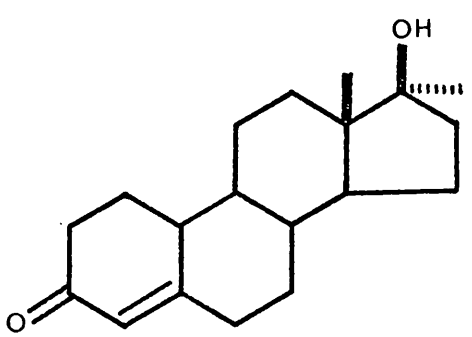
XXV



XXVI



XXVII



XXVIII

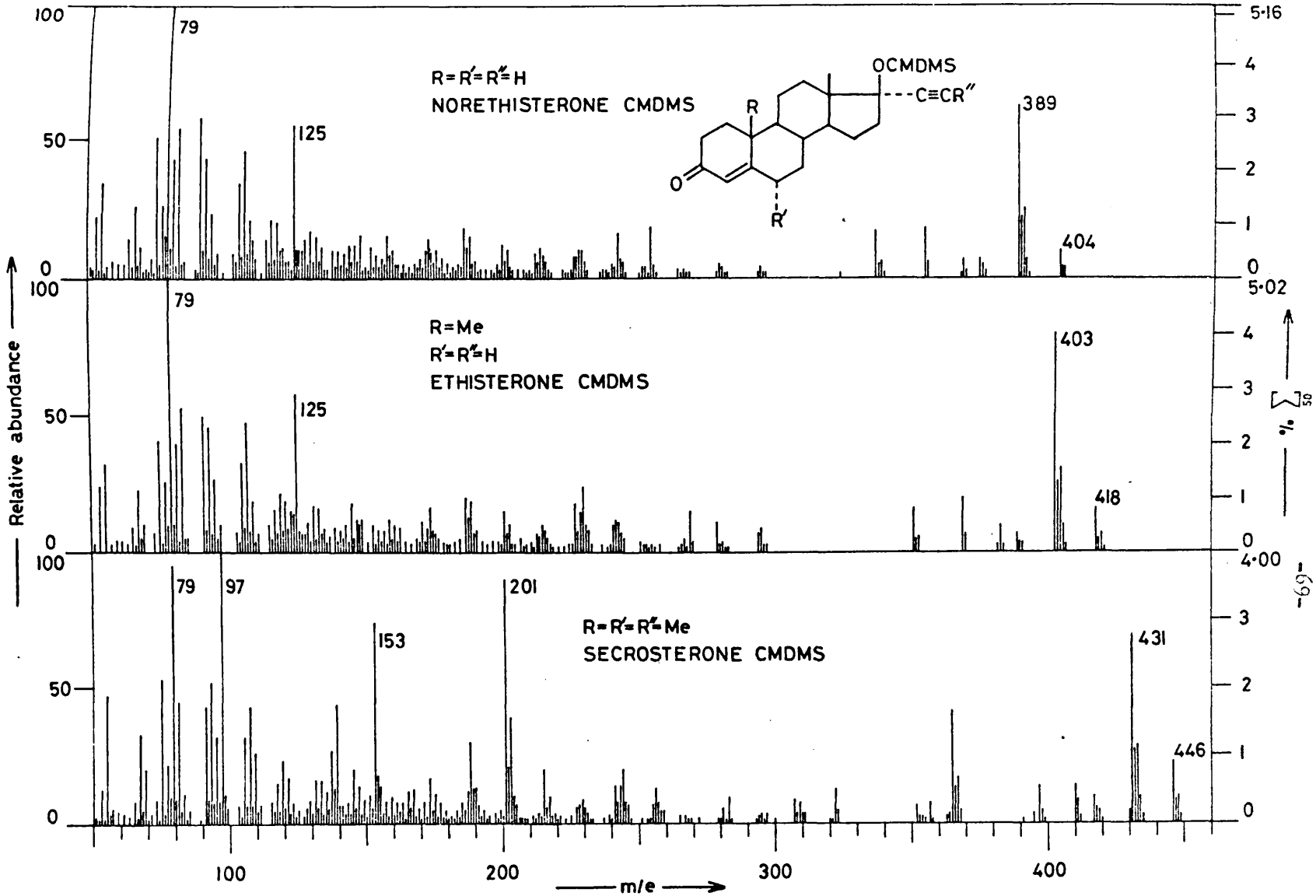

R=R′=R″=H
NORETHISTERONE CMDMS
OCMDMS
C≡CR″
R
O
R′
79
125
389
404
R=Me
R′=R″=H
ETHISTERONE CMDMS
403
418
R=R′=R″=Me
SECROSTERONE CMDMS
97
153
201
431
446
Relative abundance
m/e
100
200
300
400
5·16
5·02
4·00
%

Fig.22

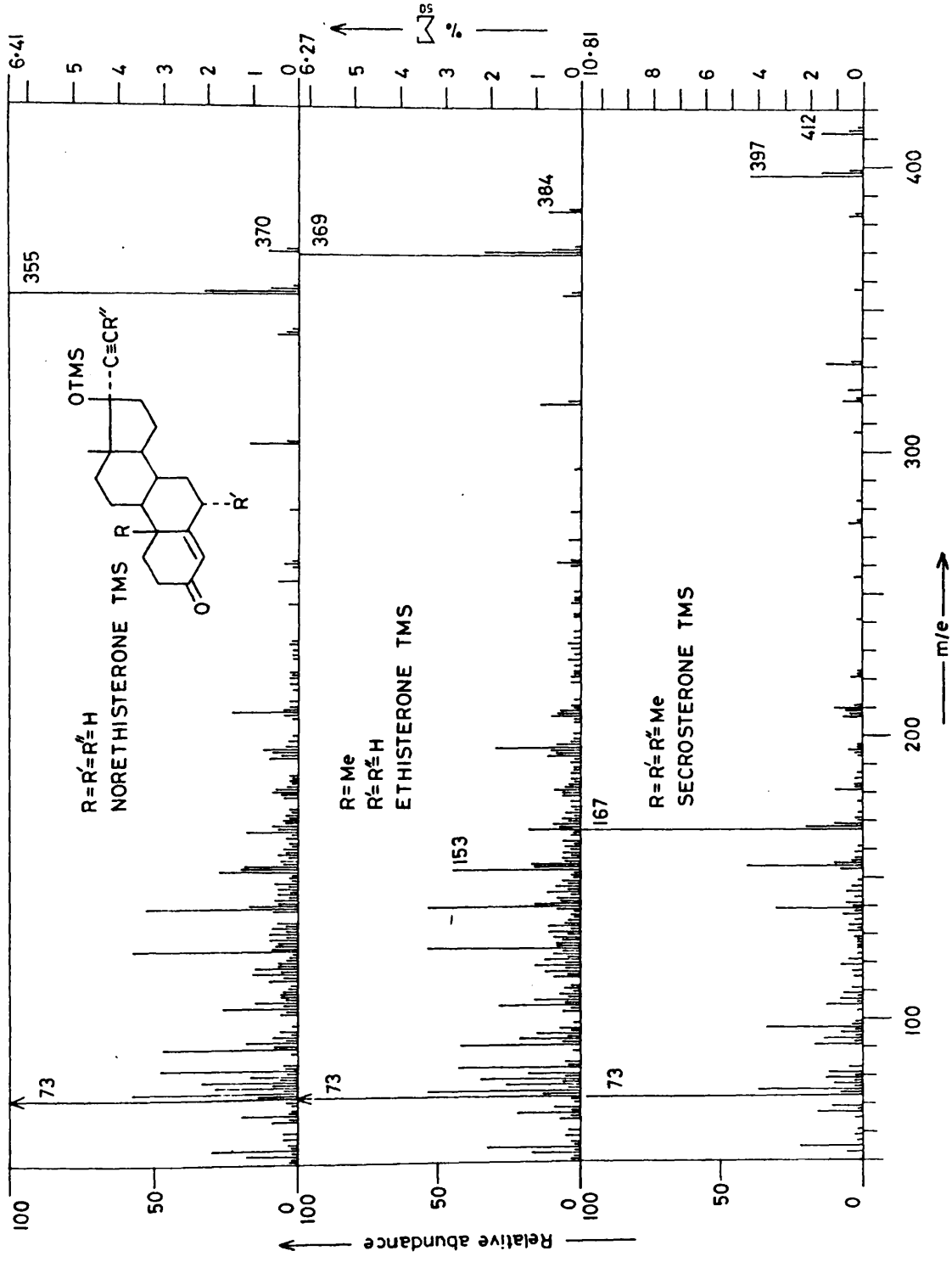
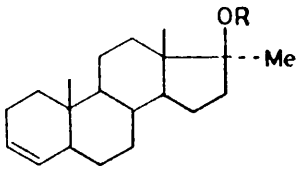
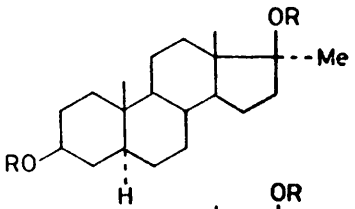
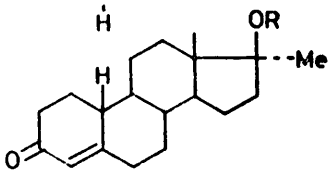
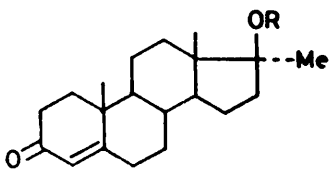
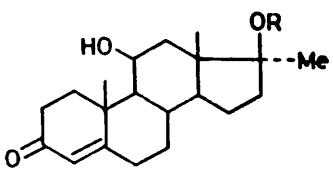
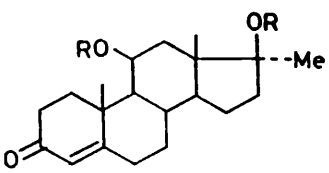
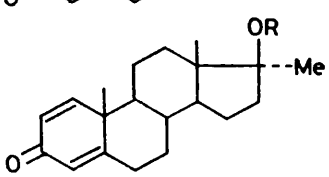
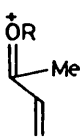

R=R'=R''=H
NORETHISTERONE TMS
OTMS
--C≡CR''
R
R'
O
R=Me
R'=R''=H
ETHISTERONE TMS
R=R'=R''=Me
SECROSTERONE TMS
355
370
369
384
397
412
73
153
167
6·41
6·27
10·81
Relative abundance
m/e
%

Fig.23

IONS IN 15eV SPECTRA

	R=TMS			R=CMDMS		
	Base peak	M⁺		Base peak	M⁺	
	m/e	m/e	%	m/e	m/e	%
	143	360	7	270	394	20
	143	450	5	177	518	3
	270	360	10	270	394	12
	284	374	5	284	408	8
	300	390	60	300	424	9
	372	462	17	406	530	21
	282	372	5	282	406	4

Base peak in all 70eV spectra: 

R=TMS m/e143
R=CMDMS m/e177

Fig 24

derivatives are represented in Fig. 25. It can be seen that the CMDMS group confers stability on the major fragment ions similar to that observed for TMS ethers.

CMDMS ether derivatives appear to possess the desirable gas chromatographic and mass spectrometric characteristics of TMS ethers and, in addition:

(i) to provide additional gas chromatographic and mass spectrometric data,

(ii) to be capable of effectively separating steroids with differing numbers of hydroxyl groups,

(iii) to provide further insight into fragmentation modes, observed by appropriate substituent shifts, and

(iv) to permit initial gas chromatographic work to be carried out using extremely small quantities of sample, with electron-capture detection.[154]

VandenHeuvel and Braly have demonstrated the utility of mixed TMS/CMDMS ethers for the characterisation of bile acid methyl esters.[200] There are, however, no reports of the application of this method.

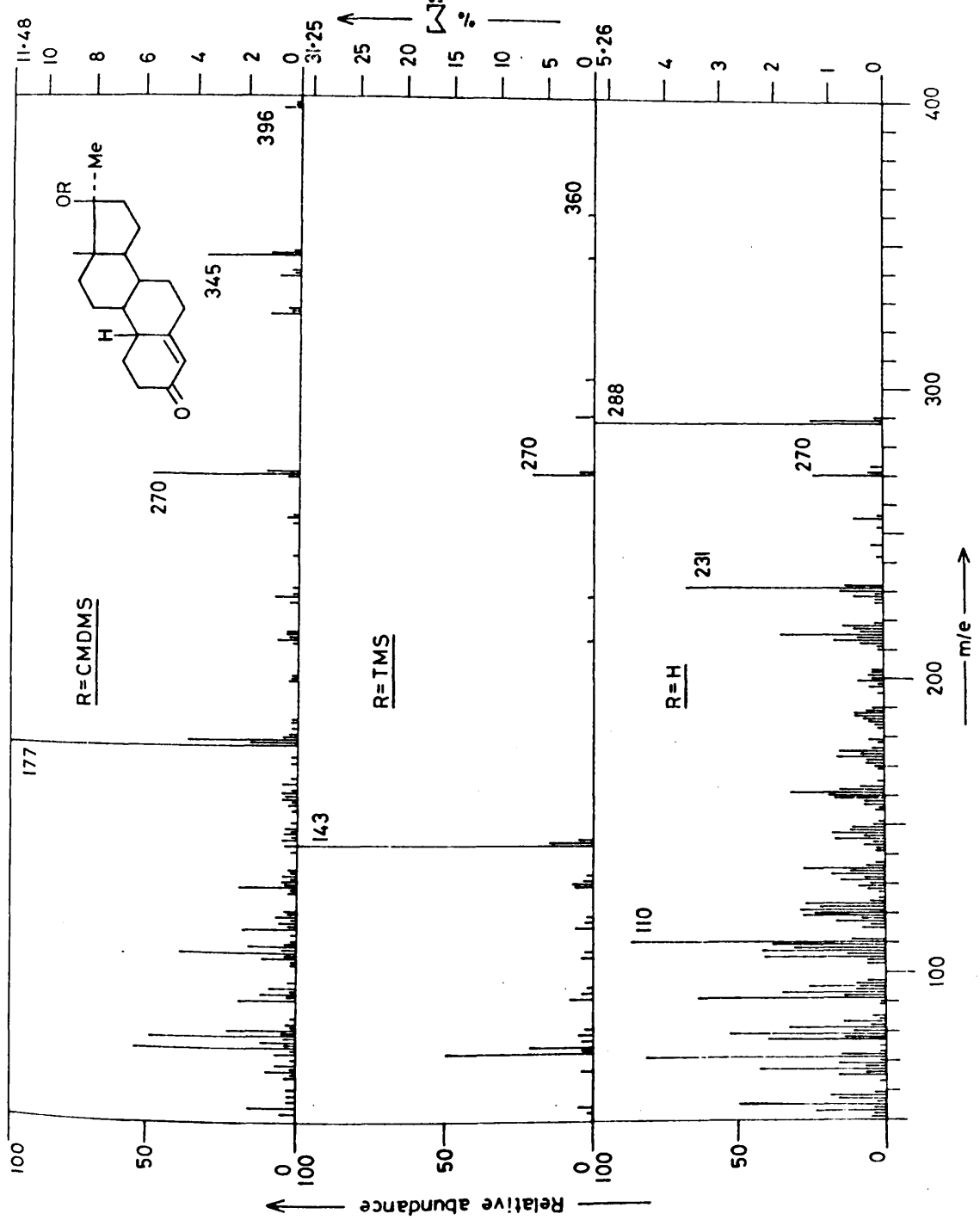
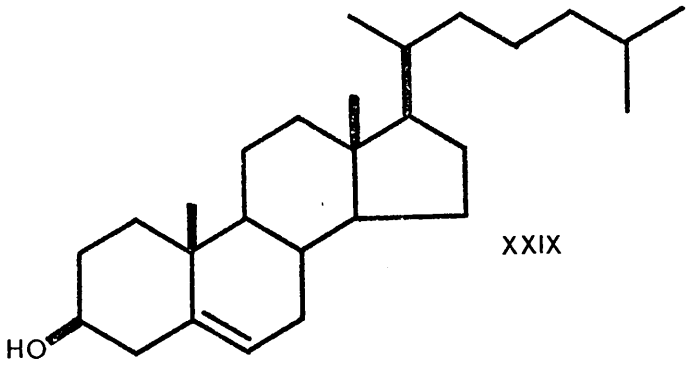

R=CMDMS
R=TMS
R=H
OR
Me
H
O
396
345
270
177
143
360
288
270
231
110
11·48
31·25
5·26
Relative abundance
m/e
100
200
300
400

Fig. 25

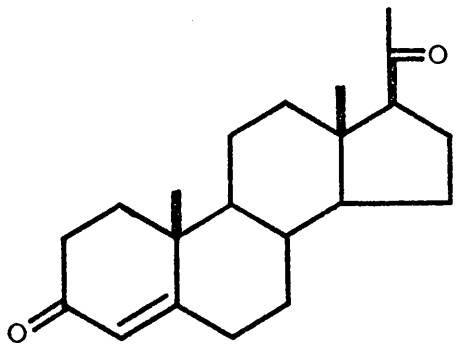
THE MASS SPECTRA OF TMS ETHER DERIVATIVES OF SOME Δ^5-3β-HYDROXY C_{19} STEROIDS

Cholesterol (XXIX) is present in practically all living organisms, and is the primary source of mammalian hormones. It can be converted to progesterone (XXX), for example, via 20,22-dihydroxycholesterol (XXXI) and pregnenolone (XXXII). Alternatively, complete oxidative removal of the side chain leads to dehydroepiandrosterone (XXXIV) and then the C_{19} hormonal steroids. The Δ^5-3β-hydroxy steroids are "inactive" precursors of hormones with the Δ^4-3-one structure, and their reduction products.[201] Consequently, Δ^5-3β-hydroxy steroids (or their conjugates) are only found in quantity if 3β-hydroxysteroid dehydrogenase is not actively converting them to Δ^4-3-keto steroids. This situation may arise, for example, in newborn mammals or under certain pathological conditions.[202]

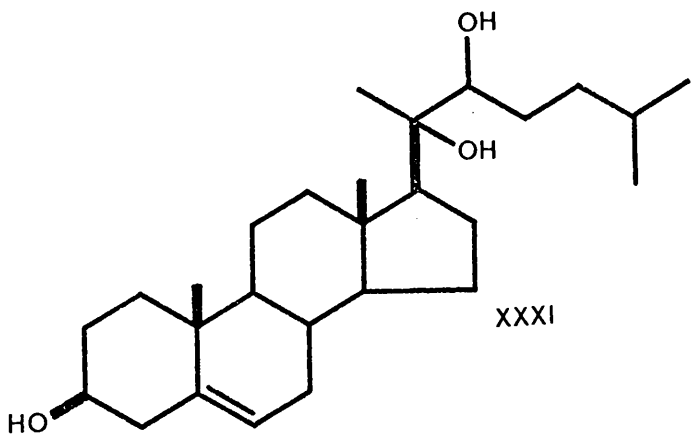
GC-MS has been used in the identification of a large number of Δ^5-3β-hydroxy C_{19} steroids, as their TMS ether derivatives, including those extracted from urine[129,136,203-205] and faeces[205-207] of newborn and infant humans, meconium of newborn humans,[205] human umbilical cord plasma,[205] human amniotic fluid,[208] human bile,[209] human peripheral plasma,[210-213] plasma and urine of an eight-year-old boy with 3β-hydroxy steroid dehydrogenase deficiency,[214] urine of a newborn chimpanzee,[215] and urine and faeces of female germ-free and "conventional" rats treated with a 3β-hydroxy-Δ^5-oxidoreductase inhibitor.[216] The mass spectra of such compounds have been considered in other reports concerning various aspects of MS and GC-MS[26,31,217-219]



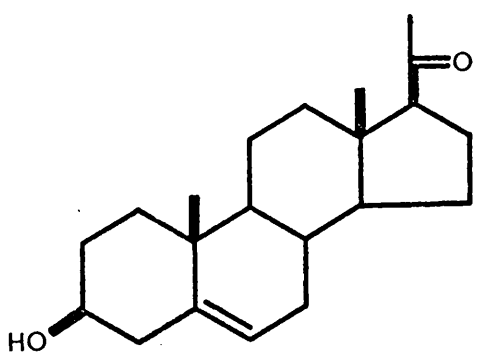
XXIX



XXX



XXXI



XXXII

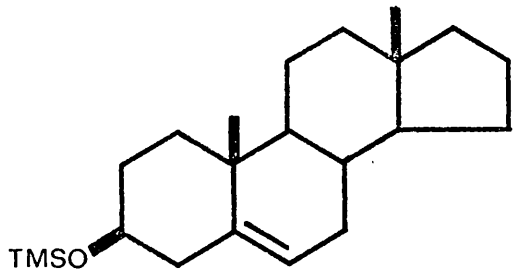
Nevertheless, despite the clinical significance[220] of androstenols, there has been no systematic survey of the mass spectral fragmentations of their TMS ether derivatives. We have accordingly examined the mass spectra of seventeen of these compounds.

The use of an electron energy of 70 eV in the measurement of mass spectra of many samples gives rise to the formation of a multiplicity of fragment ions of relatively low mass. Often, these are of limited structural significance: few informative ions in the mass spectra of steroids and their derivatives have m/e<100. Spectra obtained with a lower electron energy contain fewer ions, and these, usually being formed as products of primary, secondary, or tertiary fragmentations, often afford a more useful insight to the processes taking place in the ion source. 20 eV spectra have, therefore, been used in this study. The mass spectra of compounds XXXIII-XLIX are represented in Figs. 26-42.

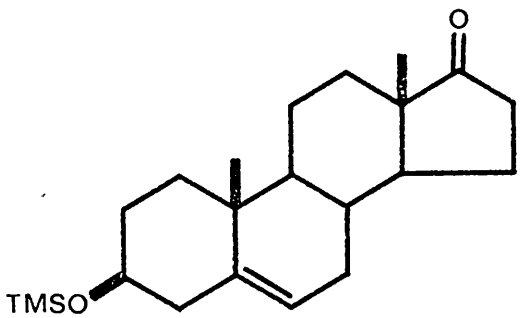
Many of the observed fragmentations are common to all of the samples studied, and to analogous compounds with side chains at C-17.[135] The investigations of Diekmann and Djerassi[221] on labelling of cholesterol with deuterium or other substituents were particularly useful in assigning origins to many of the common ions. Briefly, they can be summarised as follows (possible structures of ions viii-xxviii are given in Scheme 3).

$[M-90]^{+\cdot}$ (viii): elimination of the 3β-trimethylsilyloxy moiety with a hydrogen atom, mainly from C-4(β).

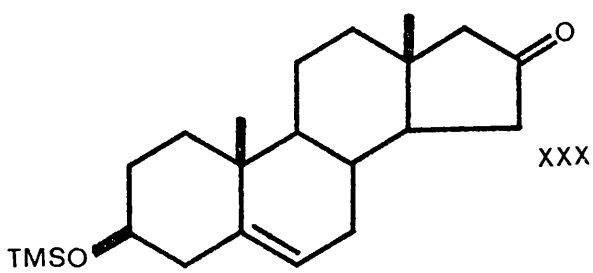
$[M-129]^{+}$ (ix): formed by scission of the C-1/10 and C-3/4 bonds



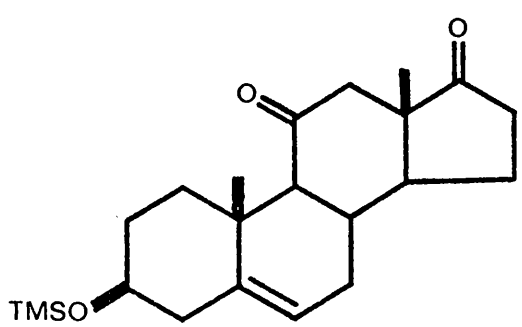
XXXIII



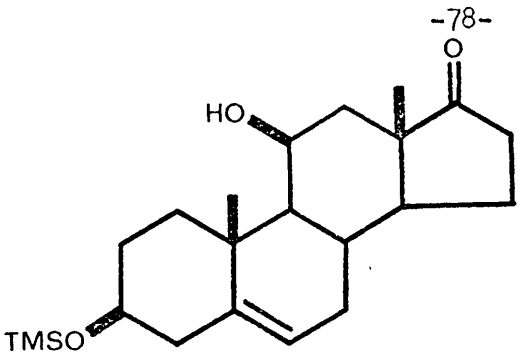
XXXIV



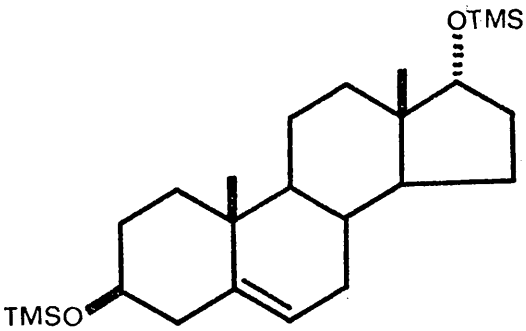
XXXV



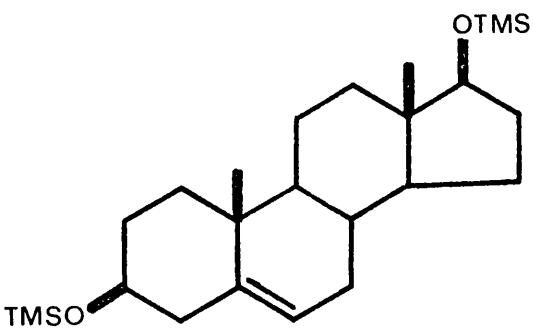
XXXVI



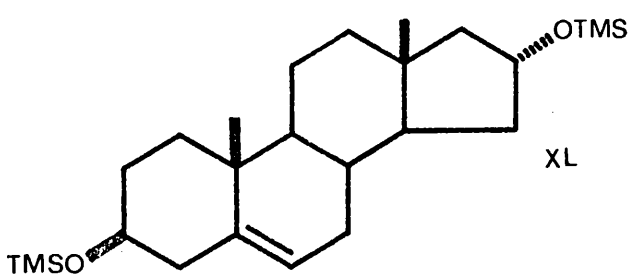
XXXVII



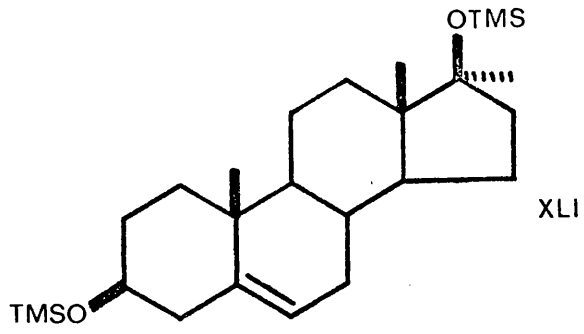
XXXVIII



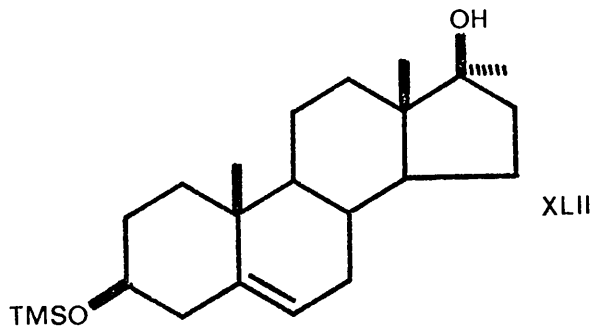
XXXIX



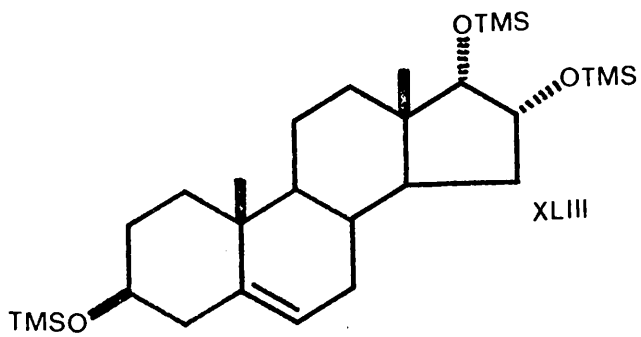
XL



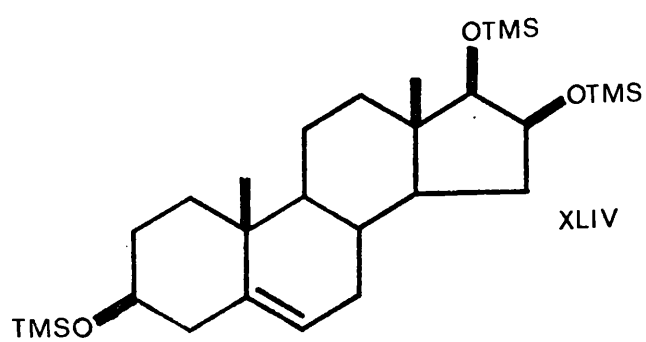
XLI



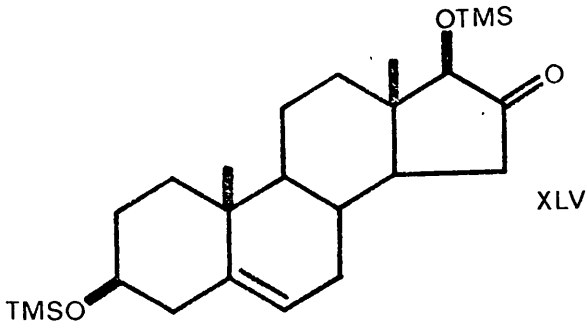
XLII



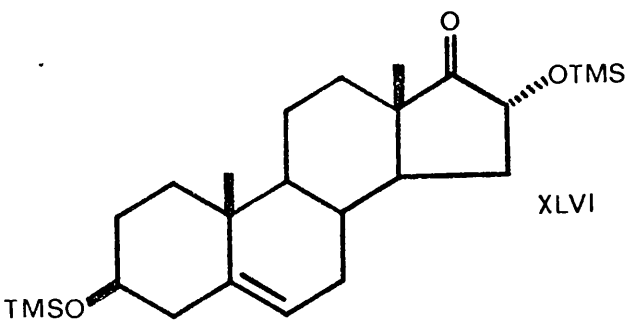
XLIII



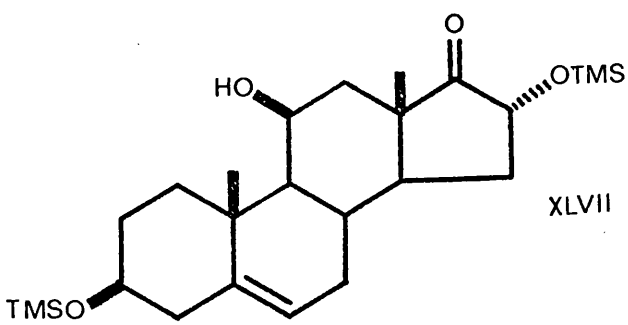
XLIV



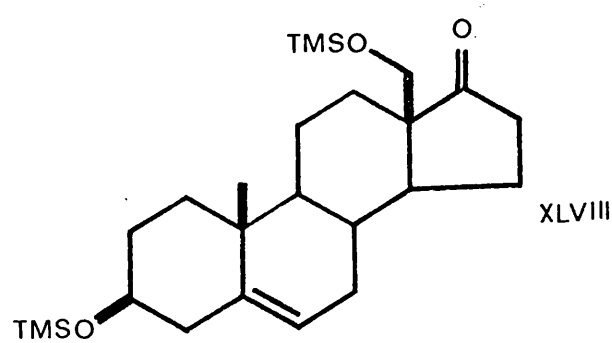
XLV



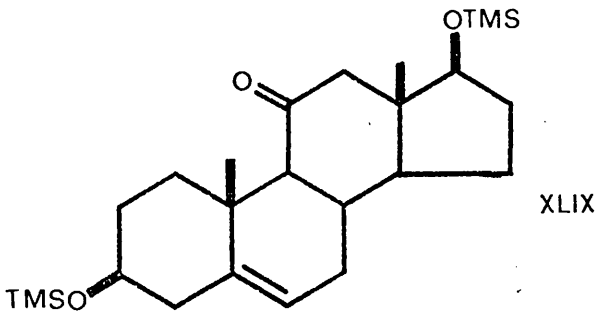
XLVI



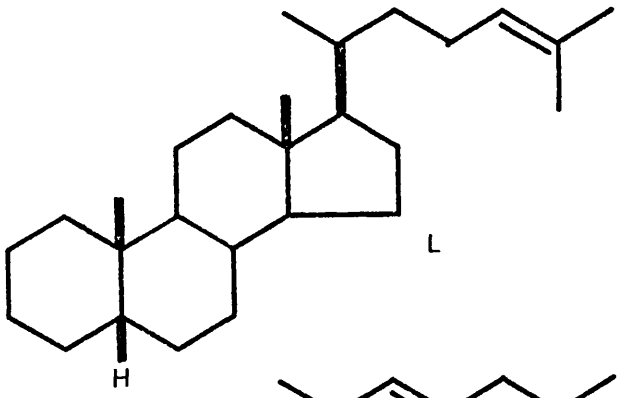
XLVII



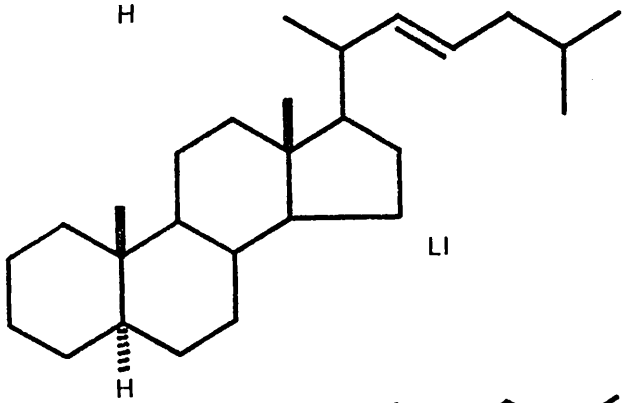
XLVIII



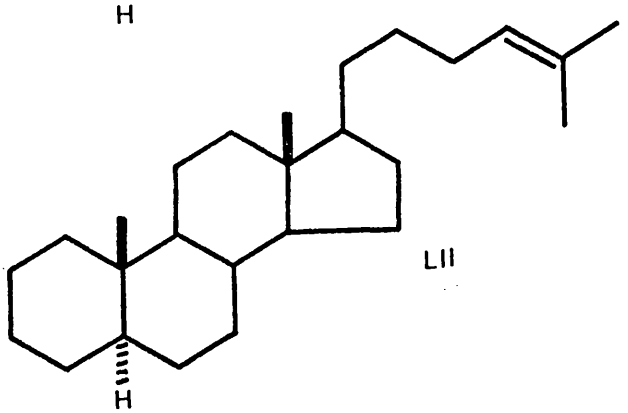
XLIX



L



LI



LII

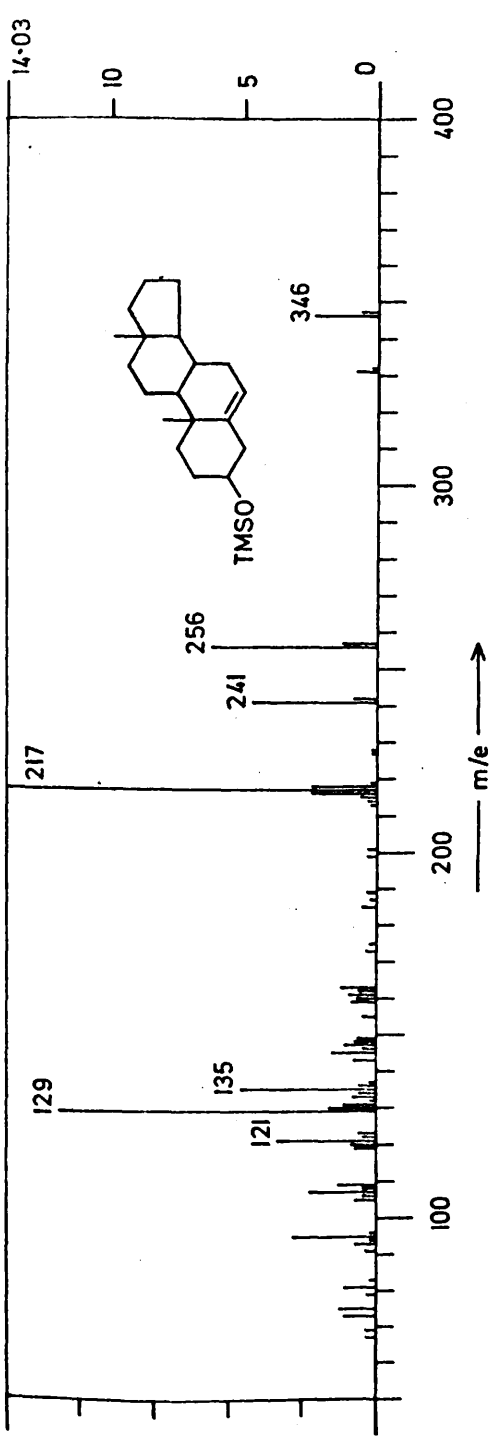

14·03
10
5
0
400
300
200
100
m/e
346
256
241
217
135
129
121
TMSO

Fig. 26

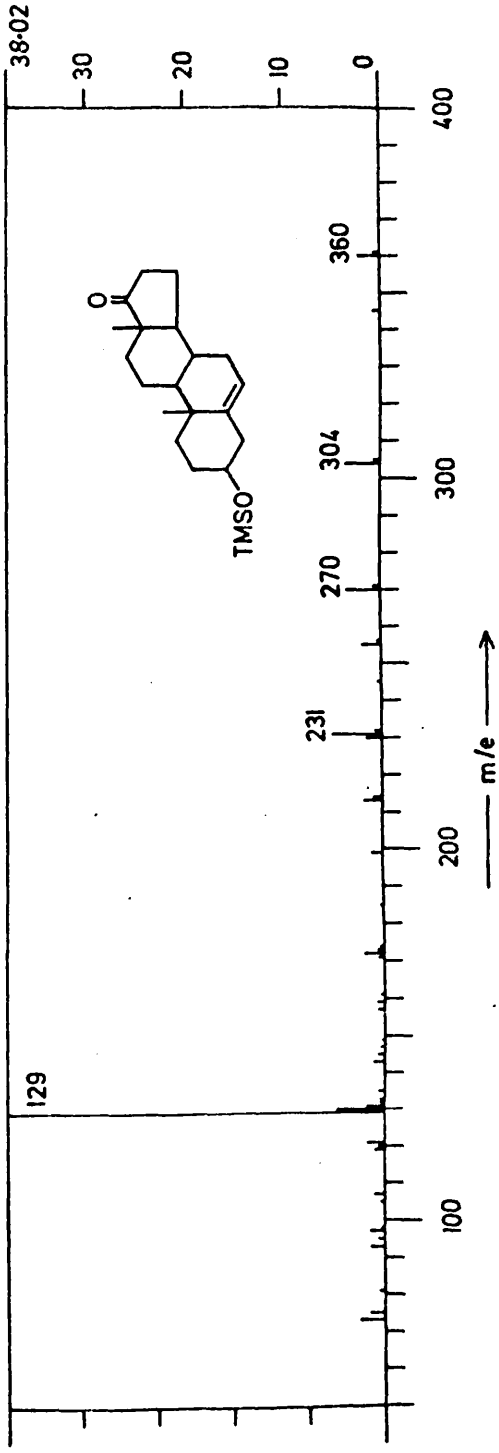

38·02
30
20
10
0
400
360
304
300
270
231
200
100
m/e
129
O
TMSO

Fig. 27

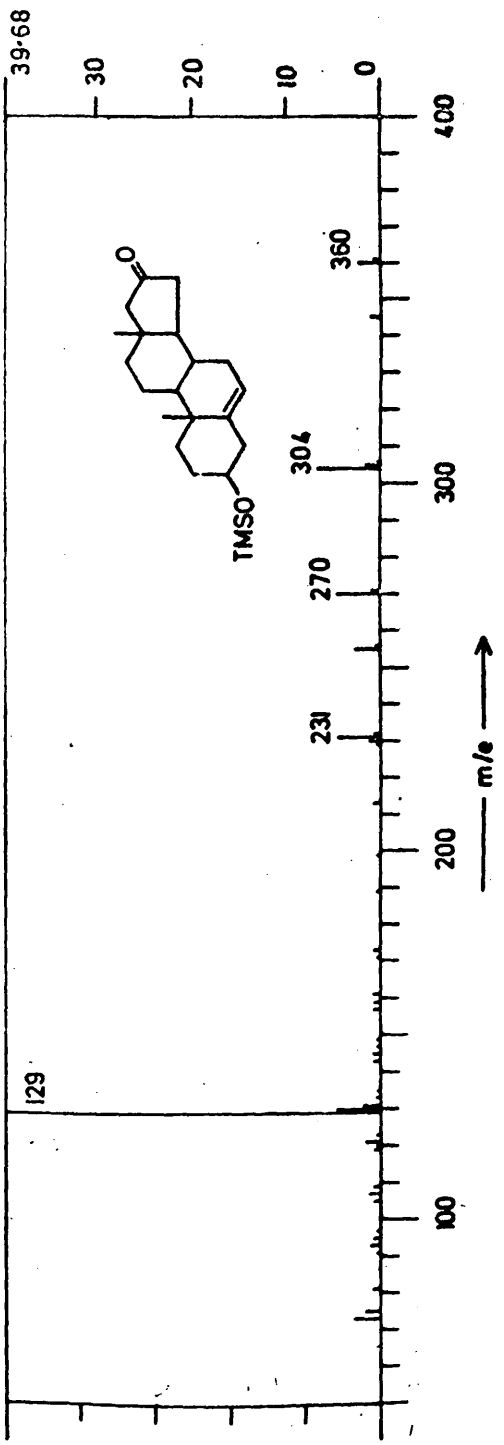

39·68
30
20
10
0
400
360
304
300
270
231
200
m/e
129
100
TMSO
O

Fig. 28

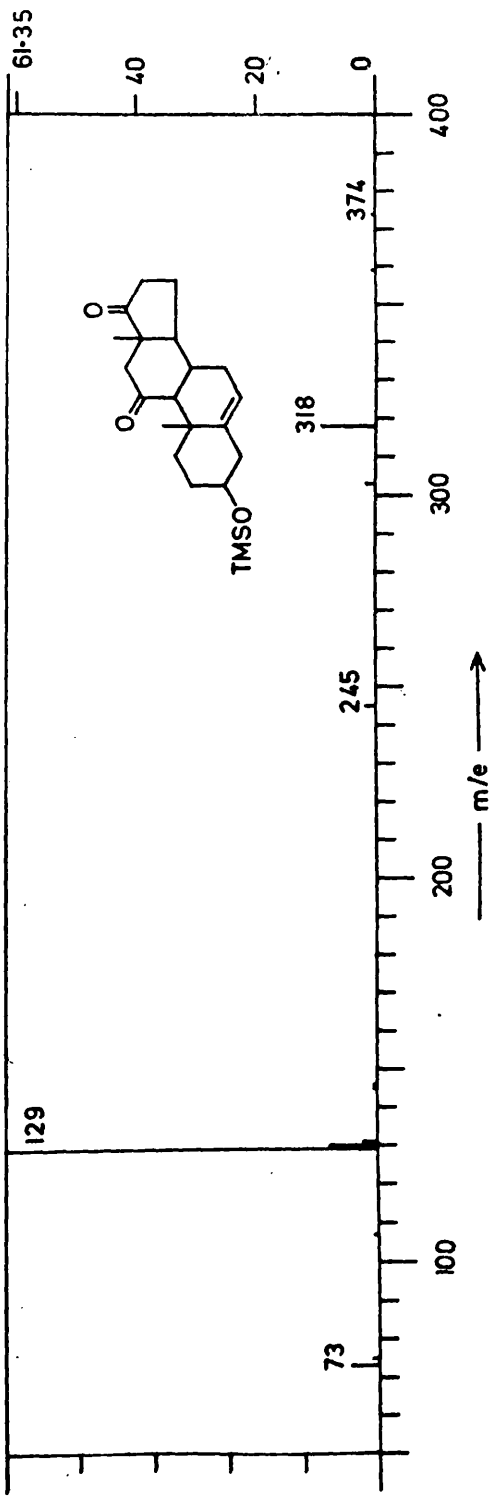

61·35
40
20
0
400
374
318
300
245
200
m/e
129
100
73
TMSO
O
O

Fig. 29

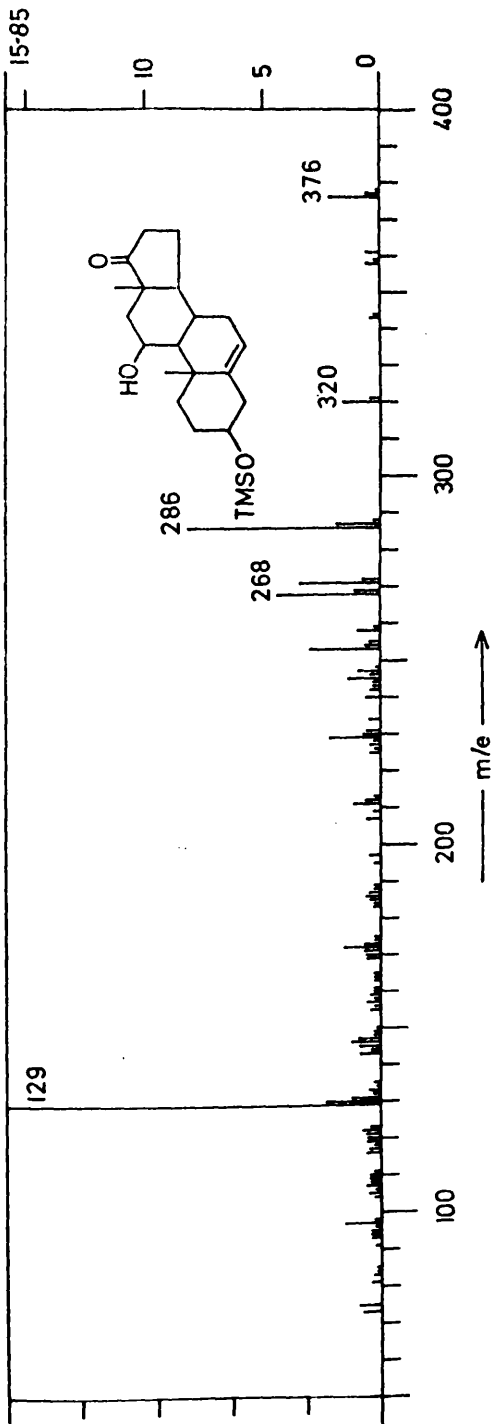

15-85
10
5
0
400
300
200
100
m/e
129
268
286
320
376
HO
O
TMSO

Fig. 30

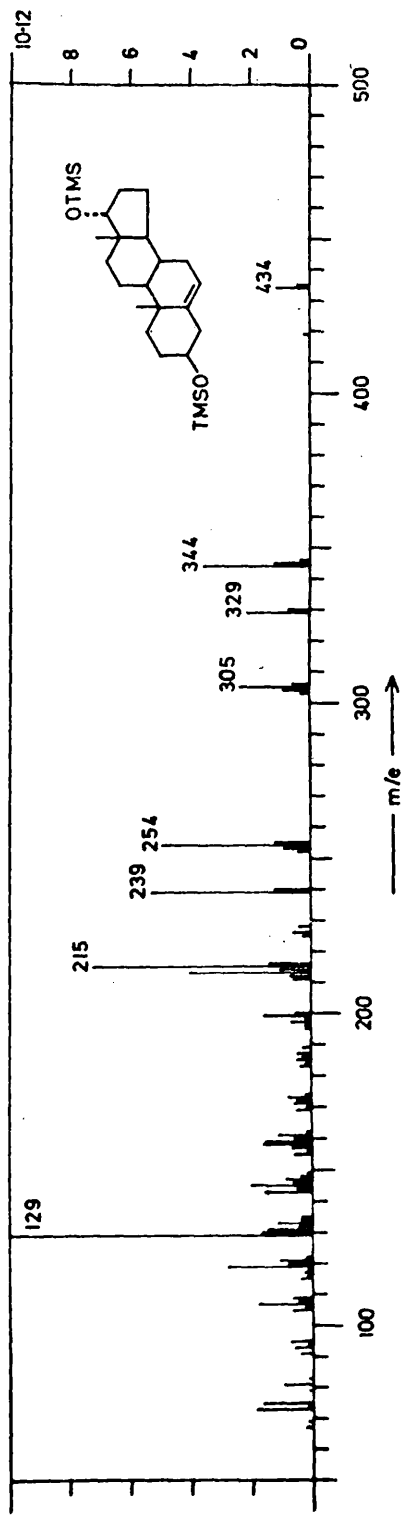

10-12
8
6
4
2
0
500
400
300
200
100
m/e
129
215
239
254
305
329
344
434
OTMS
TMSO

Fig. 31

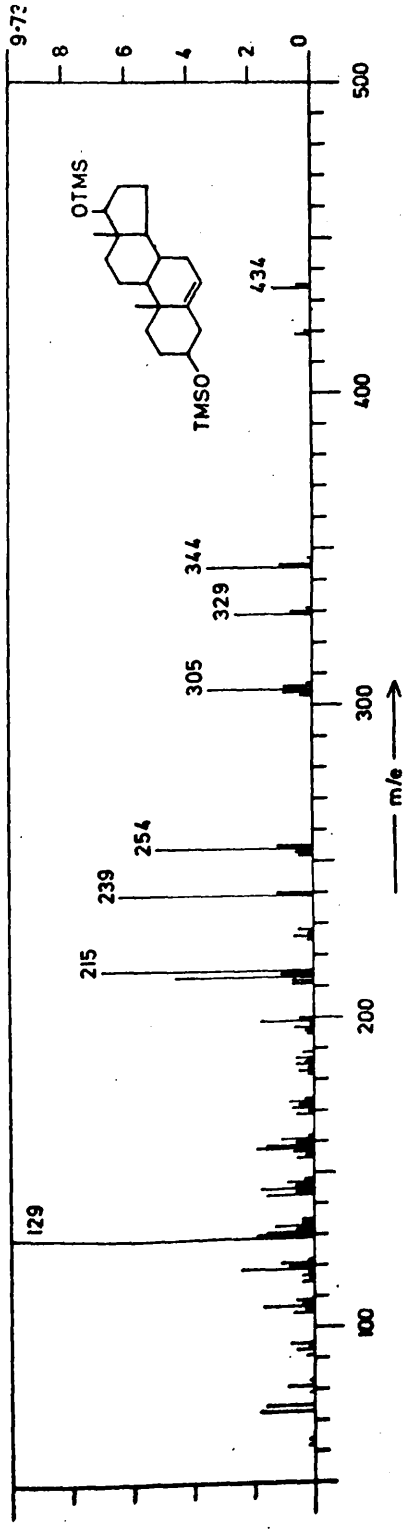

9-72
8
6
4
2
0
OTMS
TMSO
129
215
239
254
305
329
344
434
100
200
300
400
500
m/e

Fig.32

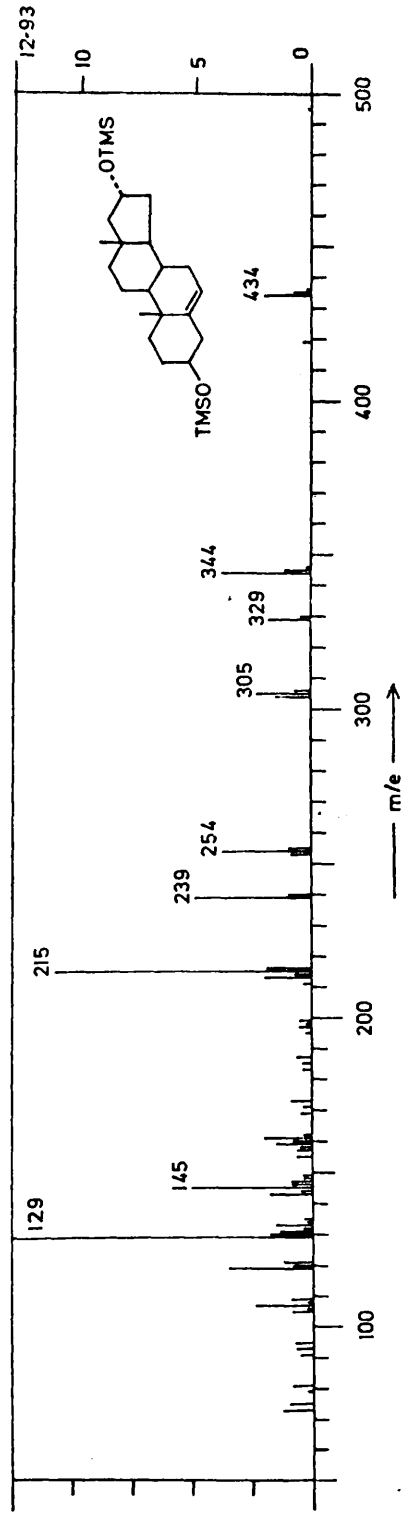

12-93
10
5
0
OTMS
TMSO
129
145
215
239
254
305
329
344
434
100
200
300
400
500
m/e

Fig. 33

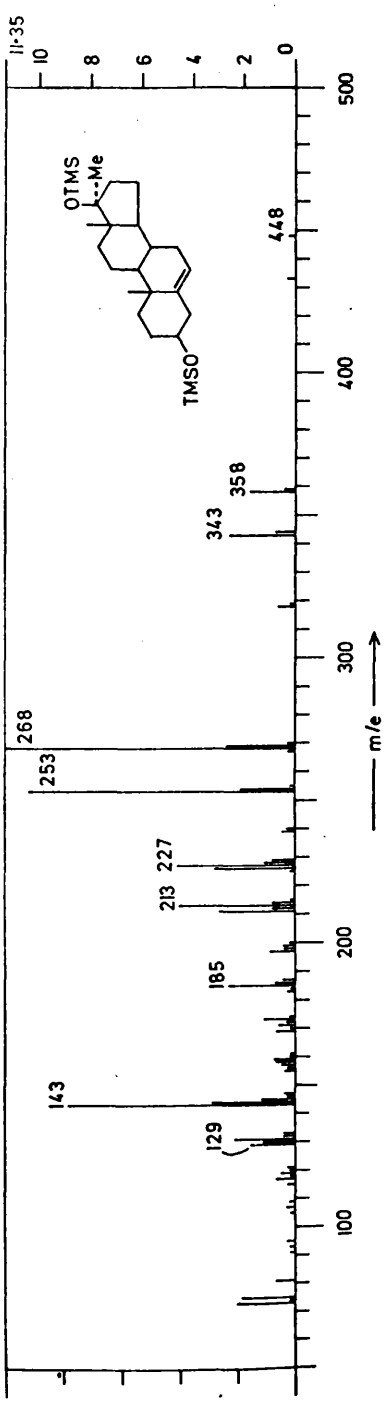

11·35
OTMS
Me
TMSO
268
253
227
213
185
143
129
343
358
448
m/e

Fig. 34

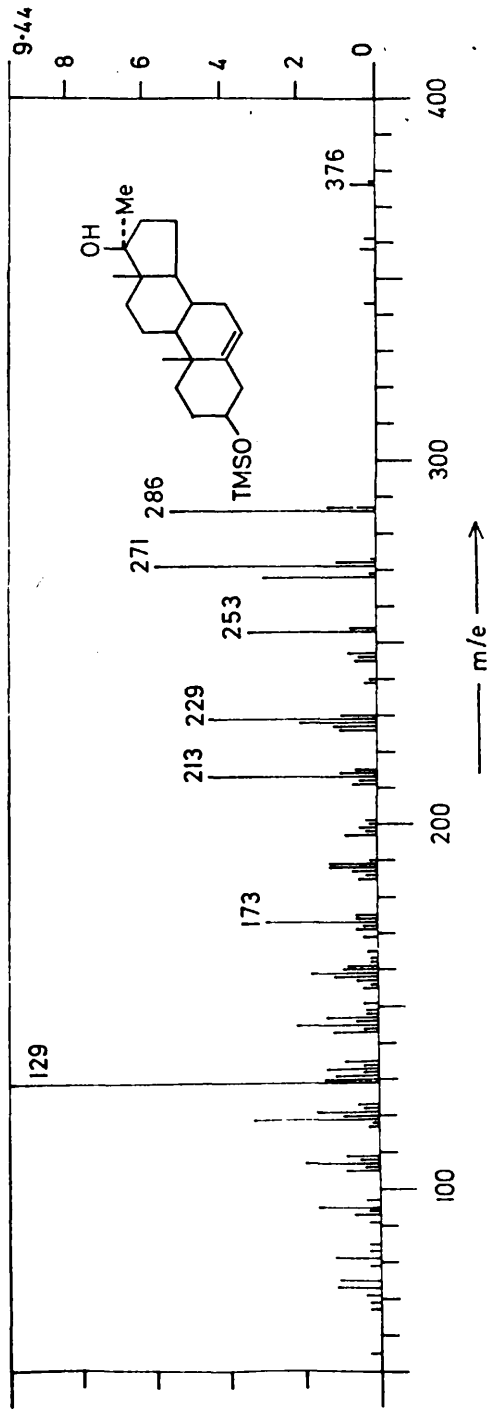

9·44
OH
Me
TMSO
286
271
253
229
213
173
129
376
m/e

Fig. 35

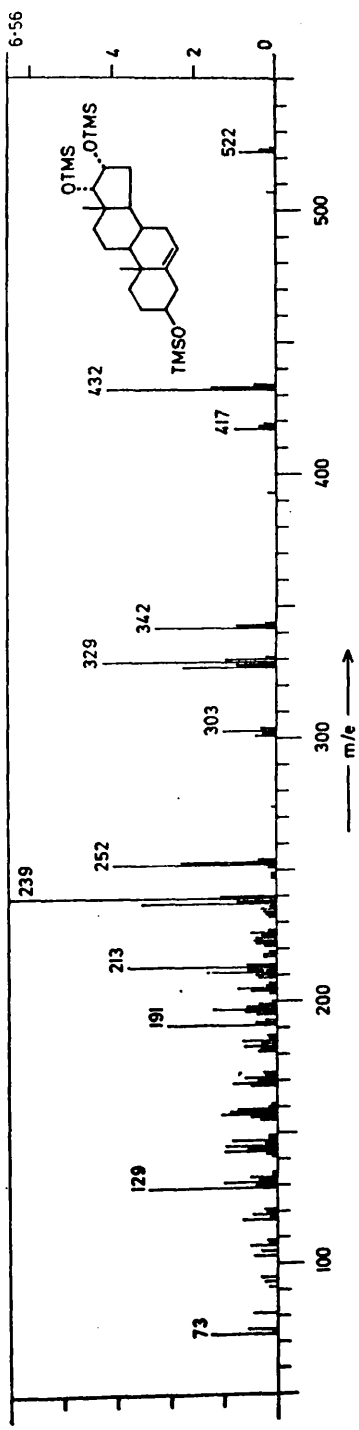

6·56
4
2
0
OTMS
OTMS
TMSO
522
432
417
342
329
303
252
239
213
191
129
73
100
200
300
400
500
m/e

Fig. 36

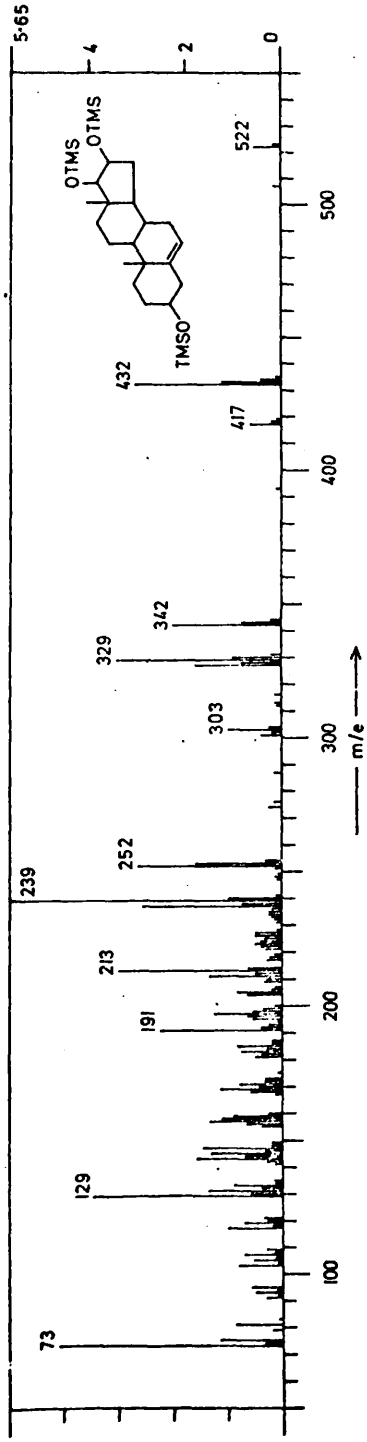

5·65
4
2
0
OTMS
OTMS
TMSO
522
432
417
342
329
303
252
239
213
191
129
73
100
200
300
400
500
m/e

Fig. 37

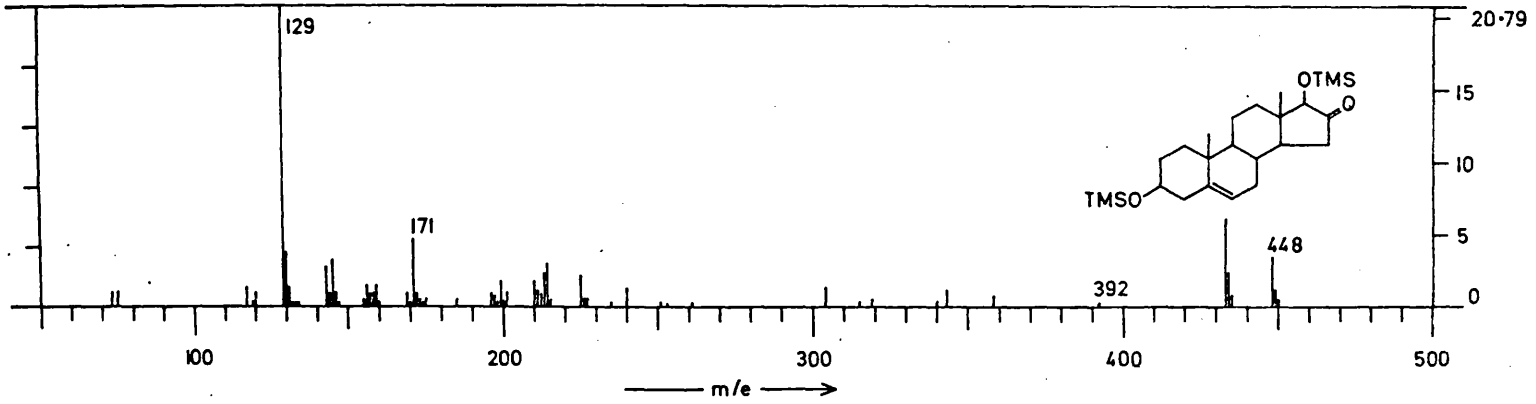

129
171
392
448
20·79
15
10
5
0
OTMS
O
TMSO
100
200
300
400
500
m/e

Fig. 38

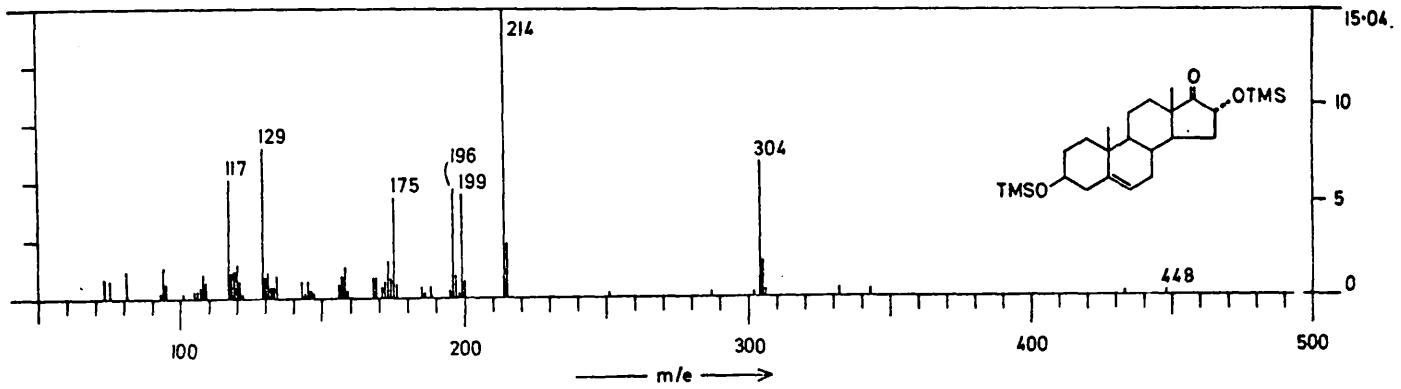

214
129
117
175
196
199
304
448
15·04
10
5
0
O
OTMS
TMSO
100
200
300
400
500
m/e

Fig. 39

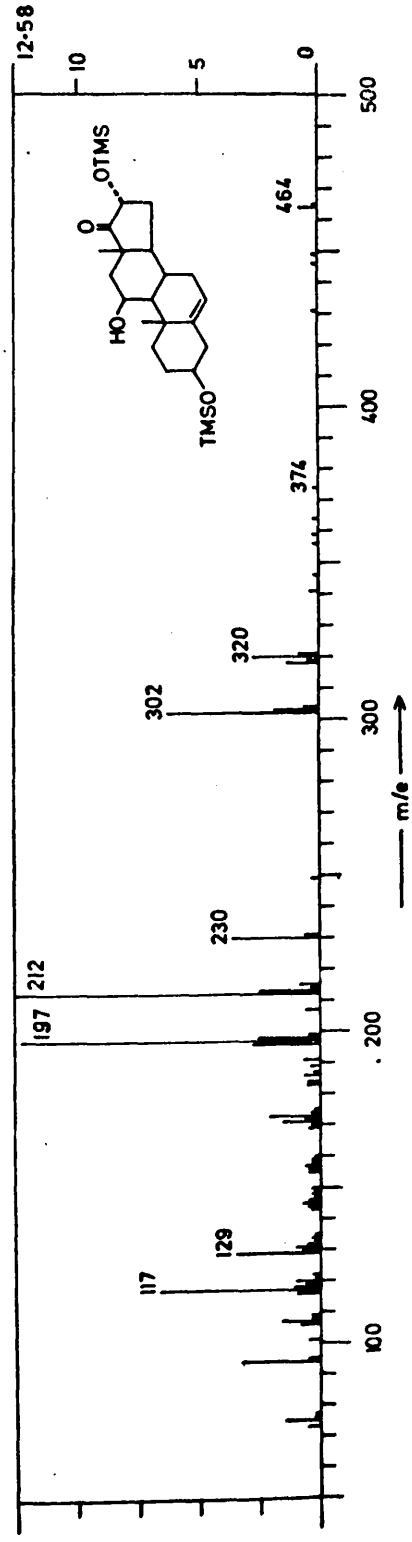

12·58
10
5
0
OTMS
O
HO
TMSO
464
374
320
302
230
212
197
129
117
100
200
300
400
500
m/e

Fig. 40

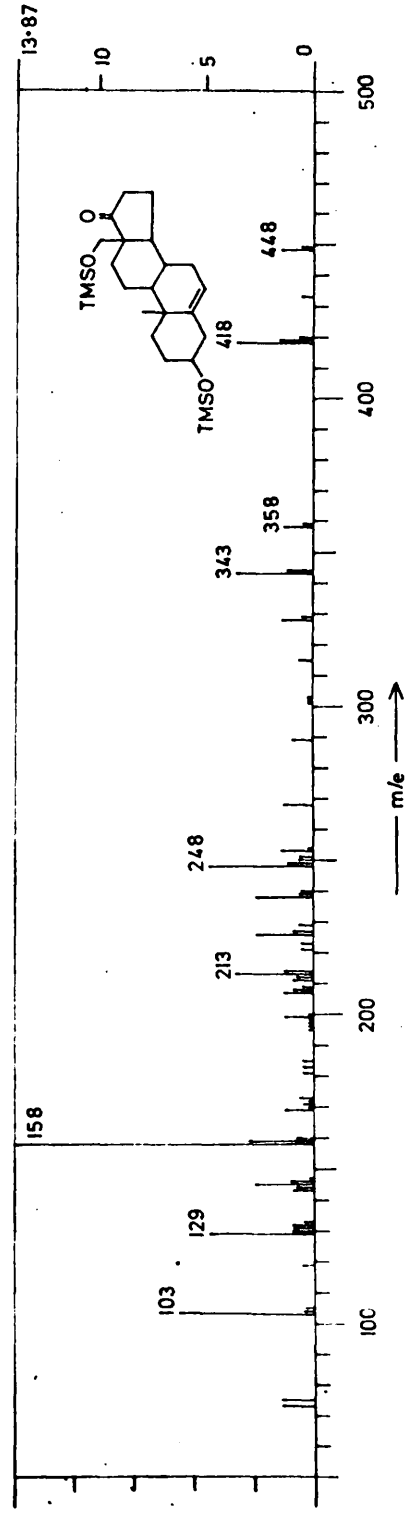

13·87
10
5
0
TMSO
O
TMSO
448
418
358
343
248
213
158
129
103
100
200
300
400
500
m/e

Fig. 41

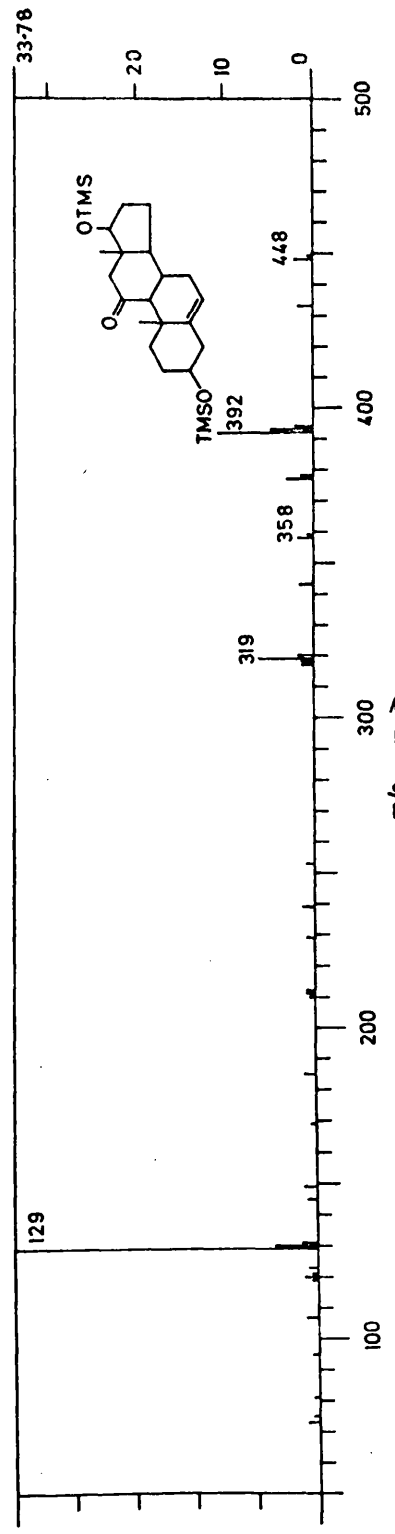
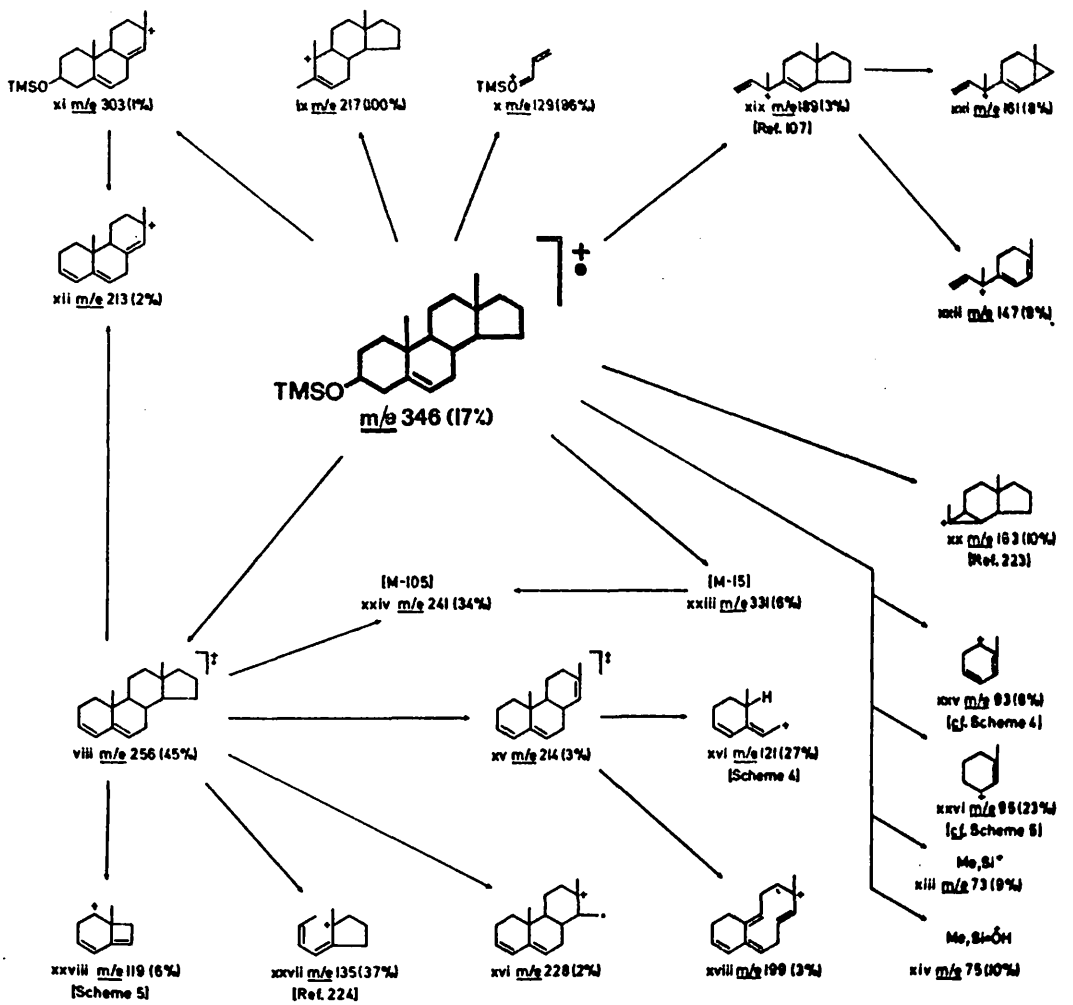
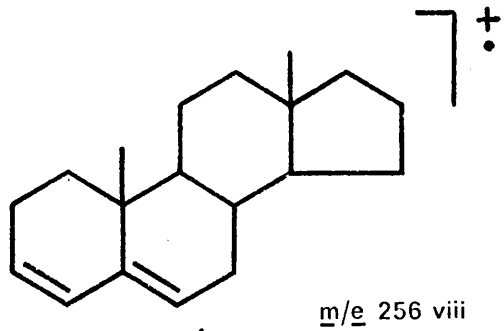

33·78
20
10
0
500
400
300
200
100
m/e
129
319
358
392
448
OTMS
TMSO
O

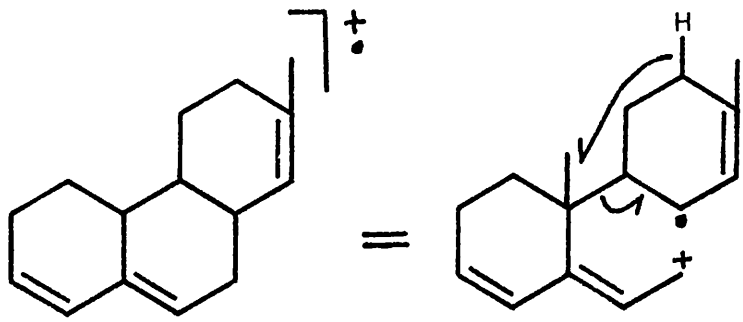
Fig. 42


TMSO
xi m/e 303 (1%)
ix m/e 217 (100%)
TMSO
x m/e 129 (86%)
xix m/e 189 (3%)
[Ref. 107]
xxi m/e 161 (8%)
xii m/e 213 (2%)
xxii m/e 147 (9%)
TMSO
m/e 346 (17%)
xx m/e 163 (10%)
[Ref. 223]
[M-105]
xxiv m/e 241 (34%)
[M-15]
xxiii m/e 331 (6%)
xxv m/e 93 (6%)
[cf. Scheme 4]
viii m/e 256 (45%)
xv m/e 214 (3%)
xvi m/e 121 (27%)
[Scheme 4]
xxvi m/e 95 (23%)
[cf. Scheme 5]
Me,Si+
xiii m/e 73 (9%)
Me,Si-OH
xiv m/e 75 (10%)
xxviii m/e 119 (6%)
[Scheme 5]
xxvii m/e 135 (37%)
[Ref. 224]
xvi m/e 228 (2%)
xviii m/e 199 (3%)

Scheme 3

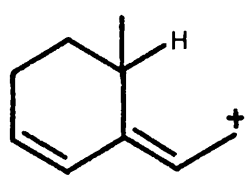


m/e 256 viii



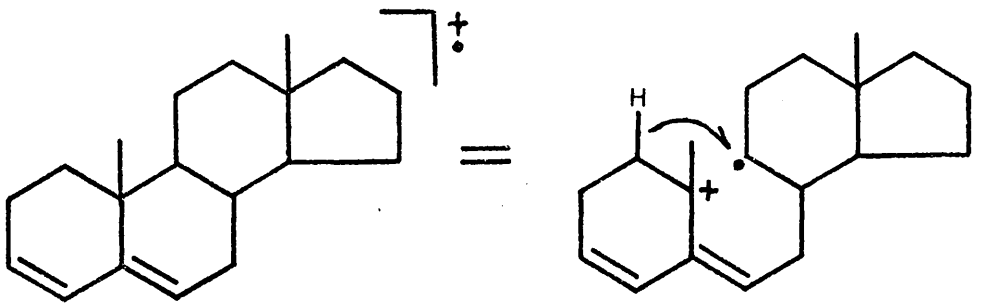
m/e 214 xv

xva

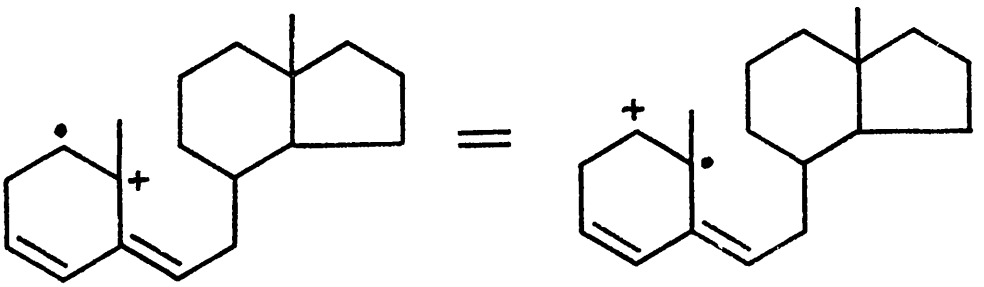


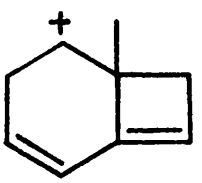
m/e 121 xvi

Scheme 4



m/e 256 viii





m/e 119 xxviii

Scheme 5

with hydrogen transfer from C-2 to C-4, and with the charge localised on the larger fragment.

m/e 129 (x): as for (ix), but with charge localisation on the smaller fragment.

The formation of these three ions can be readily rationalised by postulating that formal charge localisation is more likely at one of the following sites:

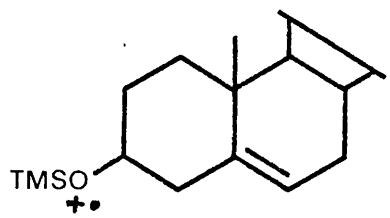
(a) the oxygen atom of the 3β-trimethylsilyloxy group by removal of an electron from a lone-pair on the oxygen atom, promoting α-fission of the C-2/3 and C-3/4 bonds (Fig. 43a),

(b) the C-5/6 bond, by removal of an electron from the π-orbital, promoting β-fission of the C-1/10, C-3/4, C-7/8, and C-9/10 bonds (Fig. 43b), or

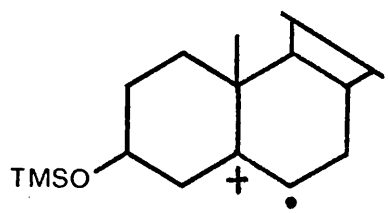
(c) one of the bonds (C-1/10 or C-9/10) adjacent to the tertiary ring junction at C-10, breaking one of these bonds by removal of an electron from the σ-orbital (Figs. 43c, 43d).

The tendency for any of these processes to take place depends, to some extent, on the influence of other substituents in the steroid nucleus. It should be noted that, whereas Δ^5-3β-hydroxy steroid TMS ethers give rise to ions at m/e 129, Δ^4-3β-hydroxy steroid TMS ethers give rise to ions at m/e 142 and 143.[217]

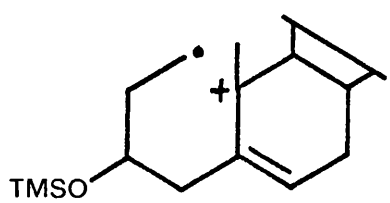
Fragmentation of ring D of steroids depends largely on the substituents therein, but also on substituents elsewhere in the molecule.[107] A major ion (xi) is formed by loss of a fragment apparently comprising



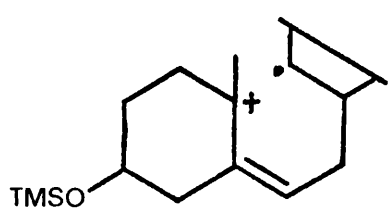
a



b



c



d

Fig. 43

C-15/16/17, with substituents, and a hydrogen atom (usually from C-8) from the molecular ion of many of these androstenol TMS ethers.[222] Loss of such a fragment ion from viii, or elimination of trimethylsilanol from xi, gives rise to a "nuclear" fragment ion (xii) of m/e 213, or of correspondingly higher mass if there are further substituents in this fragment.

Loss of a methyl radical from the molecular ion, and many of the fragment ions, is observed and could conceivably arise from several sources. For cholesteryl TMS ether, however, it has been demonstrated that the $[M-15]^+$ ion is formed mainly by loss of a methyl radical from the TMS group.[221] This should not preclude the possibility of its origin by other routes from suitably substituted androstenol TMS ethers.

Ions of m/e 73 (xiii) and m/e 75 (xiv) appear in the spectra of almost all TMS ethers but are of little diagnostic significance.

In order to gain further insight to the mechanisms of fragmentations of these compounds, it would seem logical to examine, first of all, the spectrum of androst-5-en-3β-ol TMS ether (XXXIII, Fig. 26, Scheme 3) and then to attempt to correlate the various influences of other substituents. It will, however, be seen that XXXIII is not truly representative of the series. Each of the ions viii-x is formed in relatively high abundance. Further loss of a methyl radical from viii is attested by a metastable ion: it probably originates by loss of C-19. As with cholesteryl TMS ether, it seems likely that the

$[M-15]^+$ ion is formed *via* loss of a methyl radical from the TMS group, there being no metastable ion for further loss of 90 m.u. The abundant ion of m/e 135 is probably formed in the same way as that in the spectrum of 5α-androstane: it would comprise rings C and D from which a hydrogen atom has been lost.[107] A further significant ion appears at m/e 121. This ion appears in the majority of the spectra of XXXIII-XLIX and also in many previously published spectra of samples of this type, but its significance has been obscured by the presence of other ions of similar mass, particularly in the 70 eV spectra. A possible mechanism of its formation is postulated in Scheme 4. Elimination of C-15/16/17 from viii gives rise to an ion (xv) of m/e 214 (3%). Allylic participation of the double bonds at C-5/6 and C-13/14 leads to preferential charge localisation at the C-7/8 bond (xva). This, in turn, and with the participation of the C-5/6 double bond, tends to polarise and weaken the C-9/10 bond. Fission of this bond and concomitant transfer of a hydrogen atom to C-10 produces the ion xvi. This hydrogen atom probably arises from C-12, since its bond to C-12 is weakened by the presence of the C-13/14 double bond and also because an ion of m/e 121 is present in the spectra of the 11-substituted analogues. Moreover, an ion of m/e 121 is observed in the 20 eV spectrum of cholesteryl TMS ether which is shifted to m/e 135 and m/e 149 in 4α-methyl- and 4,4'-dimethylcholesteryl TMS ether, respectively.[135] It can be seen, then, that the formation of xvi requires a delicately

balanced interplay of electrostatic effects and it is not surprising that the relative abundance of m/e 121 is much lower in the spectra of samples containing other substituents. Possible origins of these and other ions in the spectrum are shown in Scheme 3, although it should be emphasised that there is little direct evidence for the structures of these ions. It would be difficult to assign mechanisms to the formation of the few ions of lower mass because of the numerous alternative possibilities. Nevertheless, the present explanation is sufficient foundation for investigation of the influences substituents on the fragmentations of other androstenol TMS ether derivatives.

An impressive feature of the mass spectrum of 3β-hydroxyandrost-5-en-17-one TMS ether (XXXIV, Fig. 27) is its simplicity.[26,31,210,219] The base peak, at m/e 129, accounts for 38% of the total ion current. The high abundance of this ion can be accounted for on the basis that the neutral fragment is capable of removing a greater proportion of the excess energy by virtue of its possession of a keto group. The $[M-56]^+$ ion at m/e 304 appears to be formed by loss of C-15/16/17, with substituents, and a hydrogen atom from the molecular ion. The origin of this fragment ion appears, however, to be more complex. While this thesis was in preparation, it was found that 16,16-d_2-3β-hydroxyandrost-5-en-17-one TMS ether (formed by in transitu deuterium labelling) also gives an ion at $[M-56]^+$ (m/e 220). This might indicate that the fragmentation of ring D is accompanied by specific double hydrogen atom rearrangement, although there remains the possibility that the ion at m/e 220 is formed by an alternative loss of 56 m.u.

It was also found that 5α-androstan-17-one and its 16,16-d_2 analogue both gave rise to an ion at m/e 218 corresponding to the expected ring D fragmentation.[222] This observation confirms that of Djerassi and co-workers, who found that the peak at m/e 218 in the spectrum of 5α-androstan-17-one contains only traces of oxygen-containing fragments, although the peak at m/e 217 represents 80% $C_{16}H_{25}$ and 20% $C_{15}H_{21}O$.[225]

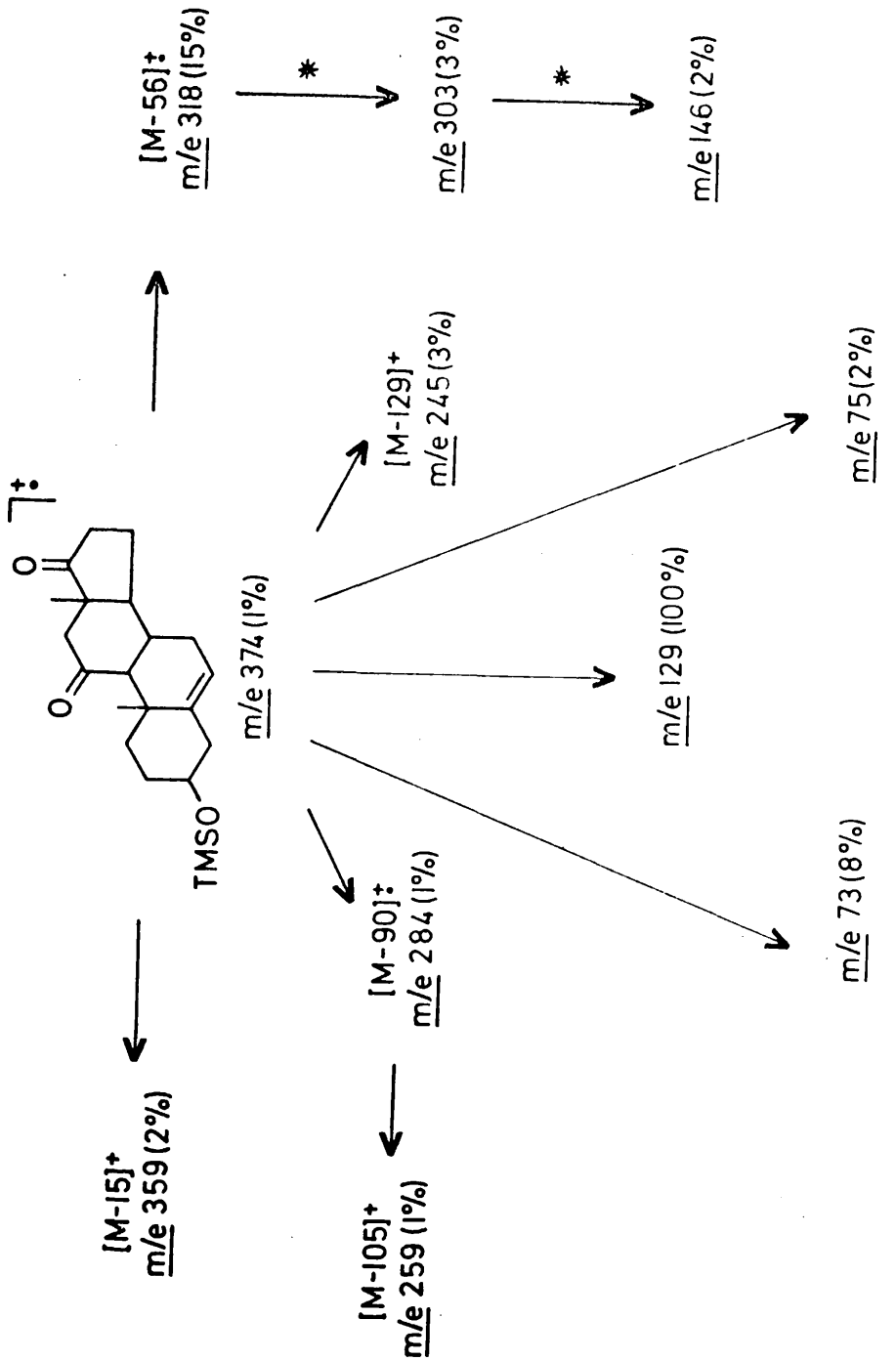
The spectrum of 3β-hydroxyandrost-5-en-16-one TMS ether (XXXV, Fig. 28) is similar in many respects to that of the isomeric 17-one, the $[M-56]^{+\cdot}$ ion being somewhat more abundant. Again, the 15,15,17,17-d_4 analogue gives rise to an ion at $[M-56]^{+\cdot}$. This would apparently preclude the formation of this ion by ring D fragmentation of the molecular ion since there have been no previous reports of specific quadruple (nor even triple) reciprocal hydrogen transfer. Further work, involving high resolution mass measurement and deuterium labelling of other keto-steroids, is to be carried out in an attempt to elucidate the mechanisms of these rragmentations.

An additional ketone function, in ring C, as in 3β-hydroxyandrost-5-ene-11,17-dione TMS ether (XXXVI, Fig.29), affords extra stability to both the neutral particles and the ions produced by certain fragmentations: the base peak (x, m/e 129) accounts for 61% of the total ion current. There is an ion at $[M-56]^{+\cdot}$. There is an intense metastable peak attesting to loss of a methyl group from this ion with the formation of m/e 303 (3%). An extremely intense metastable peak

indicates that there is a further loss of 157 m.u. with the formation of an ion of m/e 146 (1%). This is unusual in that the metastable ion is very much more abundant than both the parent and daughter ions giving rise to it. This would appear to signify that the mechanism of formation of the ion of m/e 146 has a low rate constant (the parent ion disintegrating mainly between the ion source and the magnetic analyser rather than in the ion source) presumably because of the involvement of extensive rearrangement. The fragmentations outlined in Scheme 6 account for all ions in the spectrum of XXXVI which have relatve abundance greater than 1% of the base peak.

The spectrum of 3β,11β-dihydroxyandrost-5-en-17-one mono(3) TMS ether (XXXVII, Fig. 30) is similar to that of XXXIV, but with additional ions corresponding to elimination of water from viii, ix, $[M-15]^{+}$, and $[M-90,15]^{+}$. No ion is observed at $[M-56,18]^{+}$.

The presence of a TMS ether group in ring D does not afford stability to the molecular ion, or to major fragment ions, to the same extent as a ketone group. This results in the formation of a wider variety of ions, as in the spectra of androst-5-ene-3β,17α-diol TMS ether (XXXVIII),[210,213] androst-5-ene-3β,17β-diol TMS ether (XXXIX),[210] and androst-5-ene-3β,16α-diol TMS ether (XL): the utility of low voltage spectra as an aid to the elucidation of fragmentation mechanisms is thus apparent. It is of interest to note that, whereas ions ix are formed from ring A, there is no ion corresponding to further loss of 129 m.u.. Ions of m/e 129 probably derive from both ring A and ring D since analogous ions are observed in the spectra of


[M-56]+
m/e 318 (15%)
*
m/e 303 (3%)
*
m/e 146 (2%)
[M-129]+
m/e 245 (3%)
m/e 374 (1%)
m/e 75 (2%)
m/e 129 (100%)
m/e 73 (8%)
[M-90]+
m/e 284 (1%)
[M-15]+
m/e 359 (2%)
[M-105]+
m/e 259 (1%)
TMSO
O
O

Scheme 6

many 17-trimethylsilyloxy[226] and 17-(chloromethyl)dimethylsilyloxy steroids. Thus, in the spectrum of 17α-methylandrost-5-ene-3β,17β-diol TMS ether (XLI, Fig. 34), m/e 129 (from ring A) is of relative abundance 16%, whereas m/e 143 (from ring D) is 79%. A 17-hydroxyl group (as in 17α-methylandrost-5-ene-3β,17β-diol mono(3) TMS ether, XLII, Fig. 35) directs the fragmentation of ring D to a much lesser extent than a TMS ether group. Instead, water is eliminated from the molecular ion and certain fragment ions containing a hydroxyl group, as observed in the spectrum of XXXVII.

The mass spectra of TMS ether derivatives of the trihydroxy analogues are more complex than those of the diols, and fragmentation is even more extensive: the base peaks in the spectra of androst-5-ene-3β,16α,17α-triol TMS ether (XLIII, Fig. 36) and androst-5-ene-3β,16β,17β-triol TMS ether (XLIV, Fig. 37) contribute 6.5% and 5.6%, respectively, of the total ion current. The spectra of XLIII, XLIV, and the TMS ether derivatives of the 3β,16β,17α- and 3β,16α,17β-triols are similar.[205,206] The expected fragmentations of TMS ether derivatives are observed, with sequential loss of trimethylsilanol (90 m.u.) and methyl radicals. There are additional intense ions at m/e 329 (61%), 239 (100%), and 191 (45%)(relative intensities are for XLIV). The ion of m/e 191 has been observed in the spectra of TMS ether derivatives of other 16,17-diols,[204,226-228] 17,18-diols[229] 15,17-diols,[218] and a 15,16,17-triol[136] and has been demonstrated to be a rearrangement ion containing two trimethylsilyloxy groups.[230] The ions at m/e 329 and m/e 239 are apparently formed by sequential loss of trimethylsilanol

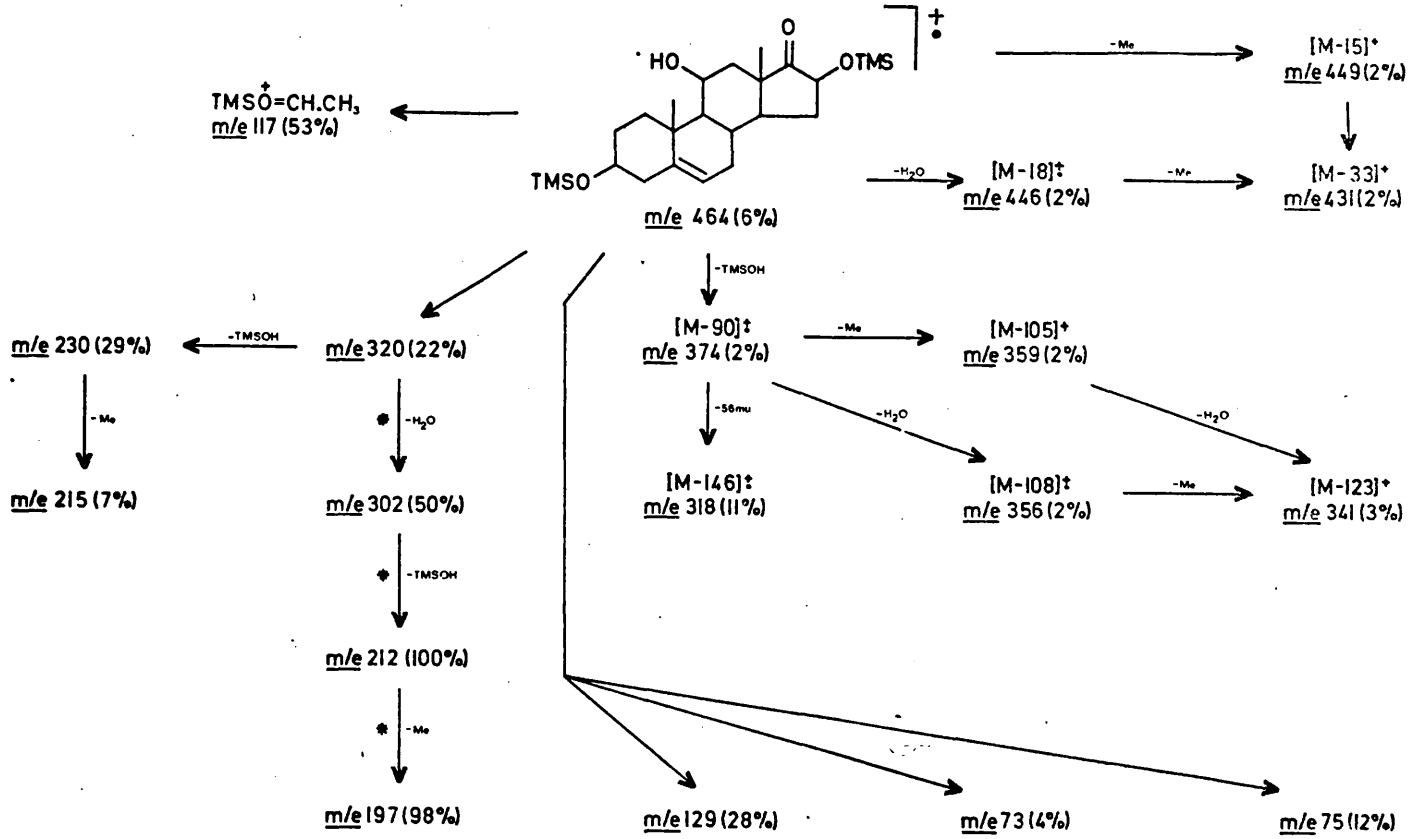
from the ion $[M-103]^+$. There is, in fact, an ion of low abundance at $[M-103]^+$ (m/e 419; 4%,2%: XLIII, XLIV). Ions at $[M-103]^+$ are typical of TMS ether derivatives of primary aliphatic alcohols[133] and also those of 21-hydroxy steroids.[231] These ions also appear in the spectra of TMS ether derivatives of steroids with vicinal hydroxyl groups.[227] They are probably formed by fission of the C-13/17 bond, transfer of a hydrogen atom to C-17, and rupture of the C-16/17 bond. This latter fission would be directed by the presence of the 16-trimethylsilyloxy group.

There are significant differences between spectra of the 16-keto-17β-trimethylsilyloxy and 16α-trimethylsilyloxy-17-keto steroids.[203,206] Spectra of the former, as in 3β,17β-dihydroxy-androst-5-en-16-one TMS ether (XLV, Fig. 38), have an ion of type ix as the base peak and afford many of the expected fragmentations. The ion $[M-56]^+$ could be formed by sequential loss of a methyl radical and C-15/16 with substituents, less one hydrogen atom but, in view of the observations on the spectra of deuteriated 16- and 17-ketones (see above), further work is to be carried out on this compound. The fragmentation pattern of 3β,16α-dihydroxyandrost-5-en-17-one TMS ether (XLVI, Fig. 39) has been described by Siegel *et al.*[208] Both XLVI and 3β,11β,16α-trihydroxyandrost-5-en-17-one di(3,16) TMS ether (XLVII, Fig. 40) give relatively weak m/e 129 peaks. The base peaks (m/e 214,212: XLVI,XLVII) are due to fragments formed by elimination of C-15/16/17 and, in the case of XLVII, a molecule of water from viii.[203,208] Abundant ions are produced by

further loss of methyl radicals. Formation of the ion of m/e 175 has been explained[203] by elimination of C-15/16/17 from x. Abundant ions are present at m/e 117, probably comprising C-15/16 with substituents and an additional hydrogen atom. Several of the proposed fragmentations of XLVII are substantiated by metastable peaks: these are summarised in Scheme 7. In all of these spectra, an ion comprising C-16/17, less a hydrogen atom, accounts for a proportion of the peak at m/e 129.[232]

The spectrum of the 18-trimethylsilyoxy compound, 3β,18-dihydroxy-androst-5-en-16-one TMS ether (XLVIII, Fig. 41), is more difficult to interpret. In particular, the ions of m/e 158 (base peak) and m/e 248 have apparently not been previously observed. It is tentatively proposed that m/e 248 comprises rings A and B, whereas the base peak is produced by elimination of trimethylsilanol from this ion. The ion of m/e 103 is presumably $TMS\overset{+}{O}=CH_2$ from C-18 (see above). The $[M-30]^{+\cdot}$ ion apparently arises via a migration of the TMS group.[138]

The apparently anomalous ion at $[M-56]^{+\cdot}$ in the spectrum of 3β,17β-dihydroxyandrost-5-en-11-one TMS ether (XLIX, Fig. 42) may have a similar origin to that tentatively proposed for XLV; loss of a methyl radical being accompanied by elimination of C-11/12 with substituents, less a hydrogen atom. The origin of this ion is to be further investigated by deuterium labelling studies. The high abundance of the ion of m/e 129 may be rationalised on the basis that energy can be readily delocalised over the ketone-containing neutral


HO
O
OTMS
TMSO
m/e 464 (6%)
TMSO=CH.CH3
m/e 117 (53%)
-Me
[M-15]+
m/e 449 (2%)
-H2O
[M-18]‡
m/e 446 (2%)
-Me
[M-33]+
m/e 431 (2%)
-TMSOH
[M-90]‡
m/e 374 (2%)
-Me
[M-105]+
m/e 359 (2%)
-56mu
[M-146]‡
m/e 318 (11%)
-H2O
[M-108]‡
m/e 356 (2%)
-Me
[M-123]+
m/e 341 (3%)
-H2O
-H2O
m/e 320 (22%)
-TMSOH
m/e 230 (29%)
-Me
m/e 215 (7%)
-H2O
m/e 302 (50%)
-TMSOH
m/e 212 (100%)
-Me
m/e 197 (98%)
m/e 129 (28%)
m/e 73 (4%)
m/e 75 (12%)

Scheme 7

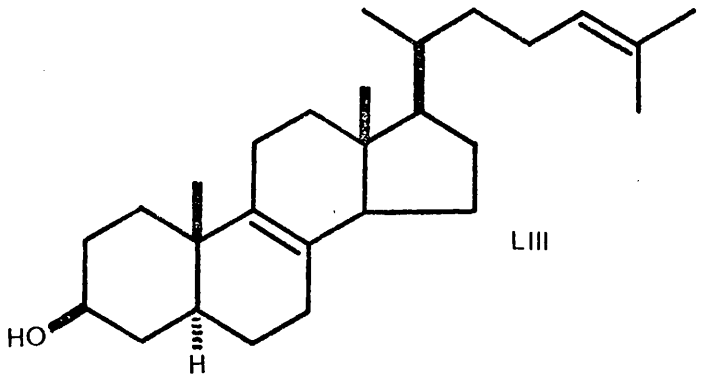
fragment.

The major fragmentations of a series of seventeen analogues of androst-5-en-3β-ol TMS ether have been examined. Many of these have been observed and explained previously:[169] their generality is now demonstrated, with the exception of $[M-56]^{+\cdot}$ ions in several spectra. It has been found desirable to carry out deuterium labelling and high resolution measurements to account for the formation of these ions. Several hitherto unobserved ions are reported and possible origins are postulated. More stable ions (hence, usually, ions of greater relative abundance) are formed if the neutral particle formed in a fragmentation contains a keto group. This observation can be explained if it is assumed that the energy content of such particles can be higher than those which do not contain keto groups, because more energy can be delocalised over the keto group. This factor is of particular relevance in the comparison of the spectrum of XXXIII with those of the substituted analogues: this illustrates the pitfalls of a "systematic" approach to the investigation of fragmentation mechanisms of complex molecules.

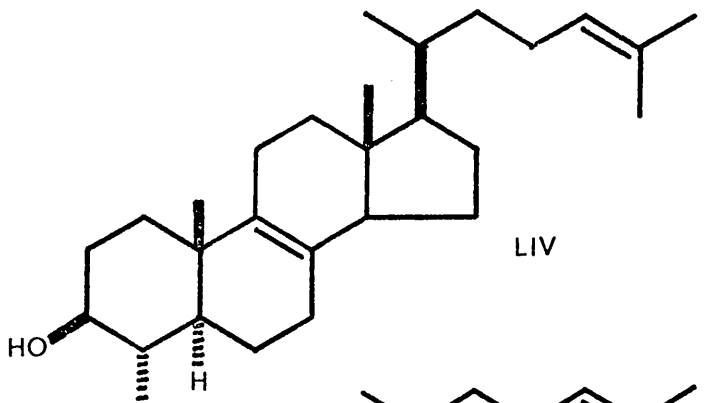
THE MASS SPECTRA OF TMS ETHER DERIVATIVES OF SOME UNSATURATED STEROLS

In mass spectrometry, the presence of a double bond in a molecule has been found to give rise to several types of fragmentation including simple β-cleavage, the McLafferty rearrangement, and the retro-Diels-Alder rearrangement. The formulation of mechanisms for these processes usually requires formal charge localisation at the double bond. The influence of any particular double bond on the fragmentation of the molecule is affected by other double bonds or functional groups which may be present in the molecule. Wyllie and Djerassi have carried out deuterium labelling experiments on three steroid hydrocarbons with unsaturated side-chains (5β-cholest-24-ene, L, 5α-cholest-22-ene, LI, and 21-nor-5α-cholest-24-ene, LII) and have proposed mechanisms for fragmentation of these and other unsaturated side-chains.[233] They reported that these fragmentations are typical, also, of sterols with unsaturated side-chains but that they are somewhat suppressed by the formation of acetates or TMS ethers. The mass spectra of TMS ethers of a large number of unsaturated sterols have been briefly discussed by Knights[223] and Brooks *et al.*[135]

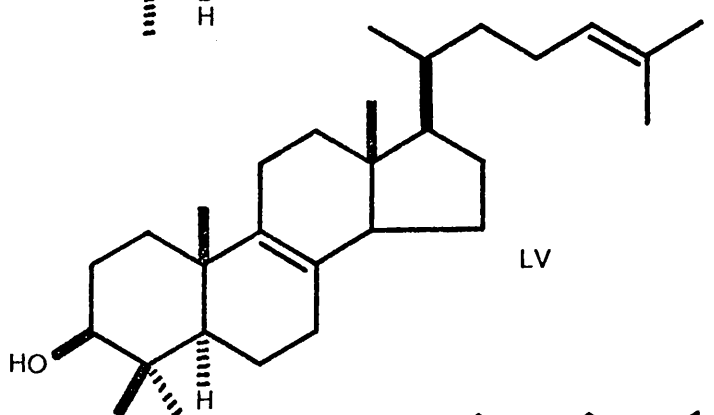
In an attempt to characterise certain sterols isolated from yeast (see following section), it was found necessary to investigate the mass spectral fragmentations of further reference steroids. TMS ethers were used because of their superior gas chromatographic properties: mass spectra of TMS ethers of seventeen authentic sterols (LIII-LXIX) were examined in detail. Partial mass spectra (70 eV) are presented in Table 5.



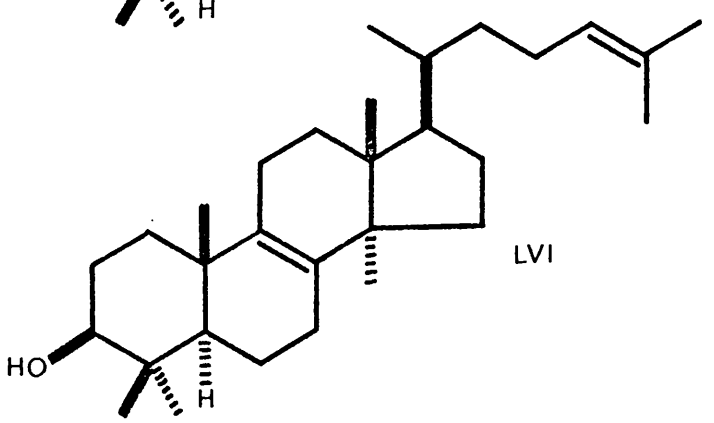
LIII



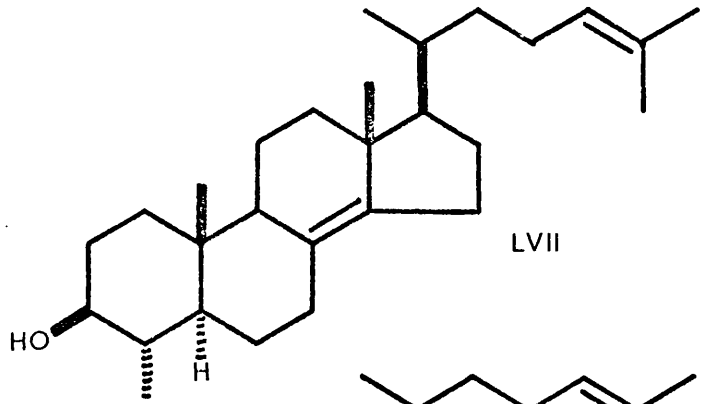
LIV



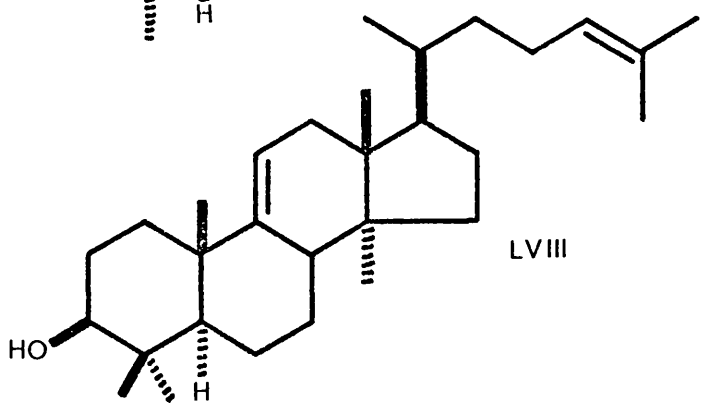
LV



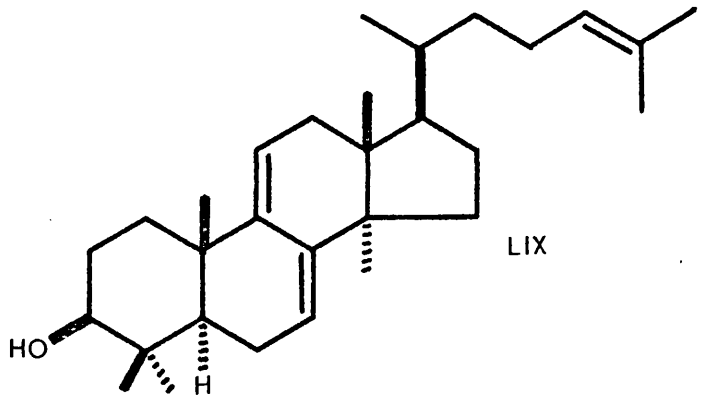
LVI



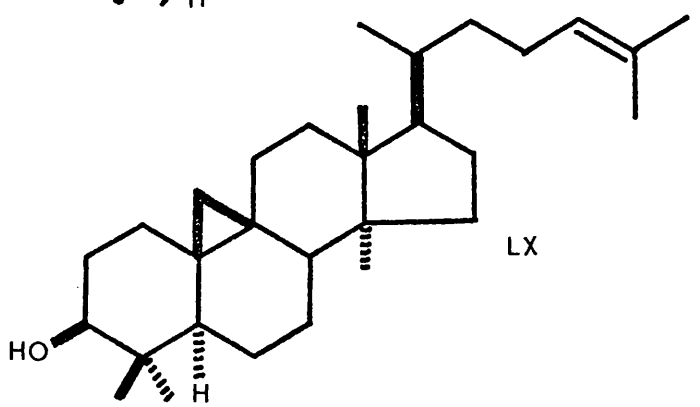
LVII



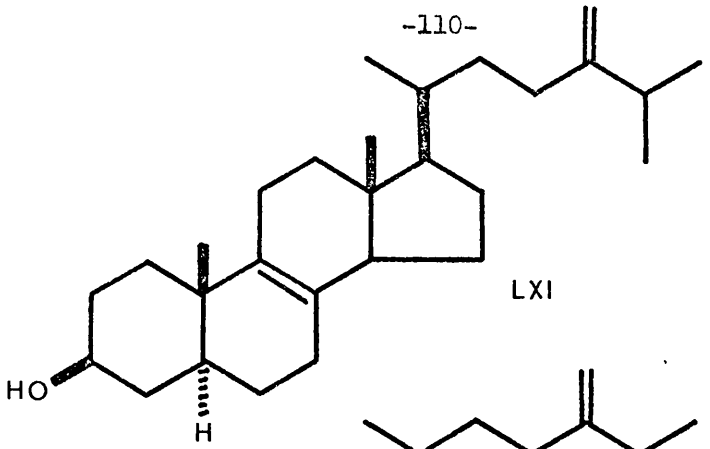
LVIII



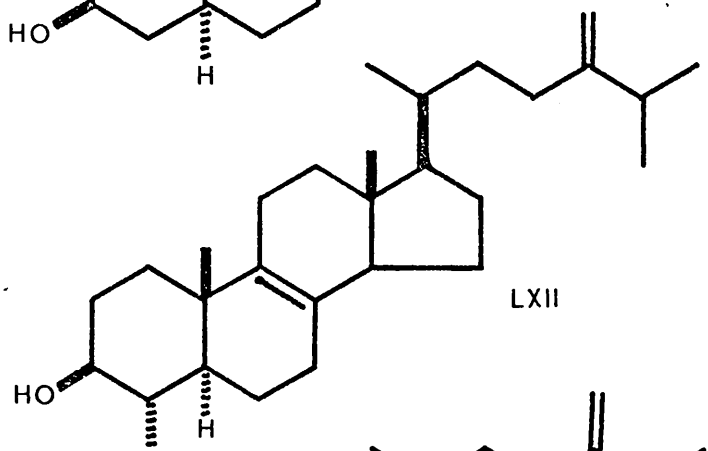
LIX



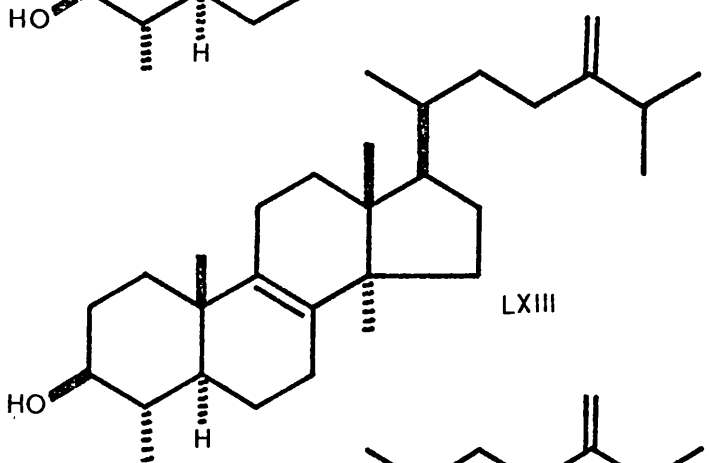
LX



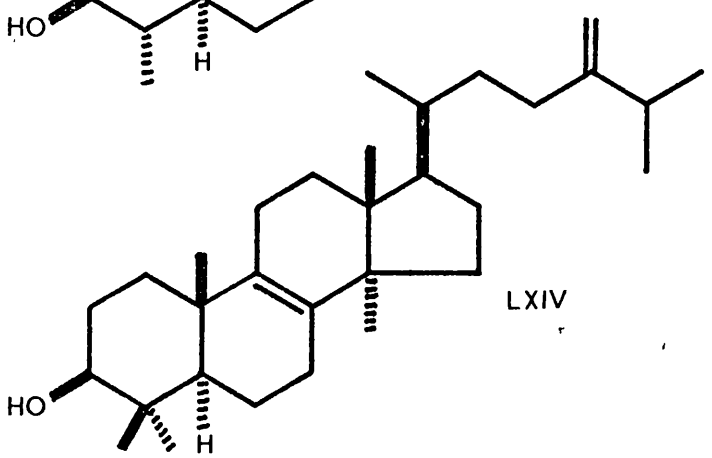
LXI



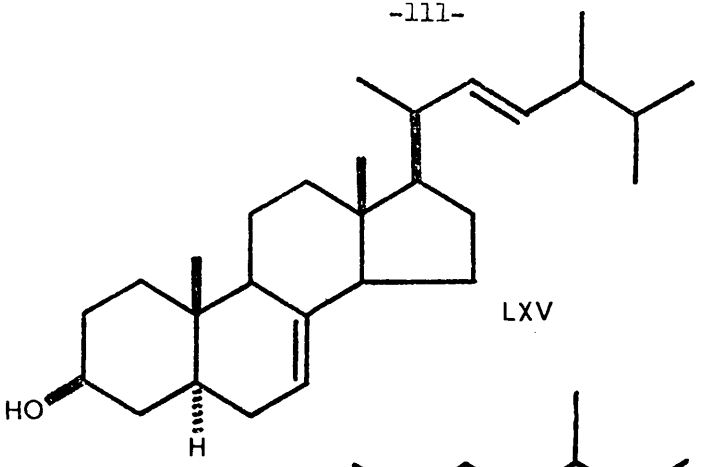
LXII



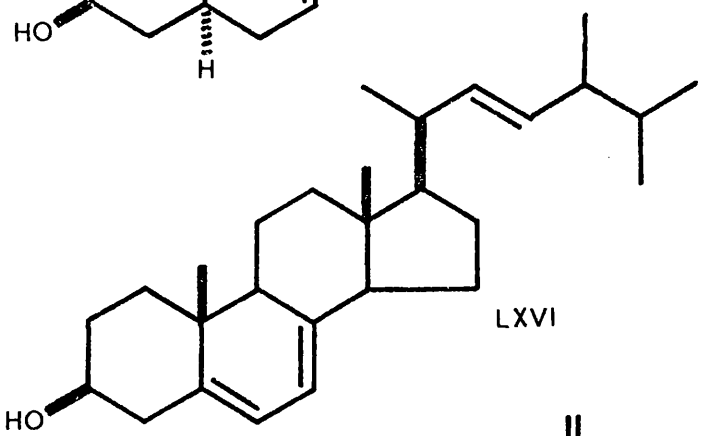
LXIII



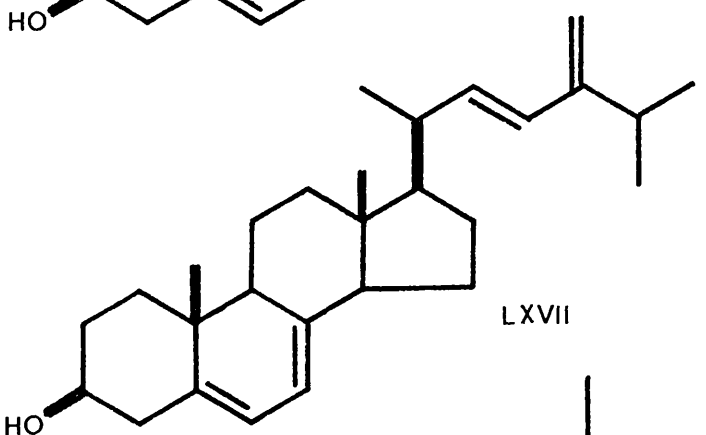
LXIV



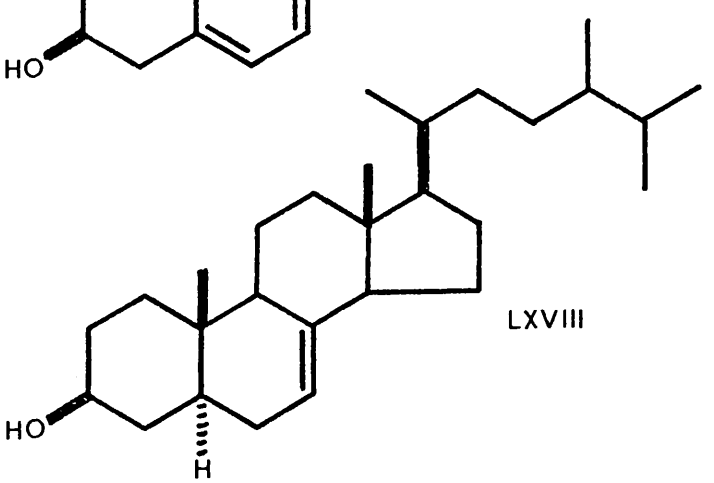
LXV



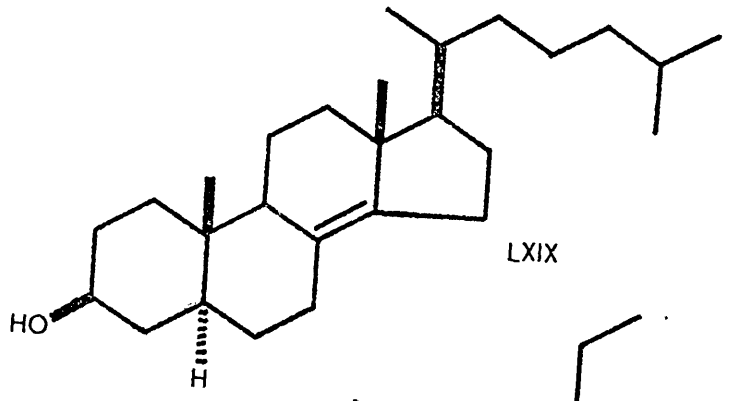
LXVI



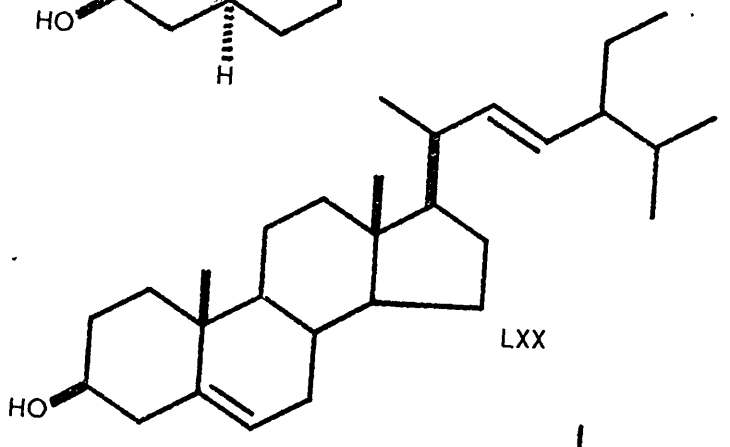
LXVII



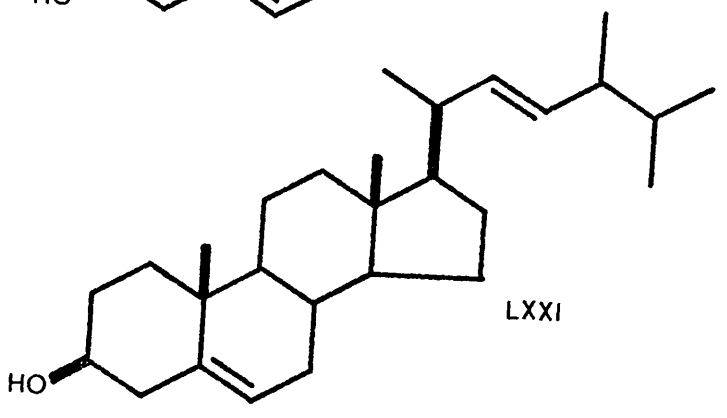
LXVIII



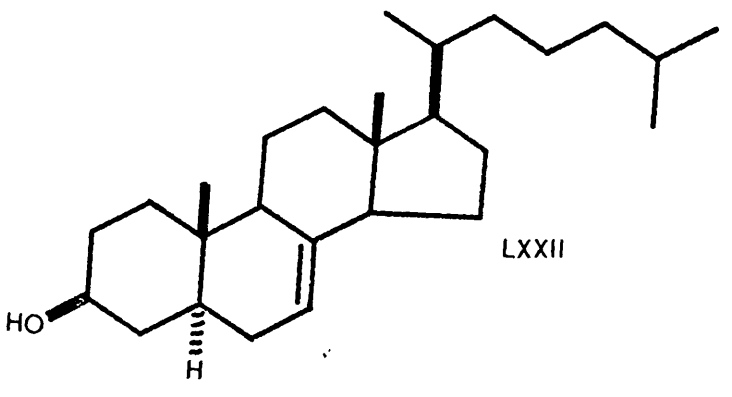
LXIX



LXX



LXXI



LXXII

Table 5. Partial mass spectral data for TMS ethers of LIII - LXIX

LIII	456	69	75	73	107	81	95	91	105	213	93
	36	100	66	59	53	40	37	33	33	30	30
LII	470	69	73	75	55	121	95	81	109	105	93
	48	100	71	60	58	51	42	42	38	38	35
III	484	69	73	135	75	81	109	105	147	93	121
	22	100	92	78	49	34	32	29	26	24	23
LIV	498	69	393	73	109	55	95	75	81	394	483
	18	100	85	52	46	40	38	34	29	28	24
LVIV	470	121	227	455	109	365	243	380	69	147	95
	100	47	33	30	30	29	28	25	24	22	22
LVIII	498	69	73	393	75	55	95	109	81	129	119
	8	100	61	49	42	39	38	31	26	25	21
LIX	496	69	73	75	55	253	95	129	81	157	109
	32	100	81	42	42	38	27	26	24	23	23
LX	498	69	73	95	55	81	109	107	93	75	393
	1	100	71	46	44	38	36	33	31	30	27
LXI	470	227	455	213	107	150	365	229	380	147	95
	100	61	55	55	53	51	46	43	33	33	31
LXII	484	55	73	75	69	121	95	81	105	379	107
	92	100	92	81	77	75	73	65	62	61	55
LXIII	498	393	483	394	95	484	97	109	227	123	69
	37	100	77	34	33	31	30	28	27	27	24
LXIV	512	407	408	497	241	95	123	109	135	83	498
	25	100	34	33	21	18	17	17	15	15	14
LXV	470	69	75	81	73	255	107	343	105	95	67
	20	100	95	68	65	61	46	41	36	34	33
LXVI	468	69	73	363	337	81	119	131	143	253	157
	17	100	97	77	56	44	38	37	35	29	28
LXVII	466	361	73	251	81	123	211	143	119	55	131
	29	100	89	68	68	55	41	40	35	35	34
LXVIII	472	75	255	73	55	107	81	95	213	105	57
	57	100	76	58	50	43	41	37	36	34	32
LXIX	472	75	107	73	55	147	229	213	57	105	81
	100	76	73	60	53	45	43	40	40	39	39

Δ^{24}-sterol TMS ethers

Zymosterol (LIII) TMS ether (Fig. 44). The molecular ion, base peak of the 20 eV spectrum,[135] is of relative abundance 36%. The base peak (m/e 69) is undoubtedly due to fission allylic to the Δ^{24} bond.[234] There is no corresponding ion at $[M-69]^+$, nor at $[M-69,90]^+$, which would result from further loss of trimethylsilanol. There is no evidence for a McLafferty rearrangement in the side-chain. In fact, it appears that the sole contribution of the Δ^{24} bond to the spectrum is in the formation of the base peak. Simple cleavage of the C-17/20 bond would produce an ion $[M-111]^+$. This ion is absent from the spectrum, although there is an ion (m/e 255, 6%) due to a further loss of trimethylsilanol. Concomitant loss of the side-chain and two hydrogen atoms, a major feature in the spectra of sterols[223,235] and steroid hydrocarbons[233] with unsaturated side-chains, gives rise to an ion of low (4%) abundance at m/e 343. The corresponding ion at m/e 253 is also of low abundance (3%). The usual fragmentations of sterol TMS ethers are observed: $[M-15]^+$ (m/e 441, 21%), $[M-90]^{+\cdot}$ (m/e 366, 8%), $[M-90,15]^+$ (m/e 351, 19%), m/e 75 (70%), and m/e 73 (58%). Other major fragment ions appear to be produced by fragmentation of ring D of the steroid nucleus. Mechanisms of fragmentation of this type are extremely complex, often involving reciprocal hydrogen transfers and several parallel pathways.[222] Loss of C-16, C-17 and the side-chain produces ions at m/e 318 (2%) and 228 (7%). A similar fission, accompanied by loss of a hydrogen atom gives no ion of m/e 319, but an

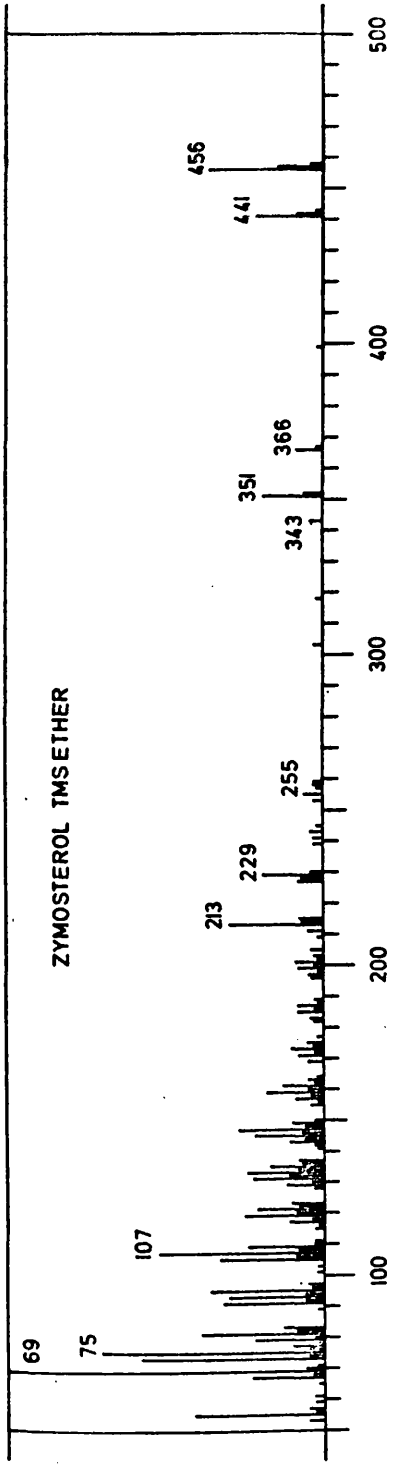

ZYMOSTEROL TMS ETHER
69
75
107
213
229
255
343
351
366
441
456
100
200
300
400
500

Fig.44

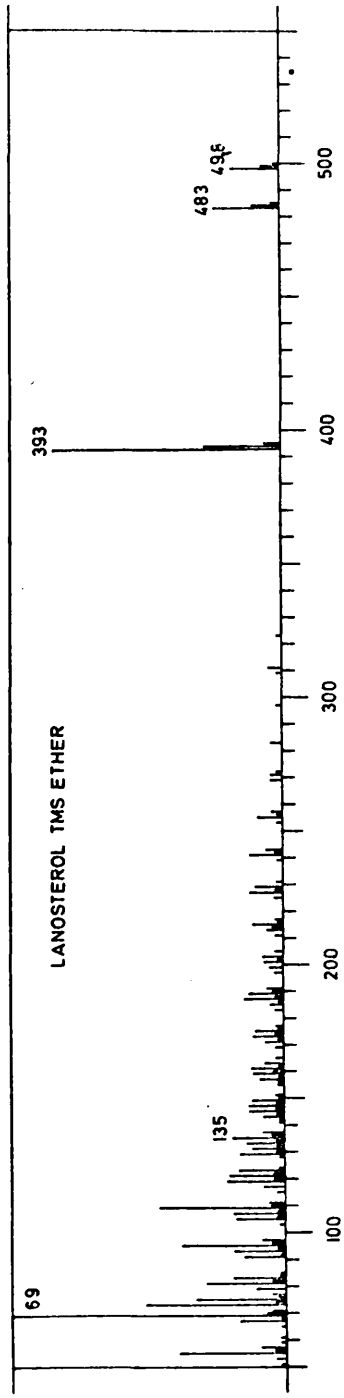

LANOSTEROL TMS ETHER
69
135
393
483
498
100
200
300
400
500

Fig.45

ion of relatively high abundance (19%) at m/e 229 is formed. Elimination of C-15/16/17 and the side-chain with an additional hydrogen atom produces ions at m/e 303 (3%) and m/e 213 (30%). 4α-Methylzymosterol (LIV) and 14-Desmethyllanosterol (LV) TMS ethers give similar spectra to that of zymosterol TMS ether. A notable trend in the spectra of this series is the relatively high abundance of ions of m/e 107 (52%), m/e 121 (50%), and m/e 135 (77%) from LII, LIII, and LIV TMS ethers, respectively, although each of these ions is present in all three spectra. This indicates that these ions comprise C-4 and substituents with an additional C_7H_9 unit. It would be rash to suggest a structure for these ions without further evidence. Lanosterol (LVI) TMS ether gives a rather different spectrum (Fig. 45). The additional methyl group at C-14 is allylic to the Δ^8 bond and appears to be readily eliminated. There are abundant ions at $[M-15]^+$ (m/e 483, 24%) and $[M-15,90]^+$ (m/e 393, 84%; base peak of 20 eV spectrum). There is no ion at $[M-90]^+$ in the 70 eV spectrum, and one of only 1% relative abundance in the 20 eV spectrum. The base peak is still at m/e 69, but fragmentation of ring D is greatly suppressed. The ion of m/e 135 is of lower relative abundance (19%) than in LV TMS ether. 4α-methyl-5α-cholesta-8(14),24-dien-3β-ol (LVII) TMS ether. The molecular ion is the base peak, demonstrating the greater stabilising influence of the $\Delta^{8(14)}$ bond as compared with the $\Delta^{8(9)}$ bond. This is further reflected in the relatively low abundance of ions of m/e 200. Allylic assistance of fission of the C-6/7 and C-9/10 bonds leads to ions of relatively high abundance (13%) at m/e 258. As expected, the

ion of m/e 121 is also formed in high abundance (46%) compared with m/e 107 (14%) and m/e 135 (21%).

Parkeol (LVIII) TMS ether gives a similar spectrum to that of LVI TMS ether. In this case, it is more likely that the 19-methyl group is eliminated in the formation of ions at $[M-15]^+$ (m/e 483, 13%) and $[M-15,90]^+$ (m/e 393, 48%). It is of interest to note that the $\Delta^{9(11)}$ bond exerts a similar, but weaker, influence to the Δ^5 bond on the fragmentation of ring A: ions are formed at m/e 129 (25%) and $[M-129]^+$ (m/e 369, 7%). Analogous ions have been observed for the corresponding 24-dihydrosterol TMS ether (III).[74,236] Elimination of the side-chain with two additional hydrogen atoms produces a relatively abundant (12%) ion at m/e 385. This appears to indicate that one of the hydrogen atoms implicated in this process arises from C-12 by allylic participation. Wyllie and Djerassi found[253] that, for several Δ^{24} steroid hydrocarbons, in the formation of similar ions, one of the two hydrogen atoms originates from C-17 and the other from C-12 (35%), C-14 (10%), C-16 (25%), or elsewhere in the molecule.

Agnosterol (LIX) TMS ether. Elimination of methyl groups from the molecular ion and $[M-90]^{\ddagger}$ ion does not take place as readily as in LVI TMS ether. The conjugated double bonds (Δ^7 and $\Delta^{9(11)}$) appear to stabilise rings B and C of the molecule. The usual fragmentations of the side-chain and ring D take place, in particular, elimination of C-15/16/17 with the side-chain and an additional hydrogen atom from the ion $[M-90]^{\ddagger}$ (m/e 253, 38%). An additional ion, also observed in the 20 eV spectrum of dihydroagnosterol,[135] appears at m/e 240 (19%).

The origin of this ion is uncertain, but it probably comprises rings A, B, and C with four methyl or methylene residues.[135] There are also ions at m/e 129 (26%) and $[M-129]^+$ (m/e 367, 4%).

Cycloartenol (LX) TMS ether. The molecular ion (m/e 498) and $[M-15]^+$ ion (m/e 483) are of very low intensity (1% and 3%, respectively). The $[M-90]^{+\cdot}$ ion (m/e 408) is of greater intensity (26%) and metastable ions attest to its decomposition to produce ions $[M-90,15]^+$ at m/e 393 (27%) and at m/e 365 (20%). The precise origin of this latter ion is uncertain. A corresponding ion (at m/e 379) is observed in the spectrum of cyclolaudenol.[238] The ion at $[M-90,69]^+$ (m/e 339) is of relatively high abundance (21%) compared with the equivalent ions from the TMS ethers of LIII-LIX (less than 4%).

$\Delta^{24(28)}$-sterol TMS ethers

The mass spectra of many $\Delta^{24(28)}$-sterols and their esters show abundant ions produced by McLafferty rearrangement in the side-chain.[223,233,238-241] These ions are, however, of relatively low abundance (less than 4%) in the spectra of the four $\Delta^{8,24(28)}$-sterol TMS ethers examined here.

Fecosterol (LXI) TMS ether (Fig. 46). The molecular ion (m/e 470) is the base peak, and there are abundant ions at $[M-15]^+$ (m/e 455, 54%), $[M-90]^{+\cdot}$ (m/e 380, 33%), and $[M-90,15]^+$ (m/e 365, 46%). Metastable ions attest to the transitions m/e 470 to 455 and m/e 380 to 365. As in the case of the Δ^{24} steroids, the side-chain is lost from the molecular ion with two hydrogen atoms (m/e 343, 31%) and from the

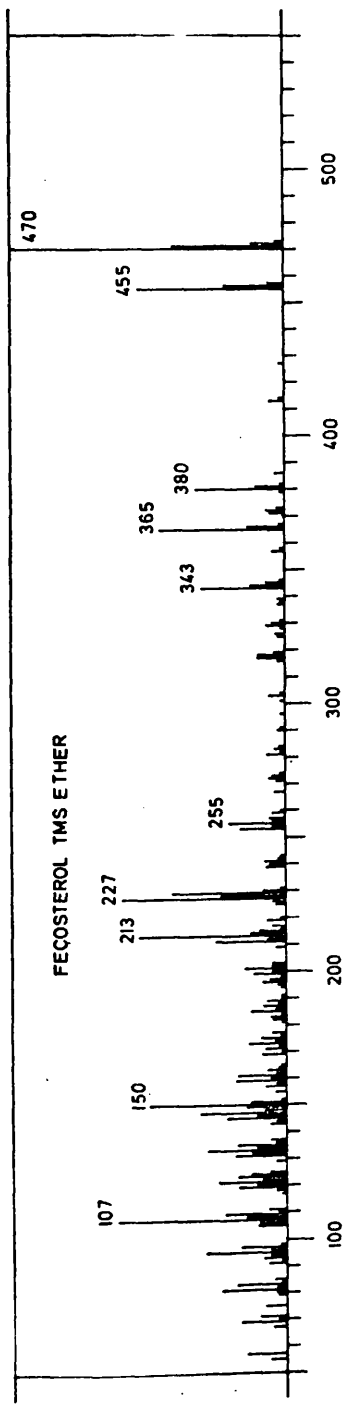

FECOSTEROL TMS ETHER
107
150
213
227
255
343
365
380
455
470
100
200
300
400
500

Fig.46

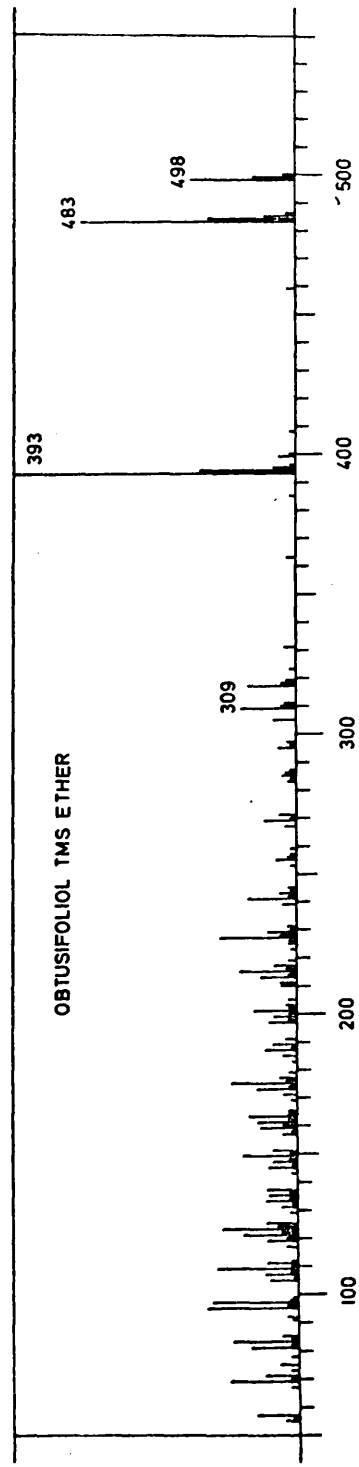

OBTUSIFOLIOL TMS ETHER
309
393
483
498
100
200
300
400
500

Fig.47

$[M-90]^{\ddagger}$ ion with (m/e 253, 17%) and without (m/e 255, 21%) hydrogen transfer, the latter predominating. Fragmentation of ring D, however, appears to be more complex. In addition to the fragmentations observed for the Δ^{24}-sterol TMS ethers, several more are observed:

(i) m/e 227 (60%). Corresponds to elimination of C-16/17 and side-chain with an additional hydrogen atom from $[M-90]^{\ddagger}$. Hydrogen transfer takes place in the opposite direction for Δ^{24}-sterol TMS ethers.

(ii) m/e 211 (26%). Can be ascribed formally to elimination of C-15/16/17 with side-chain and three hydrogen atoms from $[M-90]^{\ddagger}$, but probably involves an alternative fragmentation of ring D and loss of one or more methyl groups.

(iii) m/e 150 (50%). May comprise C-16/17 and side-chain, less two hydrogen atoms, or C-15 to C-26 and C-28 less one hydrogen atom.

The mode of formation of these ions can only be ascertained by the study of deuterium labelled or more highly substituted analogues. An ion of m/e 107 (62%) is observed, as in the spectrum of LIII.

4α-Methyl-24-methylene-24,25-dihydrozymosterol (LXII) TMS ether. The spectrum is similar to that of LXI, with the appropriate mass shifts for ions containing the 4-methyl group. There is more extensive fragmentation of the steroid nucleus, leading to more intense ions of m/e 200: the molecular ion (m/e 484, 92%) accounts for 3% of the total ion current, compared with 5.4% for LXI. The ion of m/e 150 is less intense (23%) than in the TMS ether of LXI, but quite significant as an odd-electron ion.

Obtusifoliol (LXIII) TMS ether (Fig. 47). This spectrum exhibits the same general features as observed for the other Δ^{8}-14-methyl sterol TMS ethers, namely, high abundance of $[M-15]^+$ (m/e 483, 76%) and $[M-90, 15]^+$ (m/e 393, 100%). Metastable ions attest to the sequence: M^+ (m/e 498, 37%) to m/e 483 to m/e 393. The ion $[M-90]^+$ (m/e 408) is formed in low abundance (2%). There are ions corresponding to formal loss of C-15 to C-28 and a methyl group (m/e 317, 17%) and loss of C_6H_{12}, by McLafferty rearrangement in the side-chain, from $[M-90, 15]^+$ (m/e 309, 19%). Ions of lower m/e are present only in relatively low abundance. The ion of m/e 150 (cf. LXI and LXII TMS ethers) is absent, and there is no corresponding ion at m/e 164.

24-Methylene-24,25-dihydrolanosterol (LXIV) TMS ether. The spectrum is also dominated by the molecular ion (m/e 512, 25%), $[M-15]^+$ (m/e 497, 33%), and $[M-90, 15]^+$ (m/e 407, 100%). There are no other ions of greater relative abundance than 21%.

Δ^{22}-sterol TMS ethers

5,6-Dihydroergosterol (LXV) TMS ether (Fig. 48). Fragmentation of the side-chain is more prevalent than in the examples already discussed. The ions at $[M-43]^+$ (m/e 427, 3%) and $[M-90, 43]^+$ (m/e 337, 8%) appear to be formed by loss of the terminal isopropyl group, promoted by allylic participation of the Δ^{22} bond, as proposed for the TMS ethers of stigmasterol (LXX)[241] and brassicasterol (LXXI).[223] The ion of m/e 372 (8%) is formed by the apparently unfavourable cleavage of the C-20/22 bond, with hydrogen transfer from the steroid nucleus.

Several plausible mechanisms for the equivalent fragmentation of

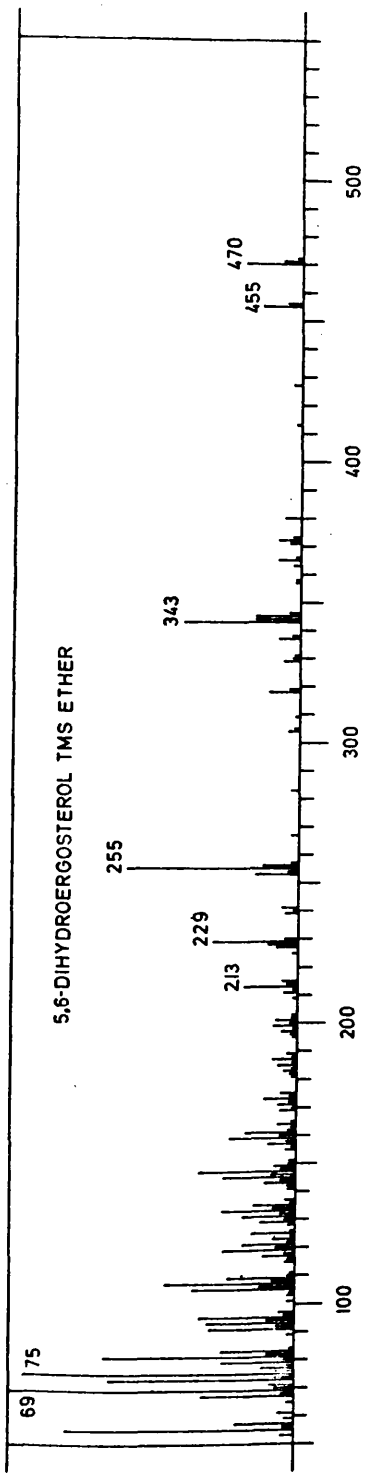

5,6-DIHYDROERGOSTEROL TMS ETHER
69
75
213
229
255
343
455
470
100
200
300
400
500

Fig. 48

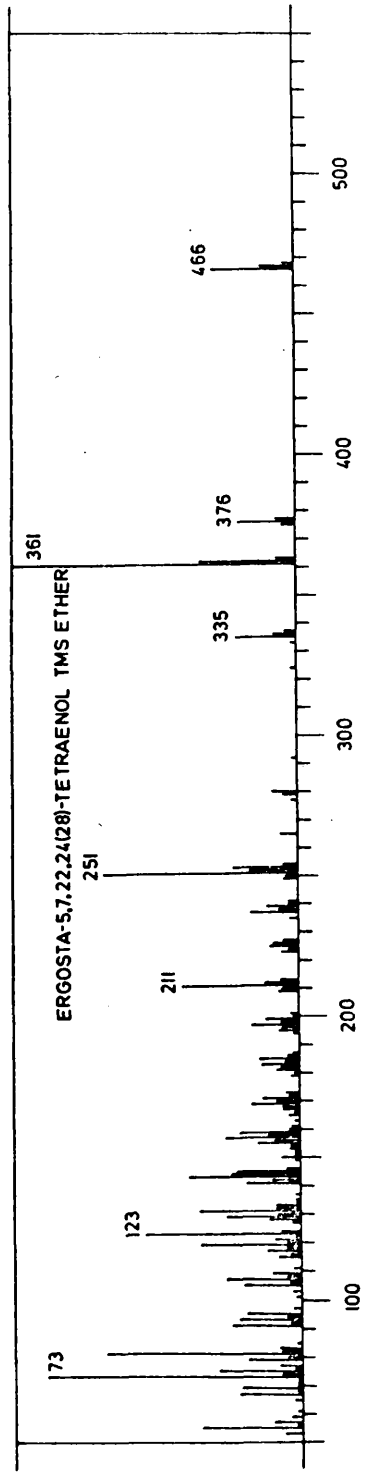

ERGOSTA-5,7,22,24(28)-TETRAENOL TMS ETHER
73
123
211
251
335
361
376
466
100
200
300
400
500

Fig. 49

5α-cholest-22-ene have been suggested.[233] Fission of the C-17/20 bond gives rise to ions of m/e 345 (16%) and m/e 255 (60%; base peak of the 20 eV spectrum[236]) from the molecular ion (m/e 470, 20%) and $[M-90]^{+\cdot}$ (m/e 380, 6%), respectively. Conversely, the corresponding ion (m/e 343, 41%) formed by loss of the side-chain and two hydrogen atoms from the molecular ion is more abundant than that (m/e 253, 15%) formed from $[M-90]^{+\cdot}$. The origin of several fragment ions may be formally ascribed to retro-Diels-Alder rearrangement in ring B of fragment ions of higher mass [m/e 161 (18%) from m/e 345, m/e 159 (24%) from m/e 343, and m/e 119 (25%) from m/e 303 (4%)]. Their relatively low abundance, however, renders them of little diagnostic significance. The base peak of this spectrum is at m/e 69. The origin of this ion may only be ascertained by deuterium labelling, but it should be noted that it is also the base peak in the spectrum of the TMS ether of LXVI and the acetate of LXVII obtained on the same instrument and under conditions similar to those employed in the present investigation.

Ergosterol (LXVI) TMS ether. The 20 eV spectrum has been discussed previously,[135] and the 70 eV spectrum is very similar, but with the base peak at m/e 69. The base peak of the 20 eV spectrum (m/e 363) is of relative abundance 76% in the 70 eV spectrum. Ions characteristic of the $\Delta^{5,7}$ structure[135] are observed at m/e 131 (36%) and $[M-131]^{+}$ (m/e 337, 55%). An ion of m/e 337 (23%) is also found in the 20 eV spectrum of LXVI TMS ether. The ion at $[M-129]^{+}$, normally observed in

the spectra of TMS ethers of Δ^5-3β-hydroxy steroids, is absent. It was previously suggested[135] that the ion at m/e 211 comprised rings C and D with the side-chain attached. It appears more likely that this ion arises by elimination of C-15 to C-28 with an additional hydrogen atom from $[M-90]^{+\cdot}$.

Ergosta-5,7,22,24(28)-tetraenol (LXVII) TMS ether (Fig. 49). The side-chain, since it contains a pair of conjugated double bonds, undergoes little fragmentation except, perhaps, elimination of the C-21 or a terminal methyl group. The $[M-15]^+$ ion is, in fact, absent, but a metastable ion attests to the formation of $[M-90,15]^+$ (m/e 361, 100%) from $[M-90]^{+\cdot}$ (m/e 376, 20%) which would be expected to be formed readily since it may take up a conjugated 3,5,7-triene structure. A relatively abundant ion at m/e 123 (54%) probably comprises C-20 to C-28, formed by fission of the C-17/20 bond, allylic to the Δ^{22} bond. There are no ions corresponding to loss of this side-chain from the molecular ion, but abundant ions exist at m/e 251 (68%) and m/e 253 (23%) by analogous processes commencing with the ion $[M-90]^{+\cdot}$. There are the expected ions at m/e 131 (35%) and $[M-131]^+$ (m/e 355, 21%). The ion of m/e 69 is of relatively low (21%) abundance.

Ergostenol TMS ethers

5α-Ergost-7-en-3β-ol (LXVIII) TMS ether (Fig. 50). The most notable feature of the mass spectra of many steroids with saturated side-chains is the loss of the side-chain without net hydrogen transfer.[106,107] Loss of the side-chain from the molecular ion of the TMS ether of LXVIII gives rise to an ion (m/e 345) of relatively low abundance (6%) but to an intense ion (m/e 255, 75%) from $[M-90]^{+\cdot}$ (m/e 382, 8%). Ions

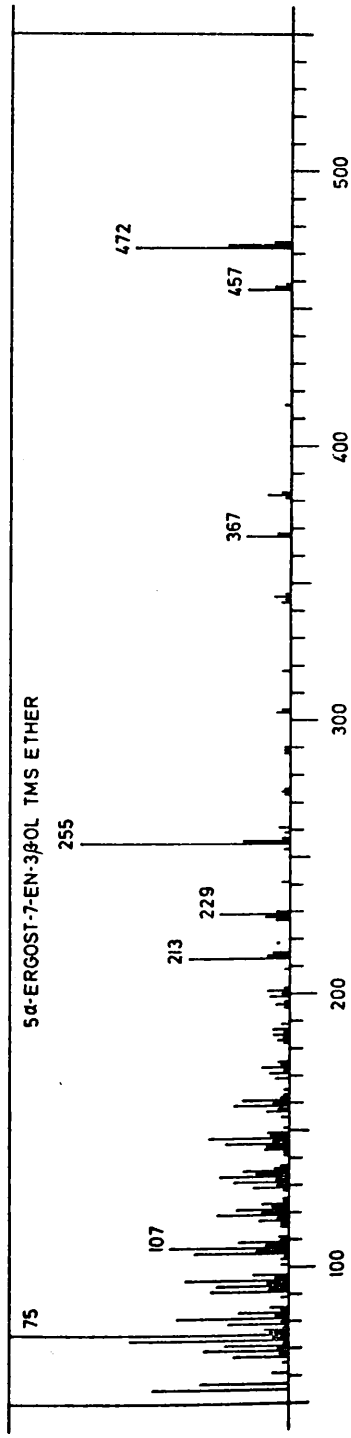

5α-ERGOST-7-EN-3β-OL TMS ETHER
75
107
213
229
255
367
457
472
100
200
300
400
500

Fig.50

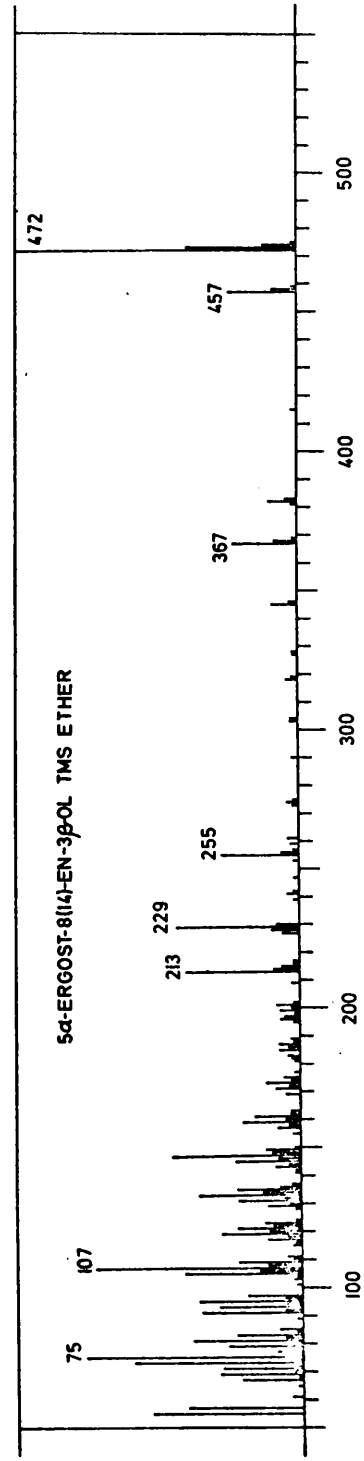

5α-ERGOST-8(14)-EN-3β-OL TMS ETHER
75
107
213
229
255
367
457
472
100
200
300
400
500

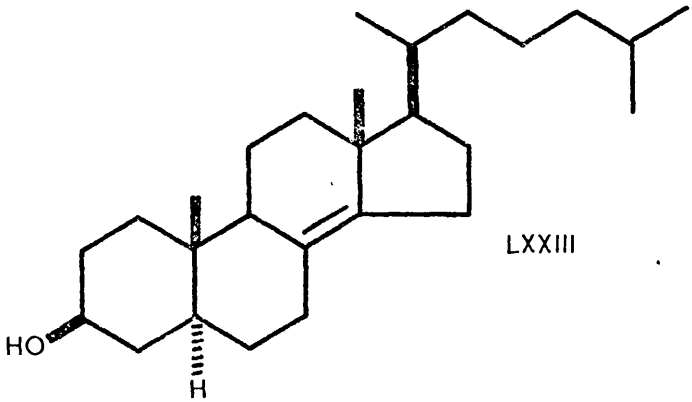
Fig.51

produced by fragmentation, with hydrogen transfer, of ring D of $[M-90]^{+\cdot}$ are formed in relatively high abundance [m/e 229 (25%) and m/e 213 (36%)]. The base peak (m/e 75) is an ion typical of TMS ethers.

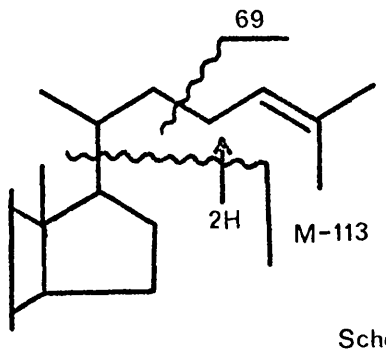
5α-Ergost-8(14)-en-3β-ol (LXIX) TMS ether (Fig. 51). The spectrum is qualitatively similar to that of the TMS ether of LXVIII, but has diagnostically signicant quantitative differences. Ions of m/e 472 ($M^{+\cdot}$, 100%), m/e 229 (43%), and m/e 107 (72%) are of notably increased relative intensity, whereas those of m/e 255 (27%) and m/e 75 (75%) are of decreased intensity in the spectrum of the TMS ether of LXIX. The reason for this relationship is uncertain, but it is appropriate to note, here, that a similar phenomenon is observed in the 20 eV spectra of TMS ethers of 5α-cholest-7-en-3β-ol (LXXIII) and 5α-cholest-8(14)-en-3βol (LXXIV).[135] In the former, m/e 255 is in nigh abundance, and in the latter m/e 107 is more prominent.

Conclusions. In spite of the inherent complexity of the fragmentation of ring D[234] and unsaturated side-chains[233] of steroids, it has been possible to recognise various features of diagnostic significance in the mass spectra (Schemes 8-12).

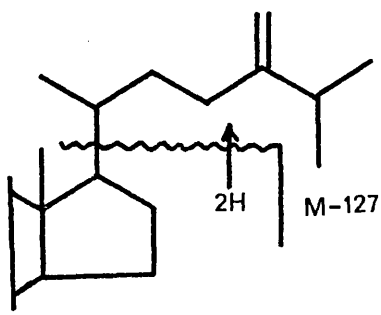
The TMS ether function, while giving rise to ions at $[M-90]^{+\cdot}$, m/e 73, and m/e 75, promotes little fragmentation of the steroid nucleus in most cases. Notable exceptions are the formation of ions at m/e 131 and $[M-131]^{+}$ in the spectra of $\Delta^{5,7}$ compounds and ions at m/e 129 and $[M-129]^{+}$ in $\Delta^{9(11)}$ and $\Delta^{7,9(11)}$ compounds. This may serve

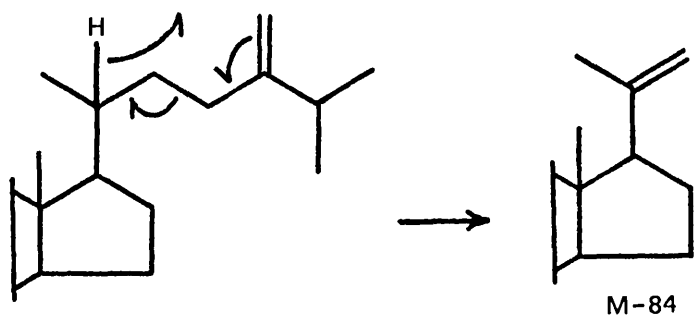


LXXIII

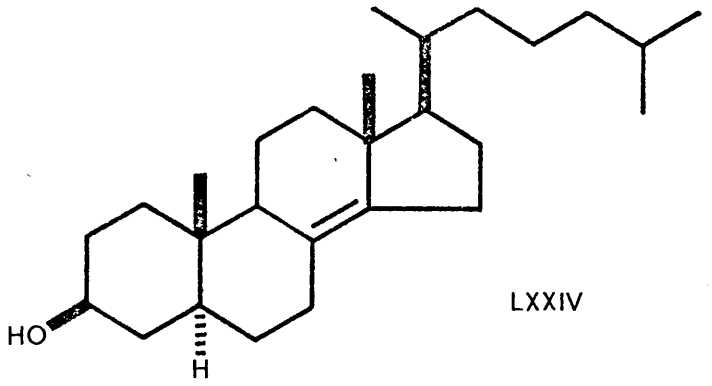


Scheme 8

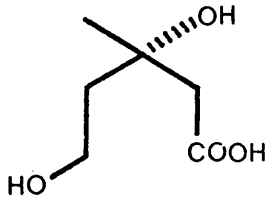




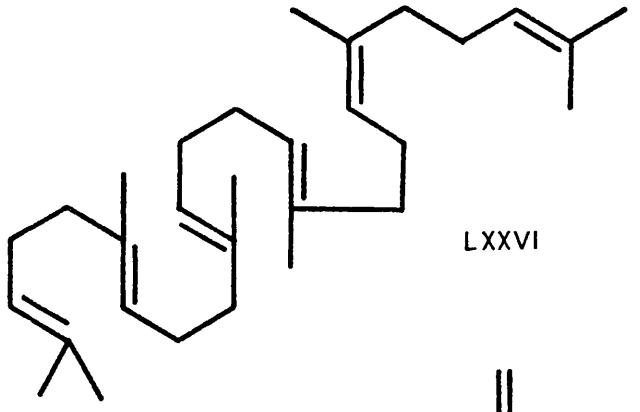
Scheme 9



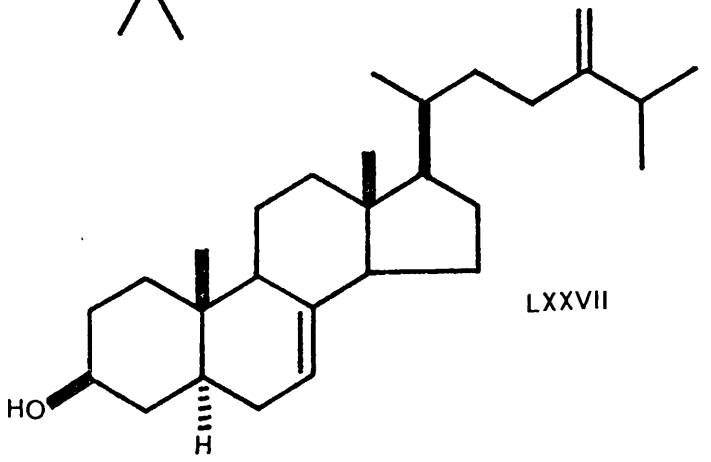
LXXIV



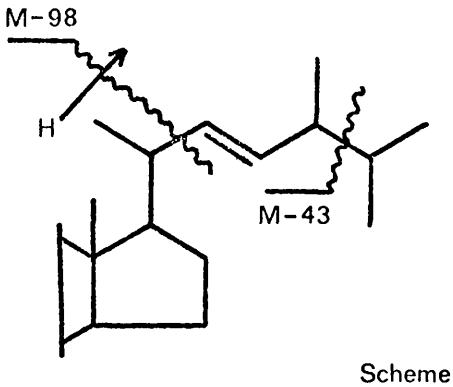
LXXV



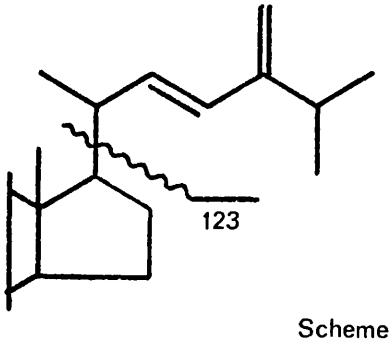
LXXVI



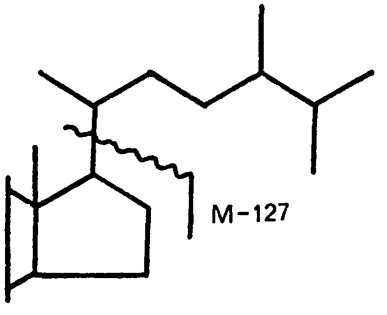
LXXVII



Scheme 10



Scheme 11



Scheme 12

to distinguish these compounds from those with double bonds in other positions in the steroid nucleus, although it should be remembered that the corresponding Δ^5 compounds usually give rise to ions at m/e 129 and $[M-129]^+$, but in greater relative abundance.

The ease of recognition of the size of saturated side-chains from steroid mass spectra has been known for some time,[104] but is not quite as simple as in the "rule" enumerated by Fitches: "For steroids containing less than three ring-keto groups the molecular weight of the side-chain at C_{17} is given as the parent mass, p, minus 42, minus the mass of the principal peak in the mass range 205-245, minus 18 for each hydroxyl group and minus 60 for each acetate group."[156] Nevertheless, in the spectra of TMS ethers of LXVIII and LXVIX, ions resulting from loss of the side-chain from, and fragmentation of ring D of, the molecular ion and $[M-90]^+$ are easily recognised. Features which serve to distinguish Δ^7 and $\Delta^{8(14)}$ isomers have been discussed. In particular, the relative abundances of ions of m/e (105, 4-substituents) and ions resulting from sequential loss of trimethylsilanol and side-chain from the molecular ion should be noted. Also, Δ^1 isomers can usually be distinguished by their higher retention indices.[135]

The majority of the Δ^{24} compounds have base peaks at m/e 69 in the 70 eV spectra (the exception being the TMS ether of LVII), presumably formed from the side-chain by fission allylic to the Δ^{24} bond (Scheme 8). The same base peak is, however, observed in the spectra of Δ^{22} compounds,

probably by McLafferty rearrangement in the cleaved side-chain. Interpretation of the significance of a base peak at m/e 69 in the spectra of "unknown" steroisd should therefore be treated with caution. Δ^{22} and Δ^{24} isomers can also be distinguished by further characteristic fragmentation of the side-chain (Schemes 8,10).

In contrast with the spectra of steroids with saturated side-chains (Scheme 12), unsaturated side-chains are eliminated from $M^{\ddagger}$ and $[M-90]^{\ddagger}$ both with and without two additional hydrogen atoms (Scheme 12). It should be noted that this hydrogen transfer is more prevalent in the fragmentation of $M^{\ddagger}$ than of $[M-90]^{\ddagger}$.

The presence of a $\Delta^{24(28)}$ bond can be recognised by the presence of an ion which formally corresponds to elimination from $[M-90]^{\ddagger}$ of C-16 to C-28 and an additional hydrogen atom. The more usually observed ion, formed by hydrogen transfer to the steroid nucleus, is also present.

An extremely abundant $[M-90,15]^{\ddagger}$ ion is observed in the spectra of compounds possessing a Δ^{8}-14-methyl moiety. Metastable ions attest to the formation of $[M-90,15]^{+}$ from $[M-15]^{+}$. There are also intense ions at $[M-90,15]^{+}$ in the spectra of the $\Delta^{5,7}$ compounds, the methyl group eliminated being, apparently, that at C-19. In this case, metastable ions indicate that $[M-90,15]^{+}$ is formed from $[M-90]^{\ddagger}$. The $[M-90,15]^{+}$ ion is of low intensity in the spectrum of the $\Delta^{7,9(11)}$-14-methyl compound.

There appears to be little direct fragmentation of ring A in most of these compounds, but it seems possible to ascertain substituents at C-4 by the intensities of ions at m/e 107 (from 4,4'-di-H), m/e 121 (from 4-methyl), and m/e 135 (from 4,4'-di-Me).. Such observations should, however, be treated with some caution since, at the present time, it is uncertain which other carbon atoms are included in these ions: methyl groups at other positions could give rise to analogous mass shifts.

Cycloartenol TMS ether gives rise to an additional fragmentation, presumably directed by the 9,10-cyclopropane ring, with elimination of a C_3H_7 fragment from $[M-90]^+$: this interpretation is supported by metastable ion evidence.

CHARACTERIZATION OF YEAST STEROLS BY GC-MS*

In recent years, much progress has been made in the elucidation of the pathways by which sterols are biosynthesised, not only in animals, but also in fungi and higher plants.[243,244] Ergosterol (LXVI) has long been known as the major sterol of yeast and other fungi - it forms crystalline inclusions in Neurospora crassa[245] - and is formed from lanosterol (LVI), which apparently originates from mevalonic acid (LXXV) via squalene (LXXVI). Details of the conversion of lanosterol to ergosterol are, at present, not clear. Various hypothetical biosynthetic pathways have been proposed and attempts have been made to identify the intermediates. In investigations of this type, it is usually necessary to isolate such sterols and to apply a wide range of physical and chemical techniques to determine their structures.[246]

In the present study, an attempt has been made to characterize sterols in relatively crude fractions of sterols of the yeast Saccharomyces cerevisiae Meyen. Column adsorption chromatography (at Imperial College) yielded three fractions, broadly representing: 4-desmethyl, 4α-methyl, and 4,4-dimethyl sterols.

GC-MS of the derived TMS ethers was carried out using a 10ft 1% OV-17 column. Because of the incomplete resolution obtained, retention indices were determinable only approximately. Spectra were recorded at an electron energy of 70 eV.

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* Preliminary fractionation of the yeast sterols was carried out by Prof. D. H. R. Barton, Dr. D. A. Widdowson, and co-workers (Imperial College of Science and Technology, University of London).

The 4-desmethyl sterol fraction was found to contain three major components, giving TMS ethers of retention index 3340, 3385, and 3420. The mass spectrum of the first (Fig. 52) corresponds to that of zymosterol (LIII) TMS ether (Fig. 44). Zymosterol is a well-known yeast sterol.[247] The retention index of the TMS ether of the second major component is closely similar to those of 5,6-dihydroergosterol (LXV) TMS ether (I = 3395) and fecosterol (LXI) TMS ether (I = 3390). The mass spectrum (Fig. 53) exhibits features characteristic of both of these compounds: there is an abundant (20%) ion of m/e 150 (cf. Fig. 46), and ions of m/e less than 100 are relatively intense (cf. Fig. 48). This component is probably a mixture of these sterols. The third component of this fraction appears also to be an ergostadienol. The retention index of its TMS ether (Fig. 54) is similar to that which would be expected (cf. Table 6) for the TMS ether of episterol (LXXVII, $I^{TMS}_{calc.}$ = 3430). No reference sample was available for comparison of mass spectra. Ergostadienols previously isolated from fungi include 5,6-dihydroergosterol,[248] fecosterol,[249] episterol,[250,251] and ascosterol (LXXVIII).[249,252] "Anasterol", previously claimed to be an ergostadienol from yeast,[253] is apparently a mixture.[254]

The 4α-methyl sterol fraction contained two major components. The TMS ether of the first (I = 3420) gave a spectrum (Fig. 55) which resembled that of the TMS ether of 4α-methyl-5α-cholesta-8(14),24-dien-3β-ol (LVII, I^{TMS} = 3400) more closely than that of the TMS ether of the Δ^8-isomer, 4α-methylzymosterol (LIV, I^{TMS} = 3400). In particular,

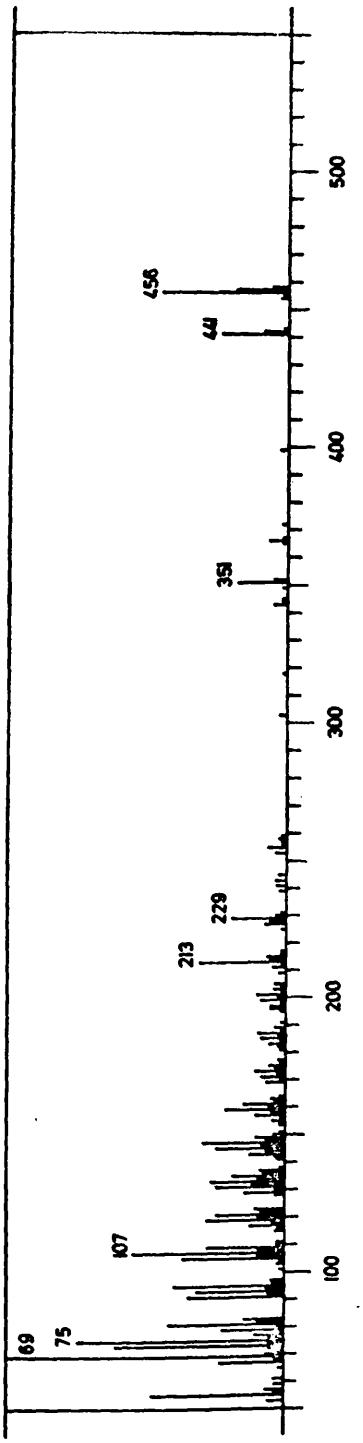
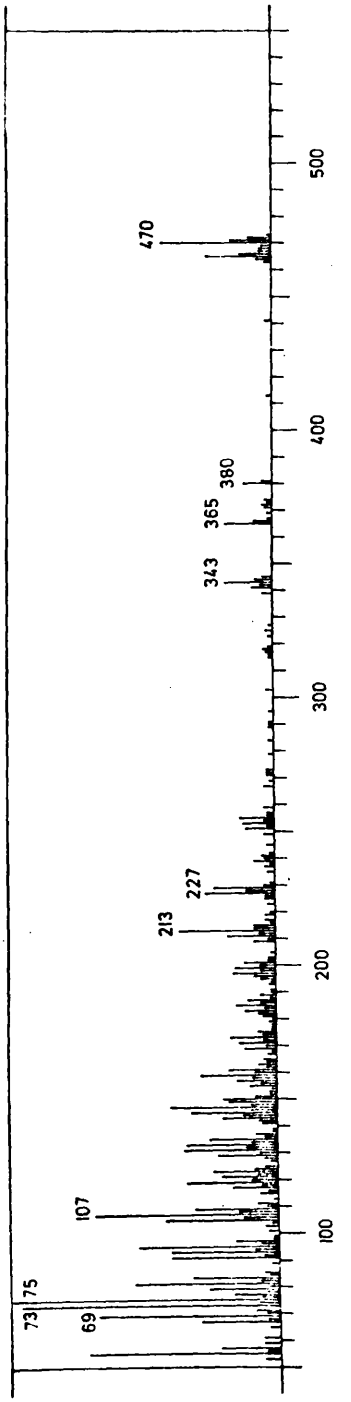

69
75
107
213
229
351
441
456
100
200
300
400
500

Fig 52


69
73
75
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213
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343
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400
500

Fig.53

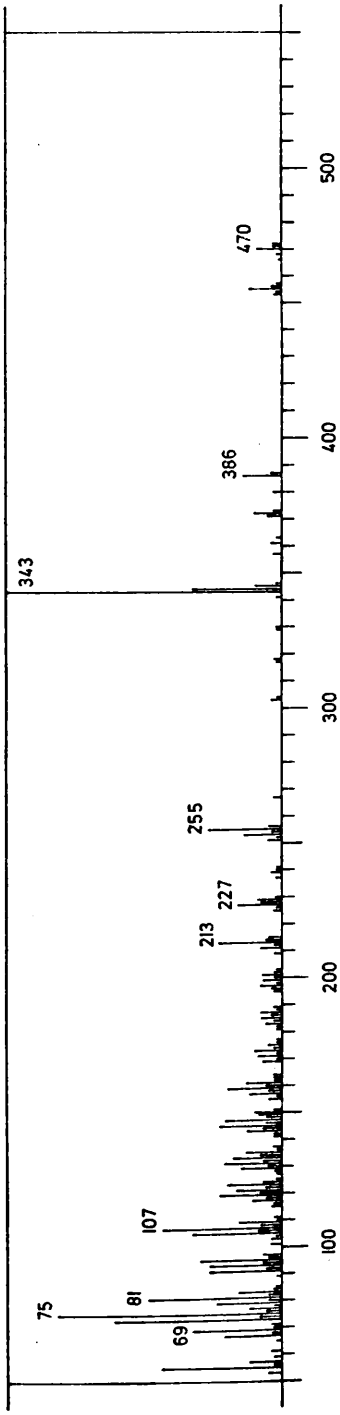

75
81
69
107
213
227
255
343
386
470
100
200
300
400
500

Fig. 54

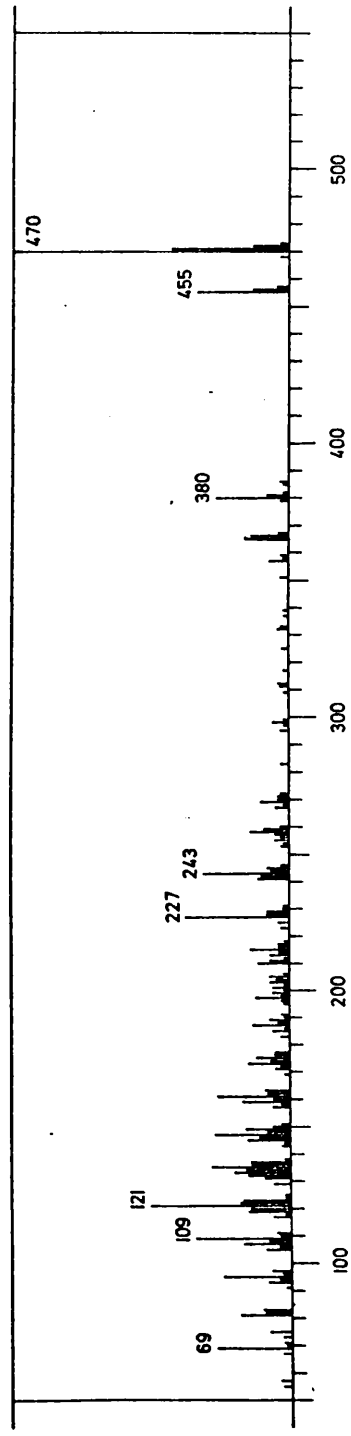
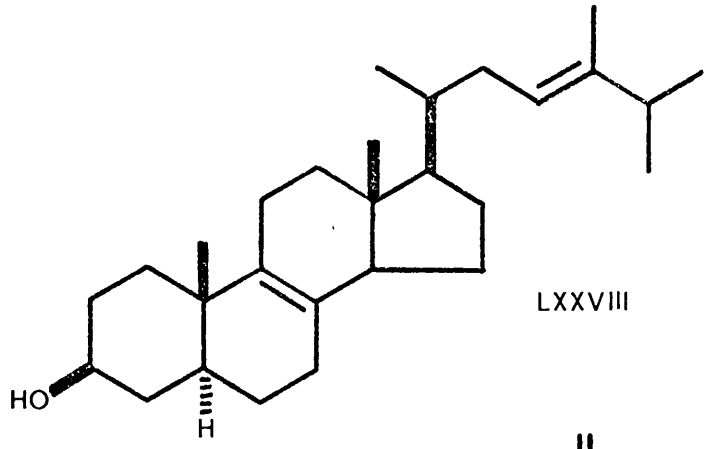
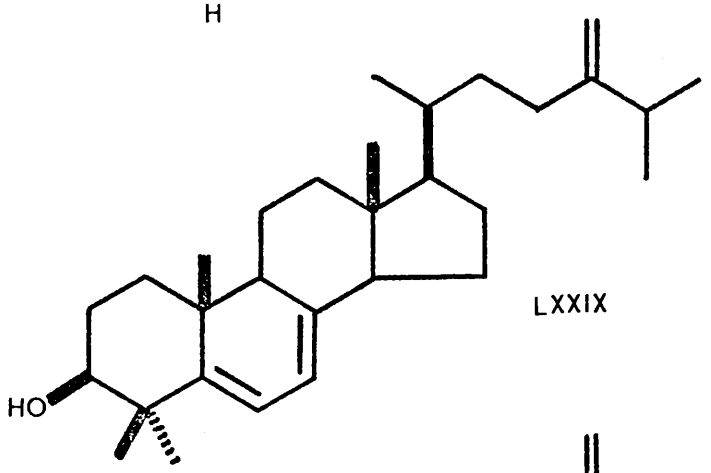

69
121
109
227
243
380
455
470
100
200
300
400
500

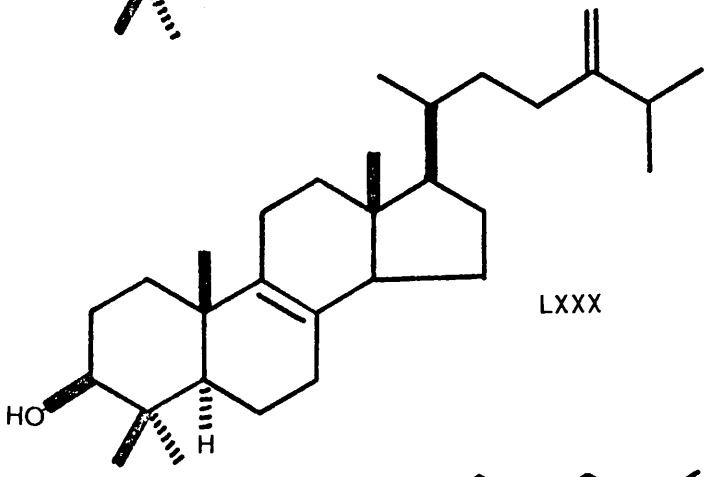
Fig.55



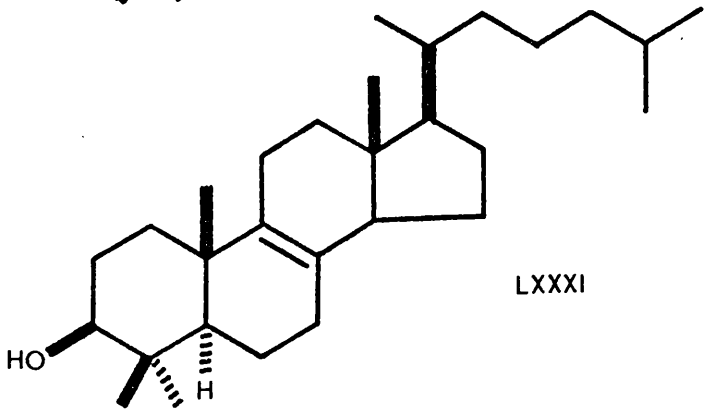
LXXVIII



LXXIX



LXXX



LXXXI

it should be noted that the ions of m/e less than 100 are less abundant in the spectrum of the $\Delta^{8(14)}$-isomer. But for the relatively high abundance of ions of m/e less than 100 in the spectrum of its TMS ether (I = 3460, Fig. 56) the second component of this fraction would resemble 4α-methyl-24-methylene-24,25-dihydrolanosterol (LXII, I^{TMS} = 3450) isolated by Barton et al.[246] This could be the the $\Delta^{8(14)}$-isomer.

The 4,4-dimethylsterol fraction also contained two major components. The spectrum of the TMS ether of the first (I = 3430, Fig. 57) has an extremely abundant ion at m/e 393 (20%Σ) characteristic of the Δ^{8}-14-methyl structure. Ions of m/e less than 100 are present in low abundance, compared with those in the spectrum of the TMS ether of lanosterol (LVI, I^{TMS} = 3420, Fig. 45). This difference may not be significant in view of the evidence for the presence of a Δ^{8}-14-methyl grouping. Closely related isomers with other nuclear double bonds have quite different retention indices ($\Delta^{9(11),24}$, I = 3480; $\Delta^{7,24}$, $I_{calc.}$ = 3470). Δ^{22}-isomers would have much lower retention indices: $\delta I(\Delta^{24} \rightarrow \Delta^{22})$ = -125. The low abundance of the ion of m/e 69 (base peak of the spectrum of the TMS ether of lanosterol) might be accounted for by a structure with a different side-chain. The TMS ether of the other major component of this fraction (I = 3465, Fig. 58) is not completely resolved from the first, but interfering ions appear only at m/e 393, 483, and 498 (cf. Fig. 57). Its mass spectrum is somewhat similar to that of the TMS ether of 14-desmethyllanosterol (LV, I^{TMS} = 3450). However, ions

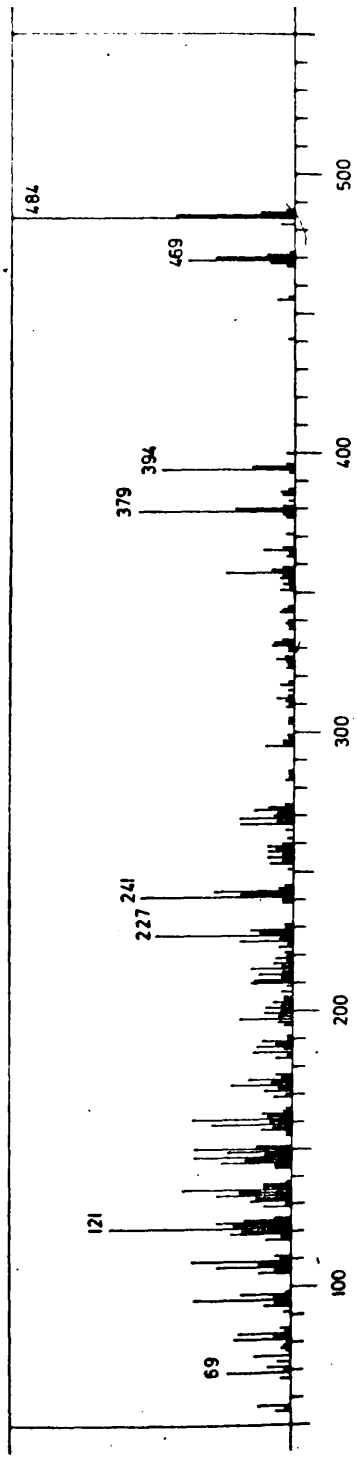

484
469
394
379
241
227
121
69
500
400
300
200
100

Fig.56

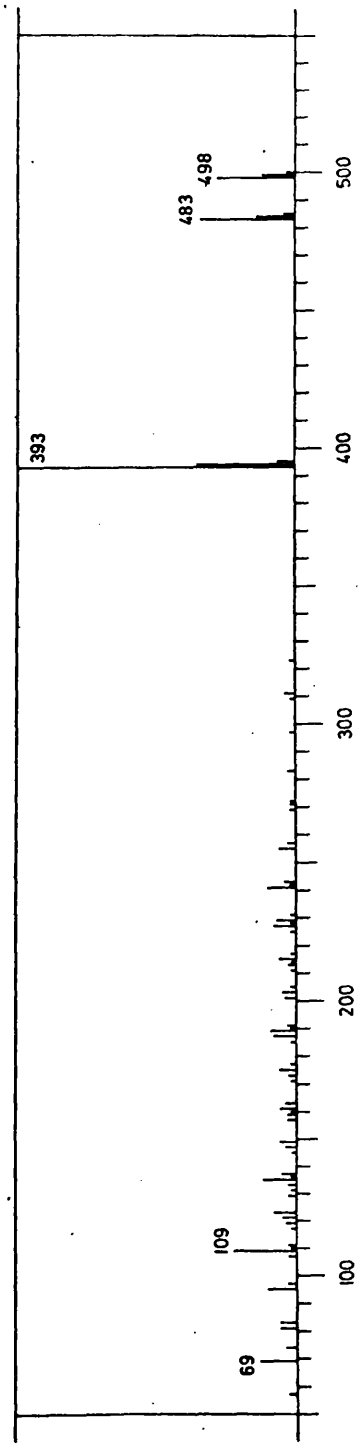

498
483
393
109
69
500
400
300
200
100

Fig.57

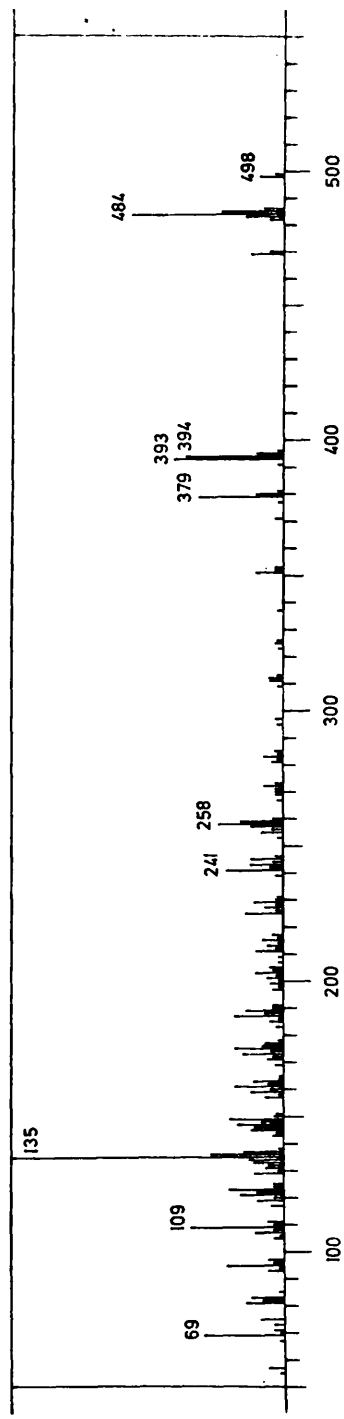
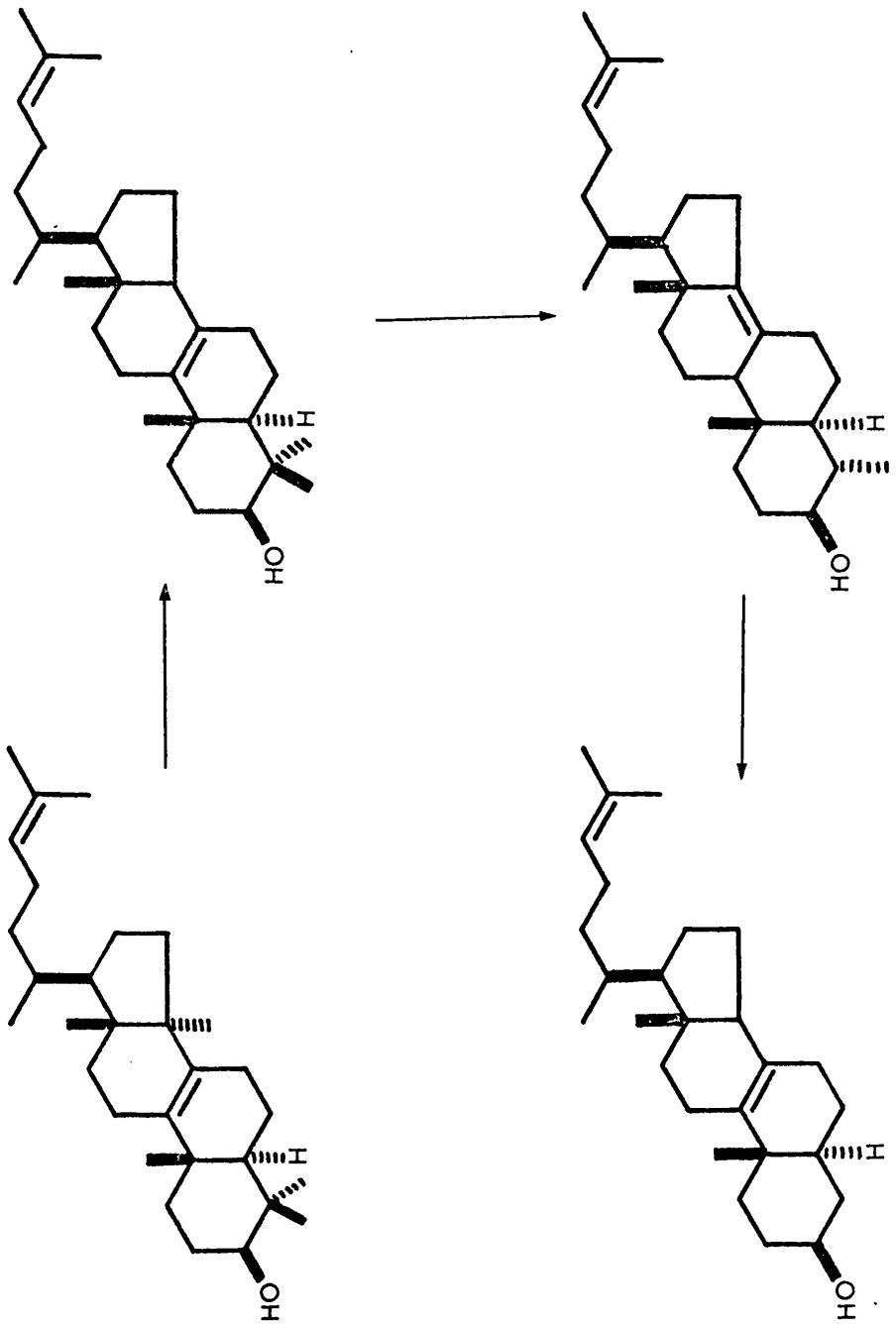


Fig.58

of m/e less than 100 are present only in low abundance in the spectrum of the TMS ether of this component. A minor component of this fraction, which affords a TMS ether (I = 3520) of molecular weight 496, could be 4,4-dimethylergosta-5,7,24(28)-trien-3β-ol ($I^{TMS}_{calc.}$ = 3530).

In this brief study, a number of yeast sterols have been characterized by GC-MS. It has been found possible to determine the masses of the nuclear and side-chain moieties in each case, but not always the locations of nuclear double bonds. In particular, differentiation between Δ^{8} and $\Delta^{8(14)}$ double bonds is difficult if suitable reference compounds are not available. It should be noted, however, that, in cases where definite identification has been made, as for zymosterol and 4α-methyl-5α-cholesta-8(14),24-dien-3β-ol, there is excellent agreement between spectra of TMS ethers of authentic samples and those of extract components. It is clearly desirable to obtain mass spectra of TMS ethers of many more unsaturated sterols of this type to aid further studies.

All of the sterols identified, albeit tentatively, in the yeast extracts can be placed on hypothetical biosynthetic pathways from lanosterol to ergosterol. Sequential elimination of methyl groups apparently leads to the formation of zymosterol (Scheme 13). Schwenk and Alexander were unable to demonstrate the conversion of zymosterol to ergosterol by yeast[255] but, more recently, Katsuchi and Bloch have shown that this process probably proceeds via ergosta-5,7,22,24(28)-

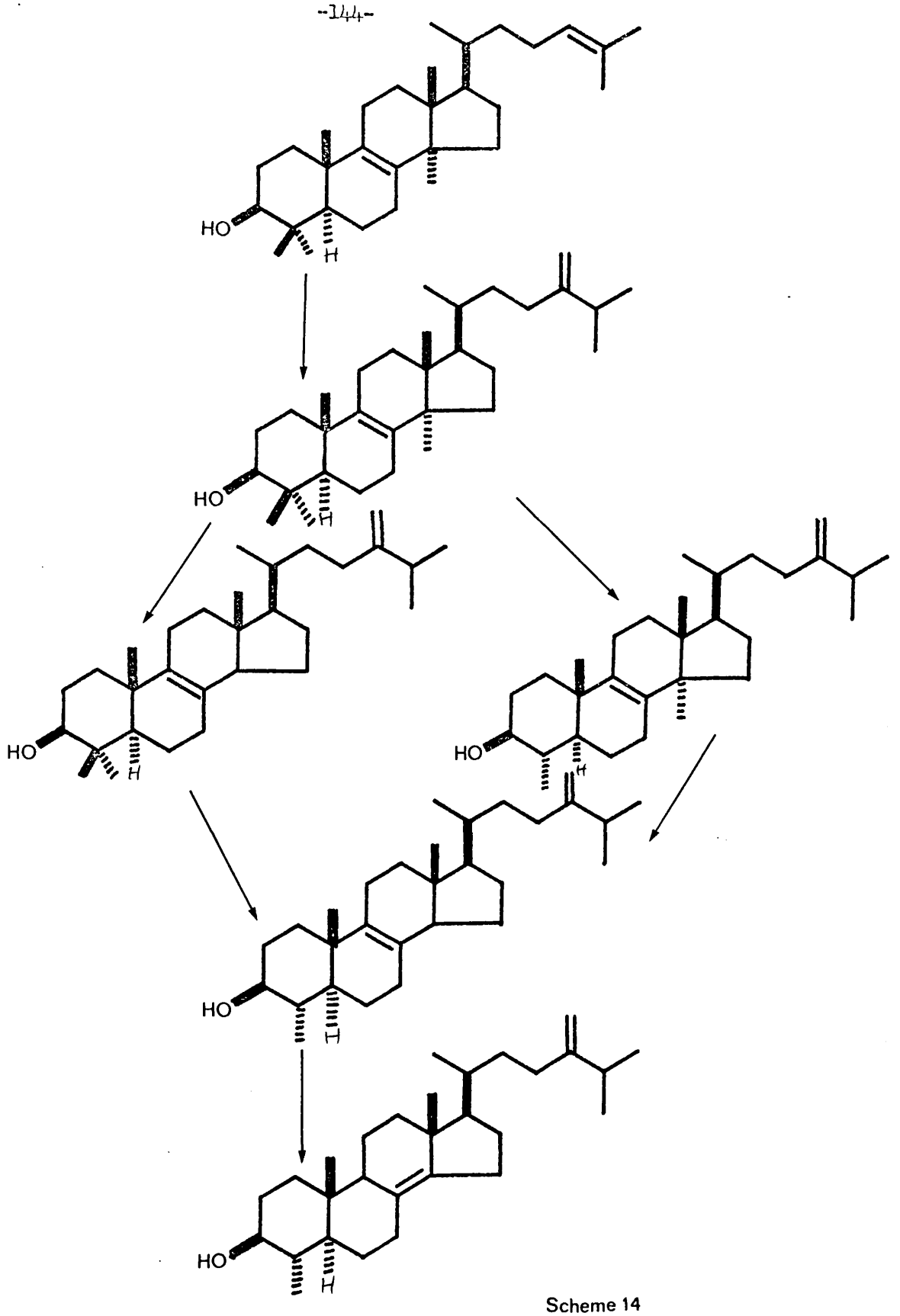


Scheme 13

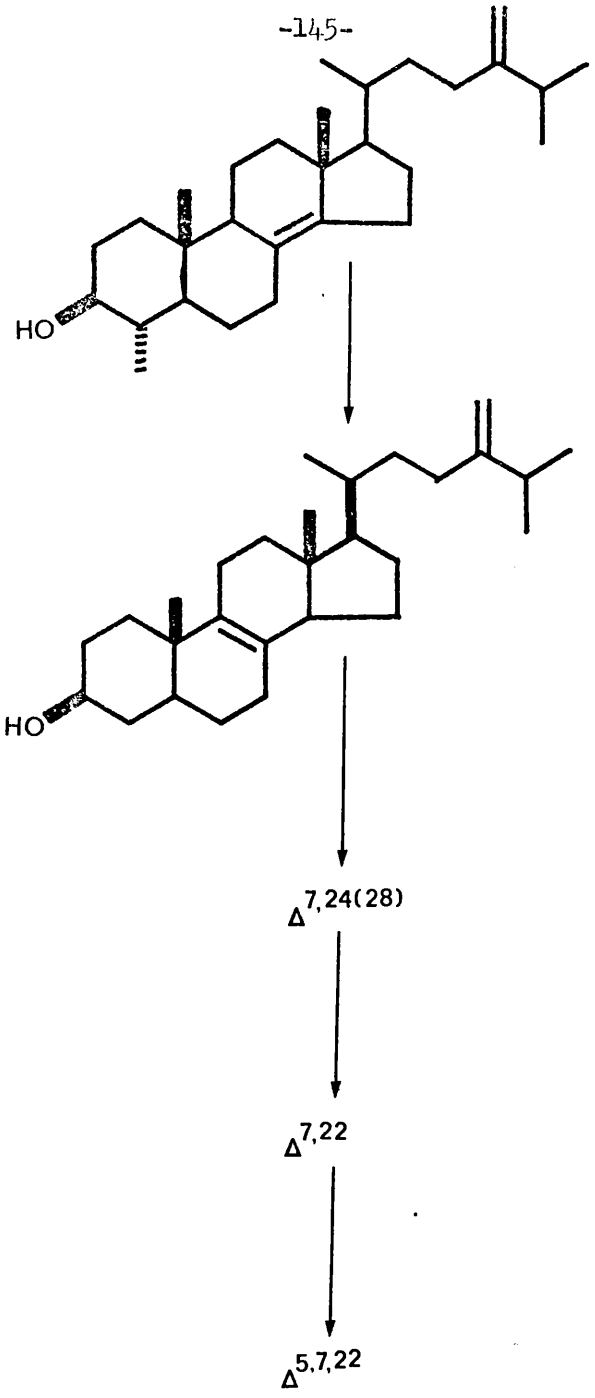
tetraen-3β-ol (LXVII).[256] Barton et al. have shown that yeast can convert 4α-methylzymosterol to ergosterol in 15% yield.[246]

Alternatively, it has been suggested that elaboration of the 24-methylene moiety can precede demethylation.[257] 24-methylene-24,25-dihydrolanosterol (LXIV) has been found in a fungus,[251] and it has been demonstrated that this compound is converted to ergosterol by yeast.[257,258] Scheme 14 illustrates two possible routes for the formation of 4α-methyl-5α-cholesta-8(14),24-dien-3β-ol from lanosterol. Both proceed via 24-methylene-24,25-dihydrolanosterol. Elimination of the 14-methyl group may lead to the formation of 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (LXXX) (tentatively identified in Phycomyces blakesleeanus by Goulston et al.[251]), whereas loss of a 4-methyl group may afford obtusifoliol (LXIII) (shown by Barton et al. to be convertible to ergosterol by yeast in 8% yield[246]). Further loss of a methyl group from either of these sterols may produce 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol (isolated from yeast by Barton et al.[246]) which presumably isomerises to the $\Delta^{8(14),24(28)}$ sterol now identified.[259] Scheme 15 shows a probable route for conversion of this sterol to ergosterol via the ergostadienols described above. The tentative identification of 4,4-dimethylergosta-5,7,24(28)-trien-3β-ol indicates a further route for conversion of lanosterol to ergosterol, possibly via ergosta-5,7,22,24(28)-en-3β-ol (Scheme 16).

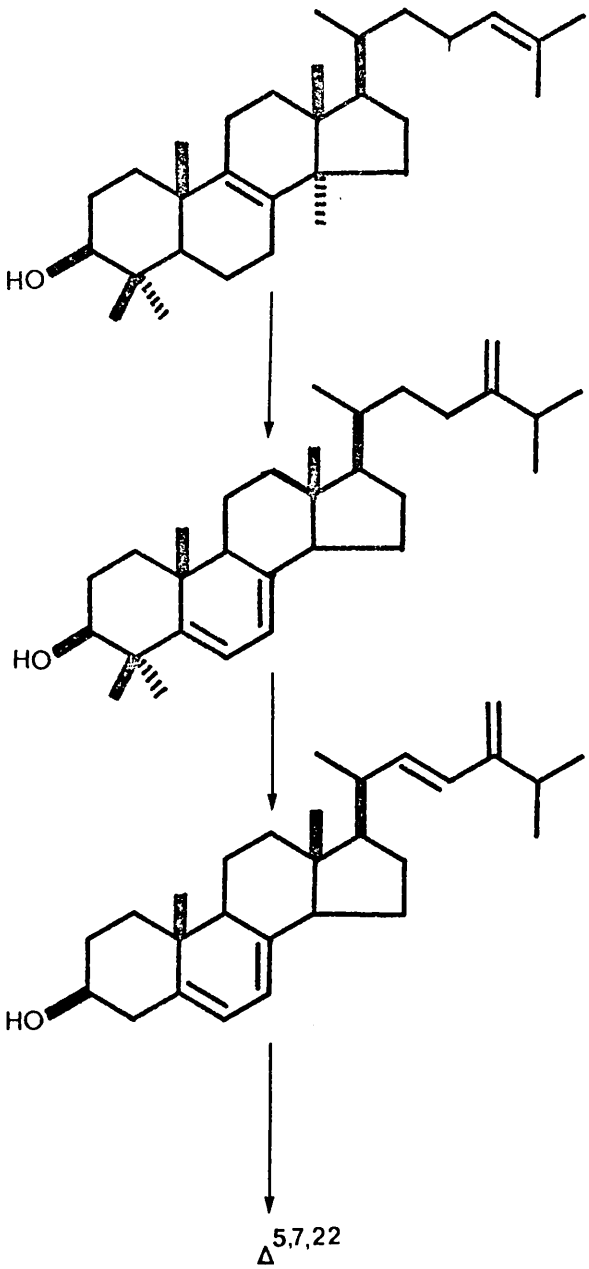
Goad has suggested[243] that different biosynthetic pathways between lanosterol and ergosterol may be operative in different fungi.



Scheme 14



Scheme 15



Scheme 16

It is possible that the full potential of GC-MS in this field will be realised in comparative studies of sterol biosynthesis. Meanwhile, GC-MS has a significant role to play in the characterization of minor sterols which often cannot be isolated in quantity

Table 6. Retention increments (OV-17)
(with repect to 5α-cholestan-3β-ol, I_{240}^{o} = 3230)

Δ^{5}	0
Δ^{7}	+65
Δ^{8}	+15
$\Delta^{8(14)}$	+15
$\Delta^{9(11)}$	+45
Δ^{22}	-50
Δ^{24}	+75
$\Delta^{24(28)}$	+15
4α-Me	+60
4β-Me	+35
14-Me	-10
24-Me	+100

CHARACTERIZATION OF STEROLS AND SQUALENE IN A BACTERIUM (METHYLOCOCCUS CAPSULATUS) GROWN ON METHANE*

The ubiquitous occurrence of steroids in nature, and their fundamental importance for plant and animal life, are well known. Until 1967, however, steroids had not been detected in prokaryotic organisms, i.e. the bacteria and blue-green algae, but only in eukaryotic (higher) organisms. Reports of the detection of sterols and squalene in prokaryotic organisms are summarized in Table 7. In general, it is found that amounts of steroids found in prokaryots are lower than those found in eukaryots. Dr. C.W. Bird, Prof. S.J. Pirt, and co-workers have observed that the bacterium Methylococcus capsulatus, grown in methane, is remarkable in containing comparatively large amounts of squalene and sterols.[260]

M. capsulatus cells were extracted with a chloroform-methanol mixture and the extract was fractionated by TLC to provide four fractions: A, B, C and D comprising, respectively, hydrocarbons, 4,4-dimethyl sterols, 4α-methyl sterols, and 4-desmethyl sterols.

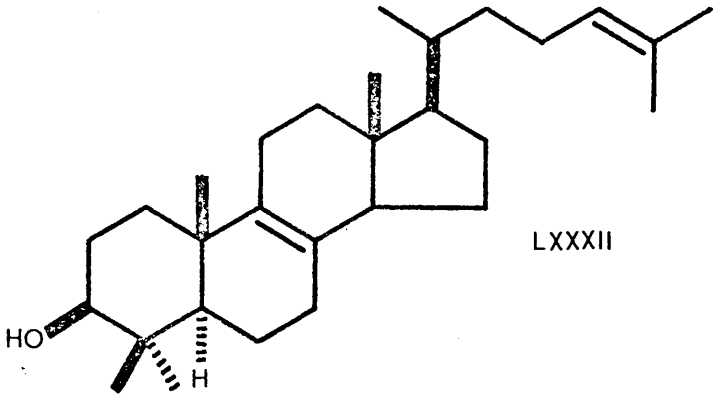
GLC of fraction A (at Queen Elizabeth College) revealed the presence of squalene (LXXVI) and a series of alkanes from $C_{20}H_{42}$ to $C_{30}H_{62}$, in approximately equal quantities. An additional component (I_{230}^{OV-1} = 3150) was characterized by GC-MS as a further triterpene

* Growth of the bacteria and extraction of the sterols and squalene were carried out at Queen Elizabeth College, University of London, by Prof. S.J. Pirt, Dr. W.W. Reid, Dr. C.W. Bird, and Mr. J.M. Lynch.

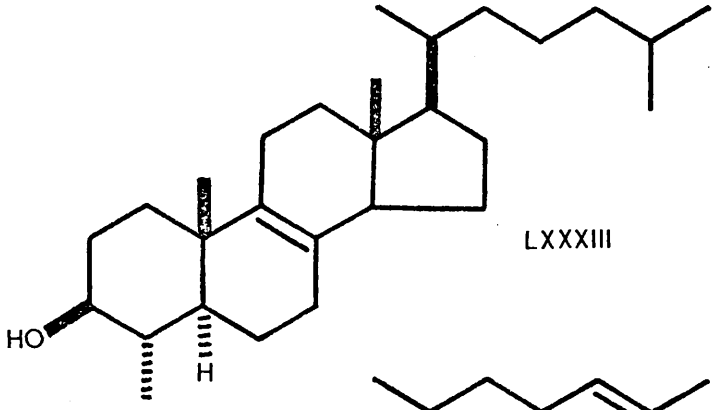
(M^+, m/e 410; base peak, m/e 69; intense ions at m/e 189 and 191). This hydrocarbon was later identified as diploptene (LXXXV).[264] Diploptene has since been isolated from another (unnamed) bacterium.[269]

Fraction B was resolved into two components by GLC: $I_{250}^{OV\text{-}17}$ ca. 3510 and 3570. The first showed a mass spectrum in which the molecular ion (m/e 414) was the base peak. An ion at m/e 301 was consistent with the loss of a C_8H_{17} side-chain. The second chromatographic peak yielded a mass spectrum in which the base peak was at m/e 412. The predominance of the ion at m/e 69 suggested the presence of a Δ^{24} double bond. The gas chromatographic retention indices, the retention index difference between the two peaks, and the mass spectrometric data were all consistent with structures 4,4-dimethyl-5α-cholest-8-en-3β-ol (LXXXII) and 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol (LXXXIII), respectively. There was insufficient sample for further study.

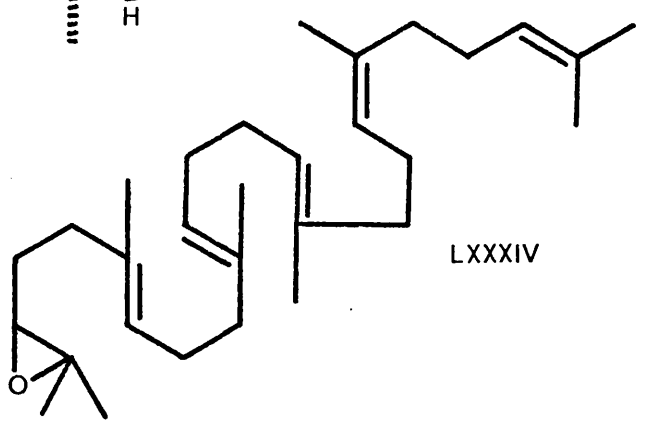
Fraction C contained substantially more material. GC-MS indicated two main incompletely-separated peaks, $I_{250}^{OV\text{-}17}$ ca. 3410 and 3480. The mass spectra paralleled those recorded for fraction B: the first component had a molecular ion at m/e 400 as the base peak, and the second showed m/e 69 as the base peak, with the molecular ion (m/e 398) of relative abundance 77%. The corresponding TMS ethers were better separated ($I_{250}^{OV\text{-}17}$ 3315 and 3385). The first, preponderant component gave a mass spectrum in which the molecular ion (m/e 472) was the base peak, both at 70 eV and 20 eV electron energy. The second



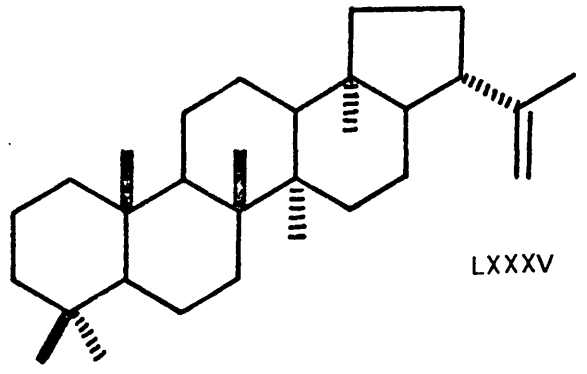
LXXXII



LXXXIII



LXXXIV



LXXXV

component yielded a molecular ion at m/e 470, amounting to 78% of the abundance of the base peak at m/e 69. The retention data and the full mass spectrum were in close agreement with those recorded for authentic 4α-methylzymosterol (LIV) TMS ether. The combined data for fraction C are thus compatible with the assignment of structures 4α-methyl-5α-cholest-8-en-3β-ol (LXXXIII) and 4α-methyl-5α-cholesta-8,24-dien-3β-ol (LIV) to the two main components. Similar mass spectra would probably be obtained from the Δ^7 and $\Delta^{8(14)}$ isomers, but the Δ^7 isomers would have distinctly longer retention times and are not clearly present as major components.

Fraction D has not yet been examined by GC-MS because of lack of material. The TLC properties of the major component of this fraction suggest that it is zymosterol (LIII).

No squalene-2,3-oxide (LXXIV) was detected in fraction A, and neither lanosterol (LVI) nor cycloartenol (LX) was detected in fraction B.

Table 7. Squalene and sterols found in prokaryotic organisms.

Bacteria:	squalene	sterols	ref.
M. capsulatus	0.55%	0.22%	260
Halobacterium cutirubrum	0.1%		261
Staphylococcus sp.	0.002%	none	262
Rhodomicrobium vannielli	detected		263
R. rubrum	detected		263
	0.0001%		264
Azobacter chroococcum		0.01%	265
Streptomyces olivacens		0.0035%	266
Blue-green algae:			
Phormidium luridum	trace	0.003%	267
Anacystis nidulans		detected	268
Fremyella diplosiphon		detected	268

REDUCTION OF 5α-ANDROSTANE-3,16-DIONE BY A CRYSTALLINE 20β-HYDROXY STEROID-NICOTINAMIDE-ADENINE DINUCLEOTIDE OXIDOREDUCTASE PREPARATION*

Pocklington and Jeffery found[284] that crystalline preparations of 20β-hydroxy steroid-NAD oxidoreductase reduced 5α-androstane-3,17-dione (LXXXVI) to 3α-hydroxy-5α-androstan-17-one (LXXXVII). The present investigation was carried out to determine whether 5α-androstane-3,16-dione (LXXXVIII) was reduced at the 3-position, or whether the preparation possessed 16-hydroxy steroid-NAD oxidoreductase activity hitherto unrecognised.

The isolated reduction product[132] was examined by GLC and GC-MS. Retention indices of the reduction product and of various reference compounds (OV-17 and OV-210) as their TMS ethers are given in Table 8. These indicate that the metabolite is not 3β-hydroxy-5α-androstan-16-one (LXXXIX). In fact, the mass spectrum (Fig. 59) of the TMS ether of the metabolite is similar to that of the TMS ether of 3α-hydroxy-5α-androstan-17-one (LXXXVII), which also had a similar retention index on OV-17, but not on OV-210. The absence of a 3-keto function in the reduction product was evident in the spectrum of its TMS/enol TMS ether (Fig. 60). 3-Enol TMS ethers of both 5α- and 5β- steroids give rise to prominent fragment ions of m/e 142 and 143.[131] Such ions

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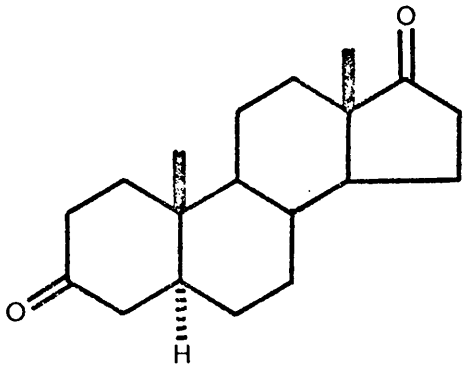
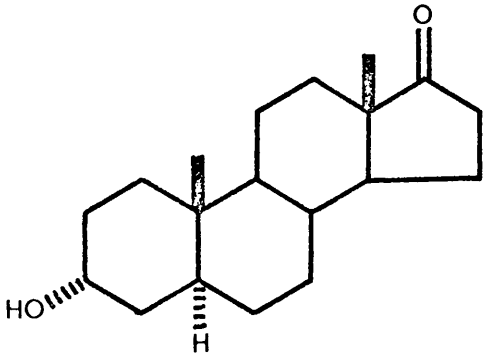
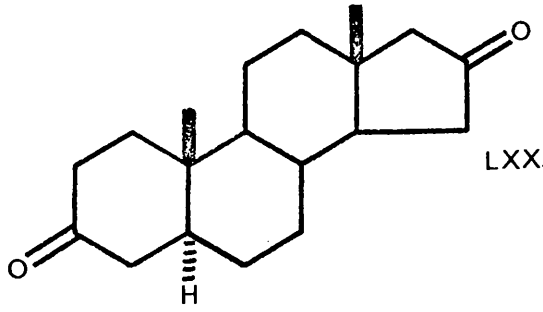
* The reaction was carried out, and the reduction product was isolated by Dr. J.d'A. Jeffery and Dr. T. Pocklington at the University of Aberdeen.



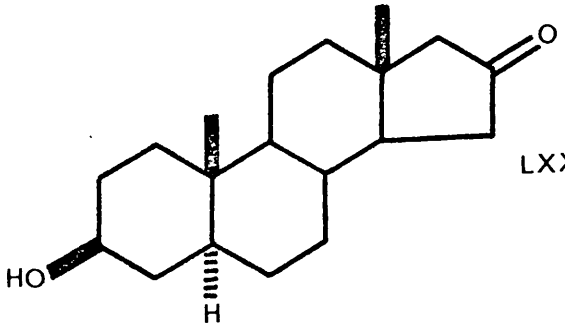
LXXXVI



LXXXVII



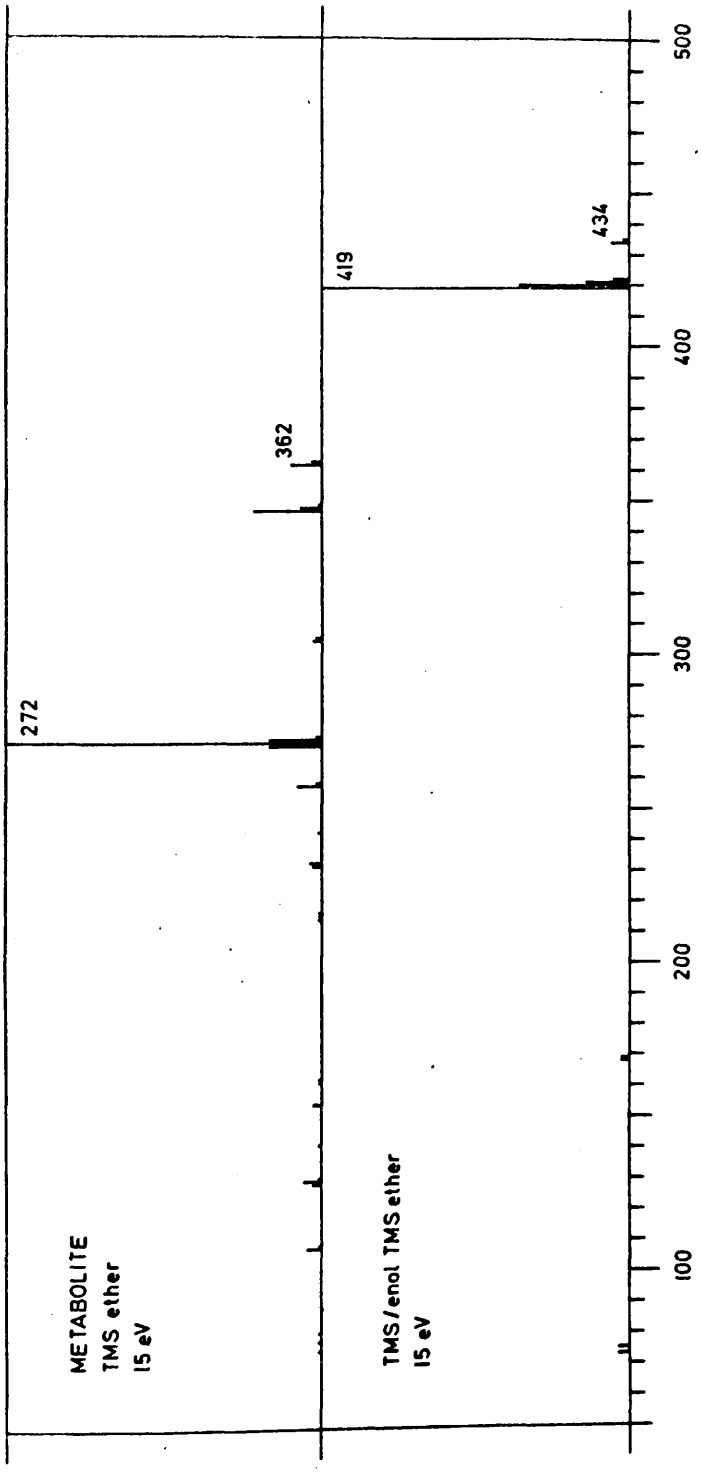
LXXXVIII



LXXXIX

Table 8. Retention indices of TMS ether of metabolite of 5α-androstane-3,16-dione and related compounds.

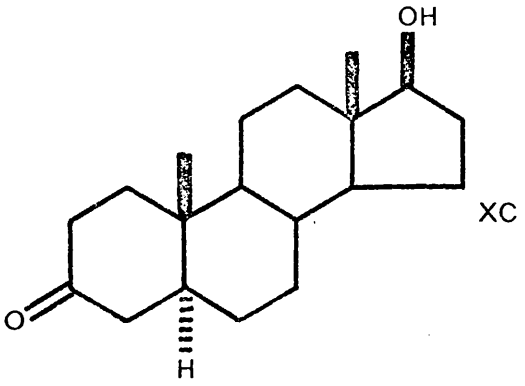
	OV-17	OV-210
Metabolite TMS	2695	3000
17β-hydroxy-5α-androstan-3-one TMS	2805	3140
3α-hydroxy-5α-androstan-17-one TMS	2695	2960
3β-hydroxy-5α-androstan-16-one TMS	2800	3130
5α-androstane-3,16-dione	2960	3540


METABOLITE
TMS ether
15 eV
272
362
TMS/enol TMS ether
15 eV
419
434
100
200
300
400
500

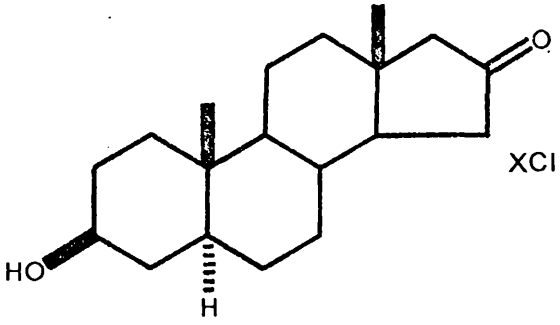
Figs 59.60

were observed in the spectra of the TMS/enol TMS ether of 17β-hydroxy-5α-androstan-3-one (XC) and of the monoenol TMS ether of 5α-androstane-3,16-dione (LXXXVIII), whereas there were no significant ions of m/e 142 or 143 in the spectrum of the TMS/enol TMS ether of the reduction product. This spectrum was closely similar to that of the TMS/enol TMS ether of 3β-hydroxy-5α-androstan-16-one (XCI).

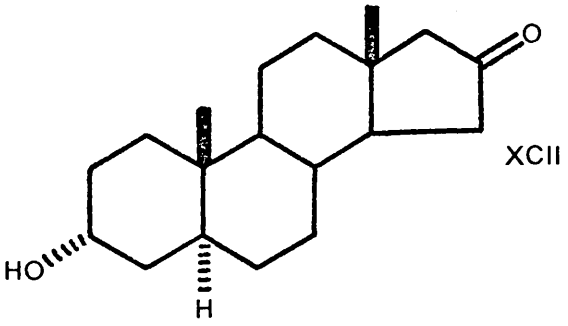
It may be concluded that the metabolite is 3α-hydroxy-5α-androstan-16-one (XCII), if the reasonable assumption is made[171] that the skeletal structure remains unaltered in the reduction.



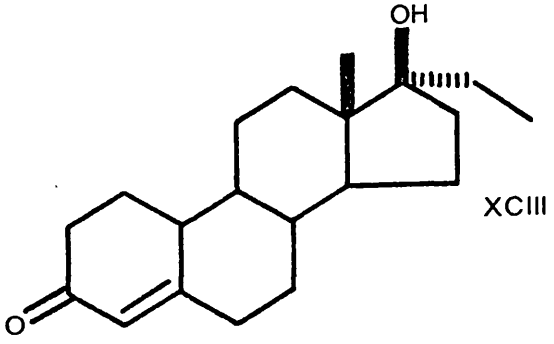
XC



XCI



XCII



XCIII

CHARACTERIZATION OF STEROIDAL DRUG METABOLITES BY GC-MS *

Structural features which distinguish steroidal drugs from natural hormones often persist in their metabolites. At the same time, the metabolic transformations of the drugs parallel, in certain respects, those of endogenous steroids. These factors provide scope for techniques based on combined GC-MS in investigations of steroidal drug metabolites. On this basis, a preliminary study has been made of neutral urinary metabolites of the anabolic steroid, 17α-ethyl-17β-hydroxyestr-4-en-3-one (Nilevar, XCIII).[270] This steroid is in widespread therapeutic[271] (and veterinary[272]) use. It was considered particularly useful for study since its possession of an ethyl substituent leads to a molecular weight 14 units above that of testosterone. It was hoped that metabolites retaining this moiety would be readily distinguished from natural metabolites by GC-MS.

Urine collections were made during the 24 hr. before and after administration of a single dose (50 mg) of Nilevar to a normal adult male volunteer. Preparation of unfractionated extracts of the enzyme-hydrolysed urine is described in the full report.[270] Three extracts were obtained containing, respectively, free steroids, steroids obtained by hydrolysis of glucosiduronates, and steroids from sulphates.

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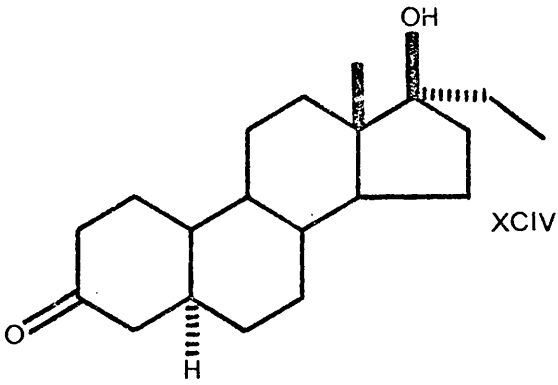
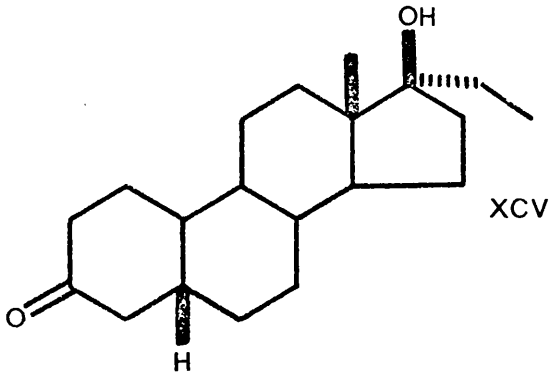
* Extraction of urinary metabolites was carried out by A.R. Thawley and P. Rocher. Preliminary examination by GLC was also carried out by G.M. Anthony and W.G. Stillwell.

The extract containing free steroids gave no indication of drug metabolites and was not further studied. Gas chromatographic examination (by W.G. Stillwell) of the extracts obtained by enzyme-hydrolysis of urine collected before and after administration of Nilevar revealed the presence of small quantities of possible metabolites. In the characterization of these possible metabolites, use was made of the full range of GC-MS facilities available to us during the first few months of 1970 (see p. 35). This involved (i) direct interpretation of retention index values, where peaks were sufficiently clearly defined by GLC alone; (ii) use of the multiple ion detector to distinguish pairs of ions characteristic of metabolites; (iii) monitoring of single ions; and (iv) repetitive scanning of mass spectra throughout the peaks suspected of containing metabolites. Each of these procedures was applied to extracts of urine collected both before and after administration of Nilevar. Glucosiduronate and sulphate hydrolysates were separately examined.

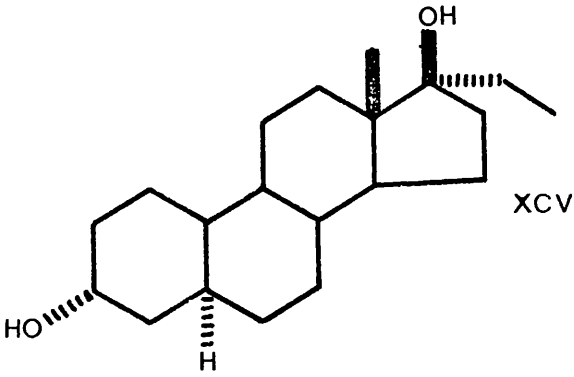
In general, Δ^4-3-oxosteroids yield tetrahydro metabolites;[273-275] the formation of phenolic metabolites appears to be a minor pathway in the human metabolism of Δ^4-estren-3-ones;[276-279] and alkyl and alkynyl substituents at C-17 are retained in major metabolites.[278-280] It was, therefore, considered likely that dihydro and tetrahydro analogues of Nilevar would be produced as metabolites. Consequently, the two dihydro (XCIV, XCV) and four tetrahydro (XCVI-XCIX)



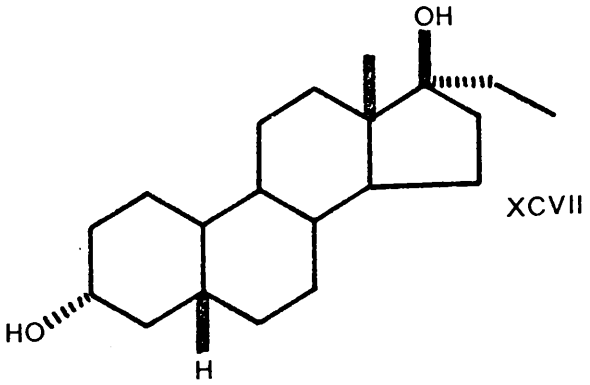
XCIV



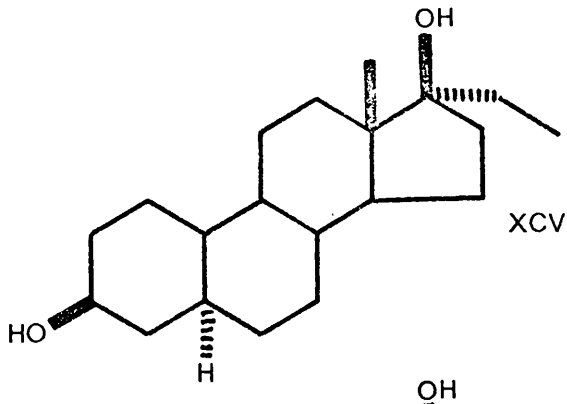
XCV



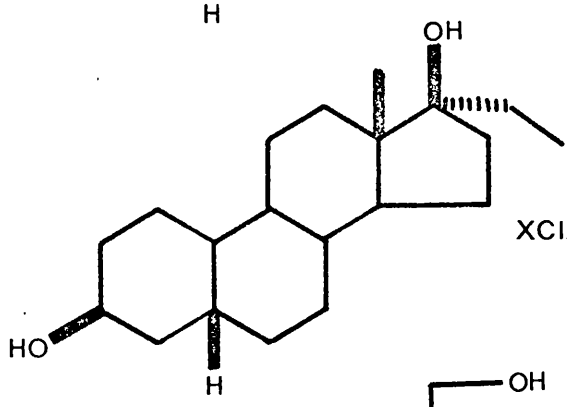
XCVI



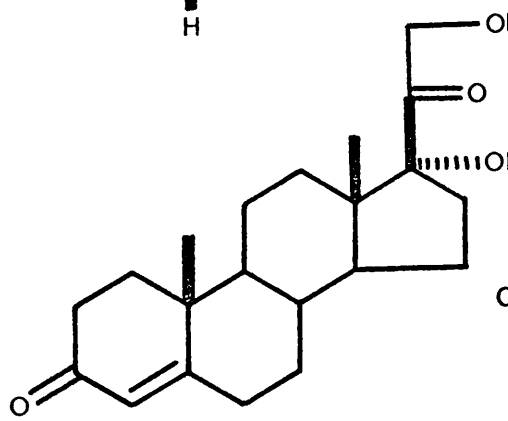
XCVII



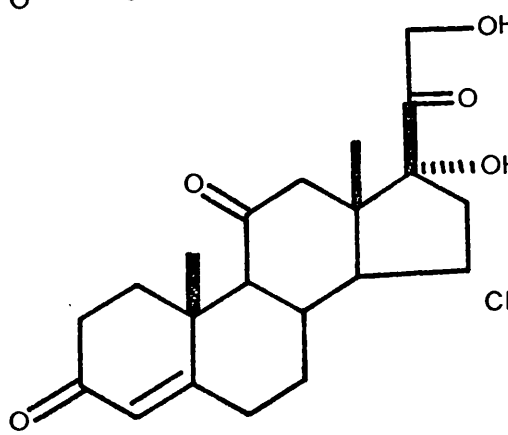
XCVIII



XCIX



C



CI

analogues of Nilevar were synthesized (by A.R. Thawley). Retention indices of these compounds, of Nilevar, and of the derived TMS ethers, on OV-1 and OV-17, are given in Table 9. The mass spectra of these analogues were recorded. In each case, a molecular ion was observed and a relatively intense ion at $[M-29]^+$ demonstrated the easy loss of the 17-ethyl substituent. The latter ion is of little diagnostic value since it coincides in mass with ions formed by loss of methyl groups from natural steroids. The ion of m/e 85, expected to arise from the 17-ethyl-17-hydroxy group, [192,281] was prominent only in the spectra of the 3-ketones. Spectra of the TMS ethers were also recorded. Again, molecular ions and relatively intense ions at $[M-29]^+$ were observed. However, highly characteristic ions of m/e 144 and m/e 157 were observed in all of these spectra. (Scheme 17). The spectra of stereoisomers, both free sterols and TMS ethers, were very similar.

It was desirable, therefore, to carry out GC-MS using single or multiple ion detection at characteristic m/e values, in an attempt to locate possible metabolites. Monitoring of free sterols at m/e 302, 304, and 306 would be expected to indicate the presence of, respectively, Nilevar and its dihydro and tetrahydro analogues. The presence of tetrahydro analogues only was indicated. On OV-1, a peak with retention index 2510 was observed for both hydrolysed glucosiduronate (Fig. 61) and sulphate fractions. On OV-17, two peaks were observed (I = 2850, 2880) which indicated the presence of 5α- and 5β-isomers. Because of the relatively low abundance of the molecular ions of the

Table 9 Retention indices of Nilevar, reduction products, and TMS ethers

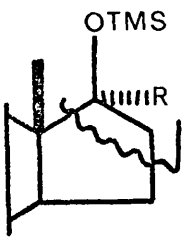
compound		OV-1		OV-1/	
		free	TMS	free	TMS
XCIII	Nilevar	2650	2775	3060	3060
XCIV	5α-dihydro	2535	2660	2915	2905
XCV	5β-dihydro	2540	2670	2925	2920
XCVI	3α,5α-tetrahydro	2515	2660	2845	2740
XCVII	3α,5β-tetrahydro	2520	2690	2865	2785
XCVIII	3β,5α-tetrahydro	2520	2735	2855	2800
XCIX	3β,5β-tetrahydro	2505	2700	2865	2765

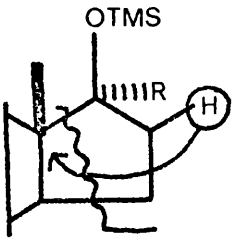
Table 10. Retention indices of possible metabolites of Nilevar, and TMS ethers.

extract	OV-1		OV-17		possible identity
	free[a]	TMS[b]	free[a]	TMS[b]	
GLU[c]	2510	2660	2850		3α,5α-diol
	2510	2690	2880	2785	3α,5β-diol
SUL[d]	2520	2655	2850		3α,5α-diol
	2520	2685	2880	2775	3α,5β-diol

a monitored at m/e 306.
b monitored at m/e 144/157.
c glucosiduronate fraction.
d sulphate fraction.

Scheme 17





R	m/e	m/e
H	(116)	129
CH_3	130	143
C_2H_5	144	157
C≡CH	140	153
$C{\equiv}C.CH_3$	154	167
$CH_2.CH{=}CH_2$	156	169

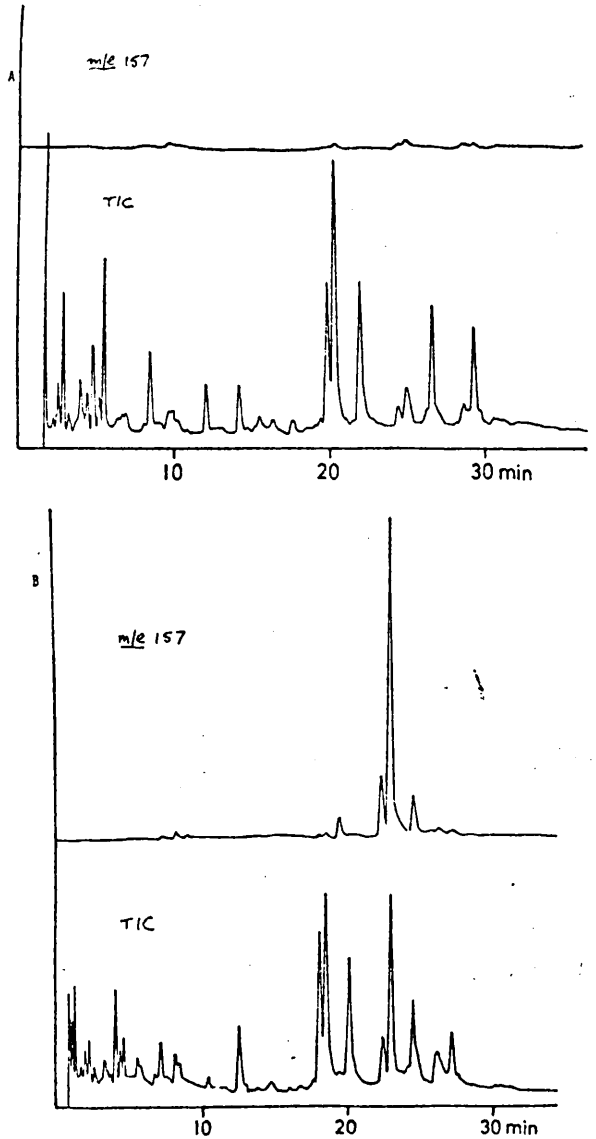


Figure 61 Simultaneous single ion (m/e 157) and TIC chromatograms of TMS derivatives of hydrolyzed sulfate fractions of urine both before (A) and after (B) administration of Nilevar: Conditions: 10 ft OV-1, 150-250° at 3°/min.

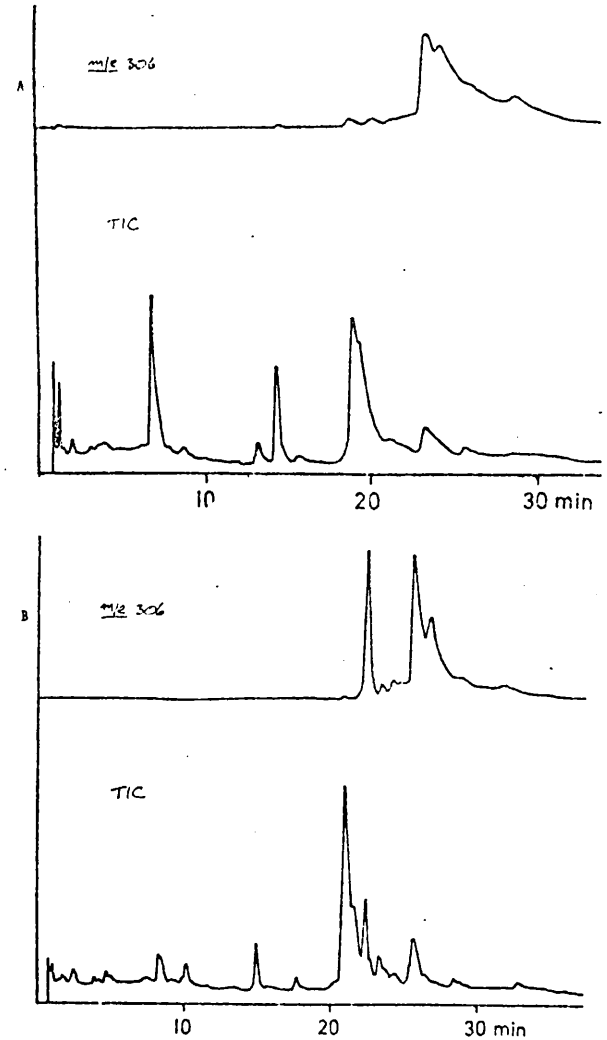


Figure 62 Simultaneous single ion (m/e 306) and TIC chromatograms of hydrolyzed glucuronide fractions of urine both before (A) and after (B) administration of Nilevar; Conditions: 10 ft OV-1, 150-250° at 3°/min.

free sterols, it was necessary to use the maximum electron multiplier voltage. Consequently, there was considerable interference from natural steroids (Fig. 61). More satisfactory results were obtained with the TMS ethers, monitoring at m/e 157 (Fig. 62). This revealed the presence of possible metabolites of retention index (on OV-1) 2660 and 2690, corresponding to the 3α,5α- and 3α,5β-diols. Their identity was confirmed by monitoring at m/e 144 and 157 and by individual addition of reference compounds which gave coincident peaks. The retention index data obtained by single and multiple ion monitoring in the search for possible tetrahydro metabolites of Nilevar are summarized in Table 10 Because of their low concentration, these possible metabolites did not yield satisfactory mass spectra. The possibilities of a preliminary fractionation by TLC have since been explored (by C.K.Y.S. Madani).

During the course of this investigation, evidence was obtained for the presence of possible triol metabolites. In particular, one of these appeared to possess a hydroxyl group on the ethyl substituent: the TMS ether gave a spectrum with ions at m/e 245 and 103. The former ion is analogous to that of m/e 157 in the spectra of the diols, but with an additional trimethylsilyloxy moiety; the latter ion is characteristic of TMS ethers of primary alcohols,[133,231] but also appears in the spectra of TMS ethers of sterols with vicinal hydroxyl groups.[221] Fig. 63 shows chromatograms obtained by monitoring at m/e 245 of TMS ethers from the hydrolysed sulphate fractions of urine,

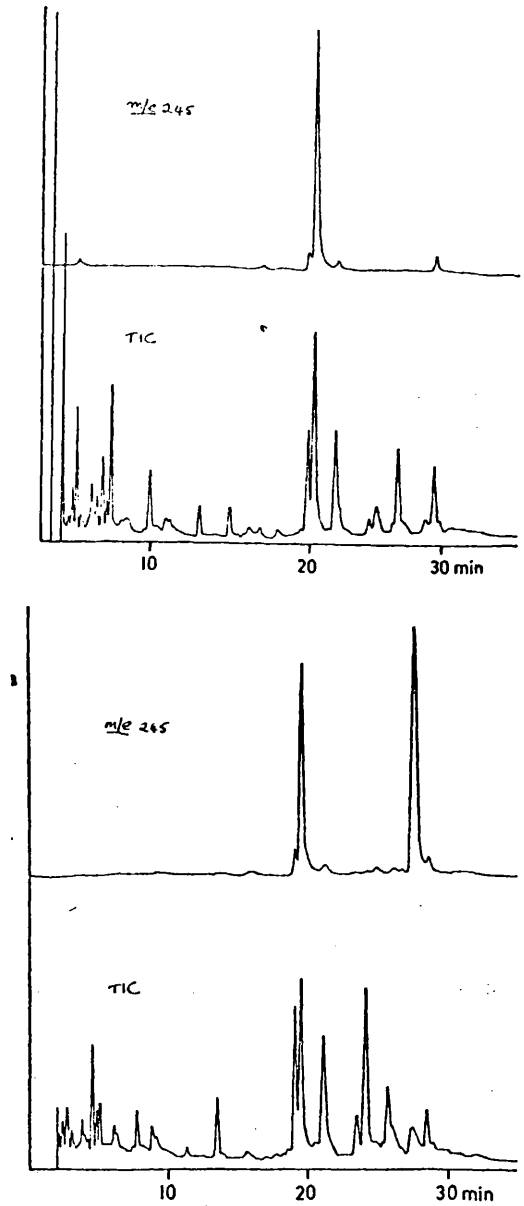


Figure 63 Simultaneous single ion (m/e 245) and TIC chromatograms of TMS derivatives of hydrolyzed sulfate fractions of urine both before (A) and after (B) administration of Nilevar; Conditions: 10 ft OV-1, 150-250° at 3°/min.

both before and after administration of Nilevar. It should be noted that ions of m/e 245 are present in the spectra of etiocholanolone and androsterone (major components of the urine extracts).

This study has indicated the potential of GC-MS in the field of drug metabolism studies. In particular, it may be possible to "screen" urine extracts for possible metabolites when relatively small quantities of the drug have been administered. Alternatively, relatively small volumes of urine, collected after administration of a larger dose, may be examined. It has been shown that quantitative GLC can be performed by single ion monitoring in the 50 - 1000 pg range.[282] A dynamic peak matching device which, it is claimed, permits determination of molecular formulae of submicrogram samples during GC-MS runs, has recently been described.[283]

III

BORONATES

THE MASS SPECTRA OF SOME CORTICOSTEROID BORONATES*

In the course of our recent studies on the analytical utility of boronate derivatives[285-293] we have obtained mass spectra of a large number of methyl, n-butyl, t-butyl, phenyl, and cyclohexyl boronates of various classes of corticosteroid. These derivatives possess good gas chromatographic properties[286,292] and the mass spectral fragmentations are usefully characteristic of the structure of the side-chain of the parent steroid.[286-288,292] This latter aspect is now discussed in more detail.

Line diagrams of low-resolution mass spectra of representative corticosteroid boronates are shown in Figs. 64-73. Selected data for other compounds discussed are given in Tables 11-15. Accurate mass measurements** substantiating the elemental compositions of many of the ions discussed in the text are presented in Table 16. Tabulated mass spectral data (low-resolution) have been submitted to the Mass Spectrometry Data Centre.

Boronates of 17α,21-dihydroxy-20-ketones

In a preliminary survey[286] of n-butyl and phenyl boronates it was noted that the mass spectra gave prominent molecular ions, and ions at $[M-15]^+$. Derivatives containing free hydroxyl groups yielded ions also at $[M-18]^+$. There were no other noteworthy ions containing boron in the higher mass range. The base peaks were due to "nuclear" fragments

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* This work was carried out in collaboration with Dr. D.J. Harvey, now at the Institute for Lipid Research, Baylor College of Medicine, Houston, Texas, U.S.A.

** High-resolution mass spectrometry was carried out on a CEC 21-110B instrument fitted with a gas chromatographic inlet system.[1]

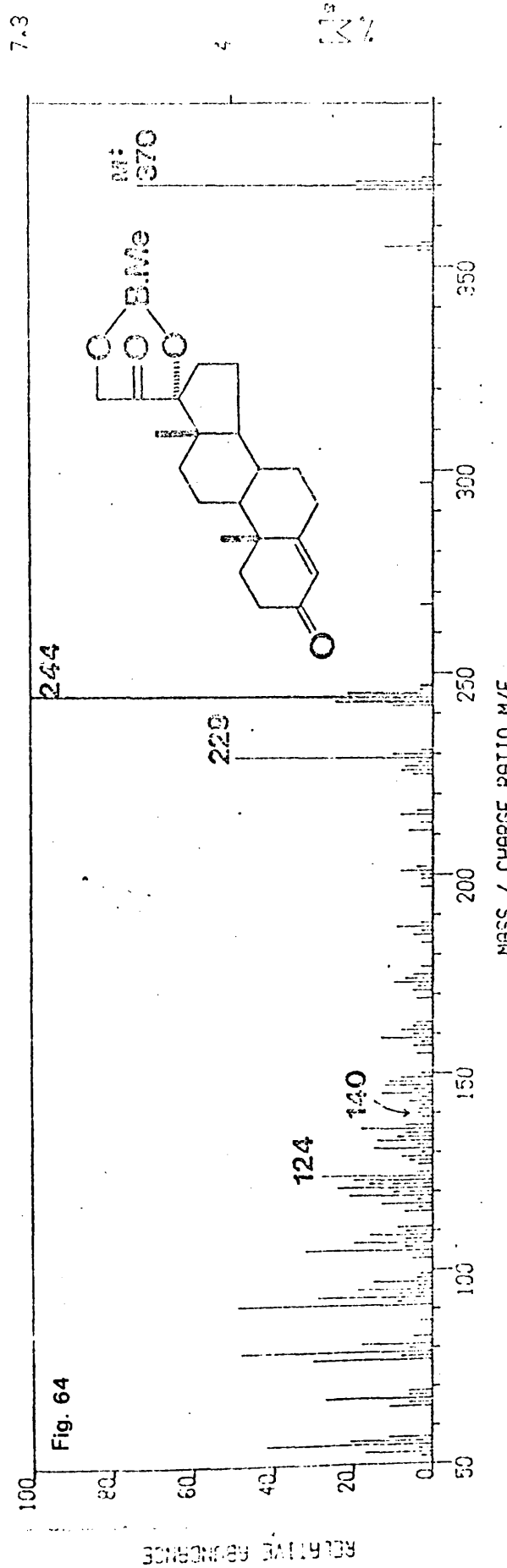

B.Me
M: 370
244
229
140
124
RELATIVE ABUNDANCE
MASS / CHARGE RATIO M/E

Fig. 64

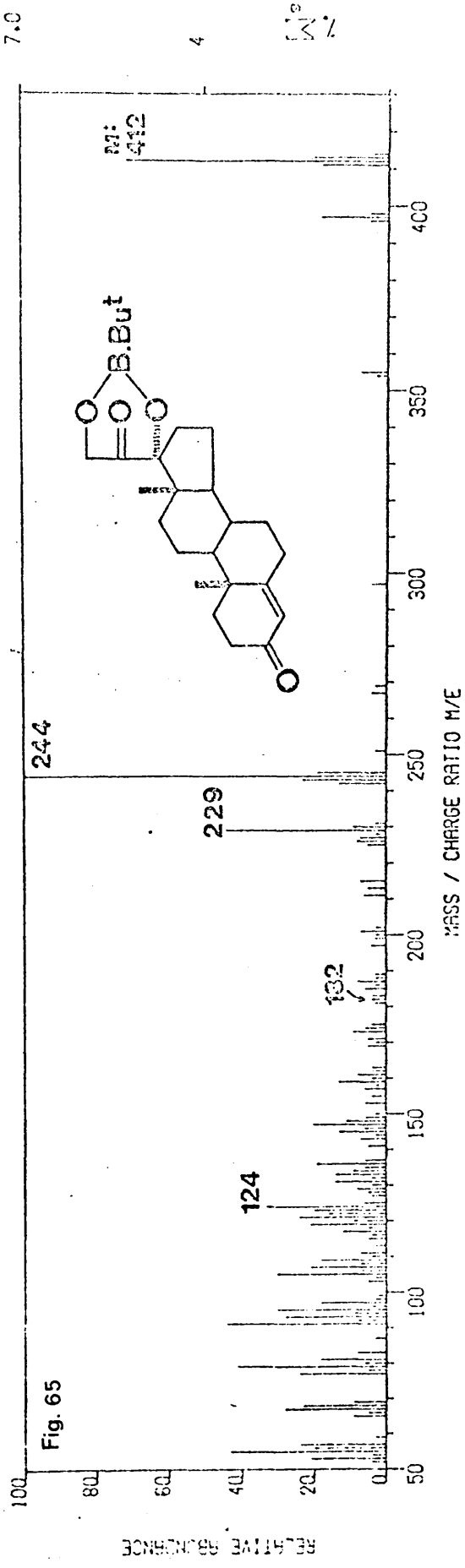

B.But
M: 412
244
229
132
124
RELATIVE ABUNDANCE
MASS / CHARGE RATIO M/E

Fig. 65

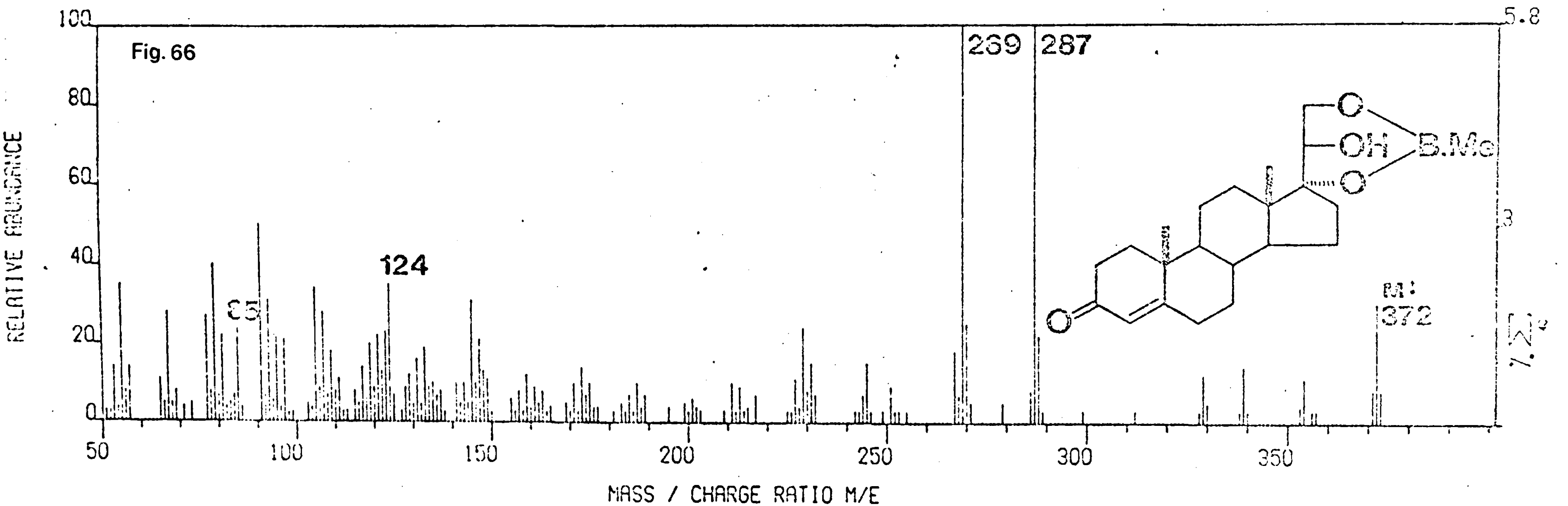


Fig. 66
RELATIVE ABUNDANCE
269
287
124
85
B.Me
OH
M:
372
5.8
3
MASS / CHARGE RATIO M/E

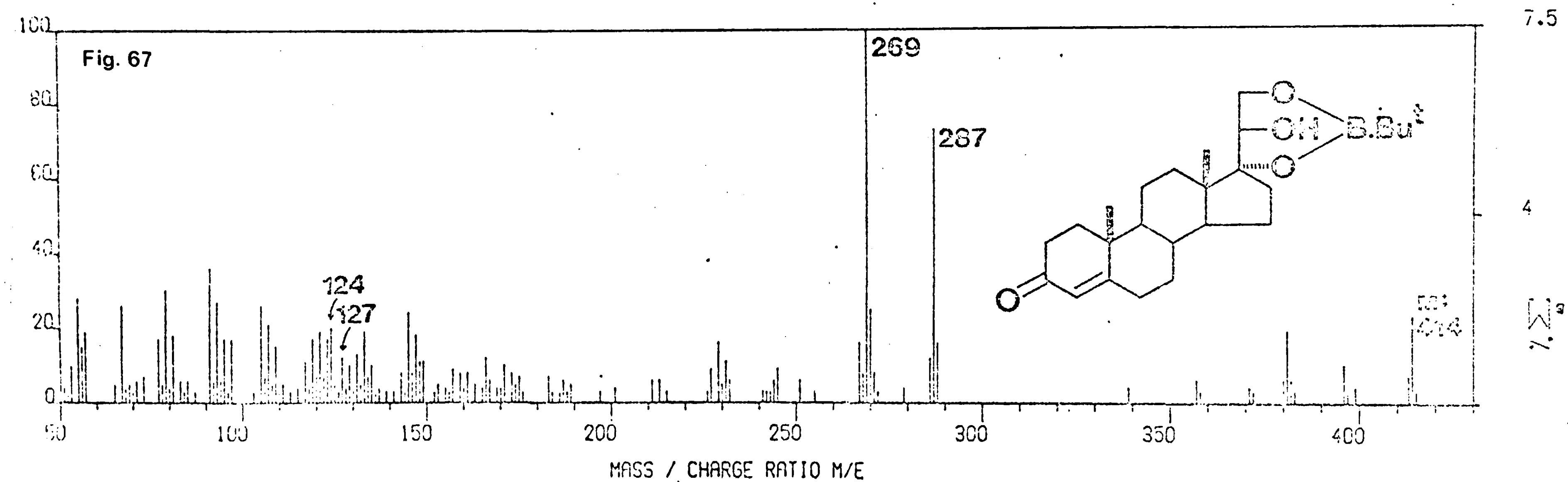


Fig. 67
269
287
124
127
B.Bu
OH
7.5
4
MASS / CHARGE RATIO M/E

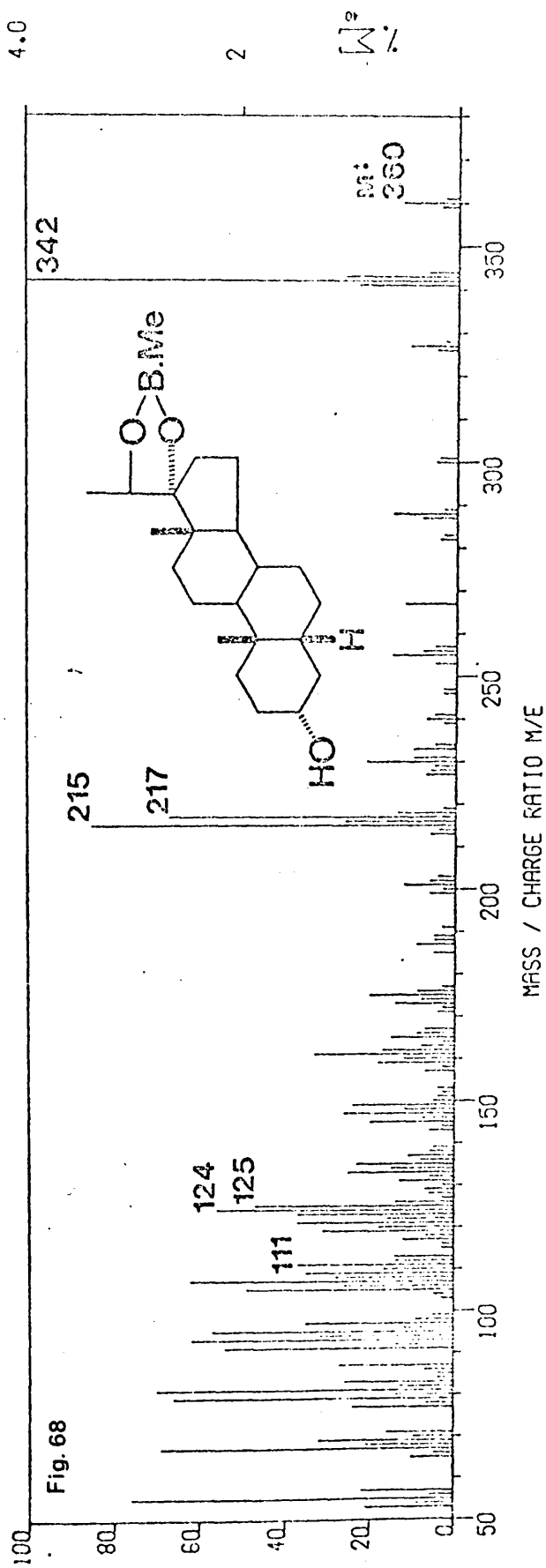


Fig. 68
M: 360
342
215
217
124
125
111
O-B.Me
HO
H
RELATIVE ABUNDANCE
MASS / CHARGE RATIO M/E

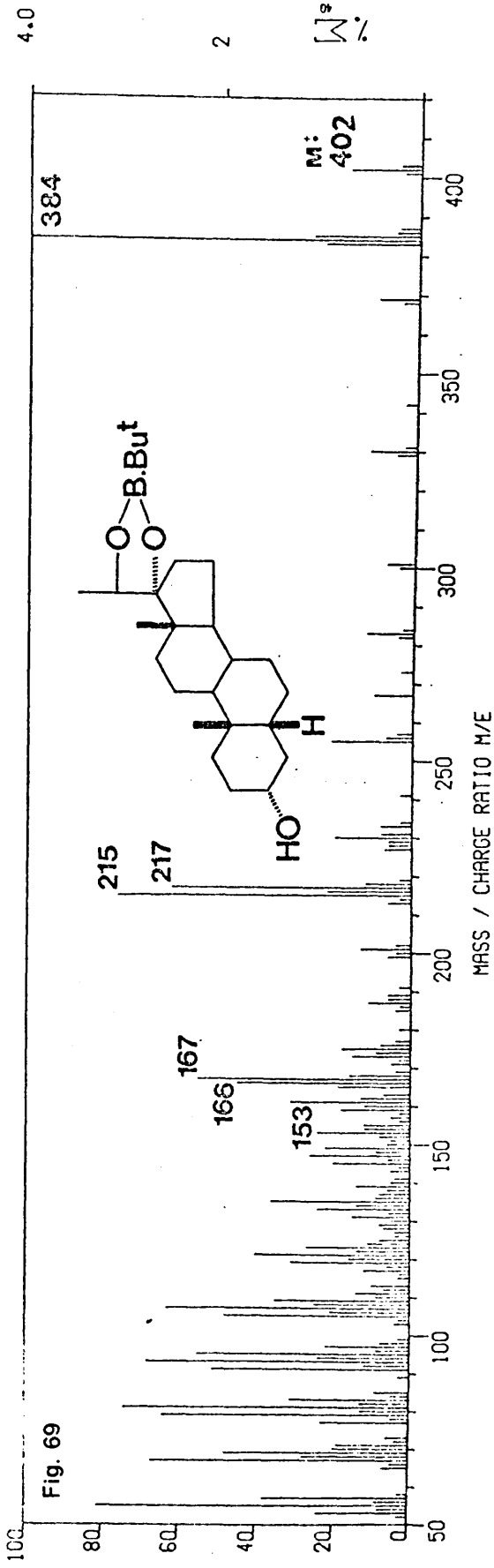
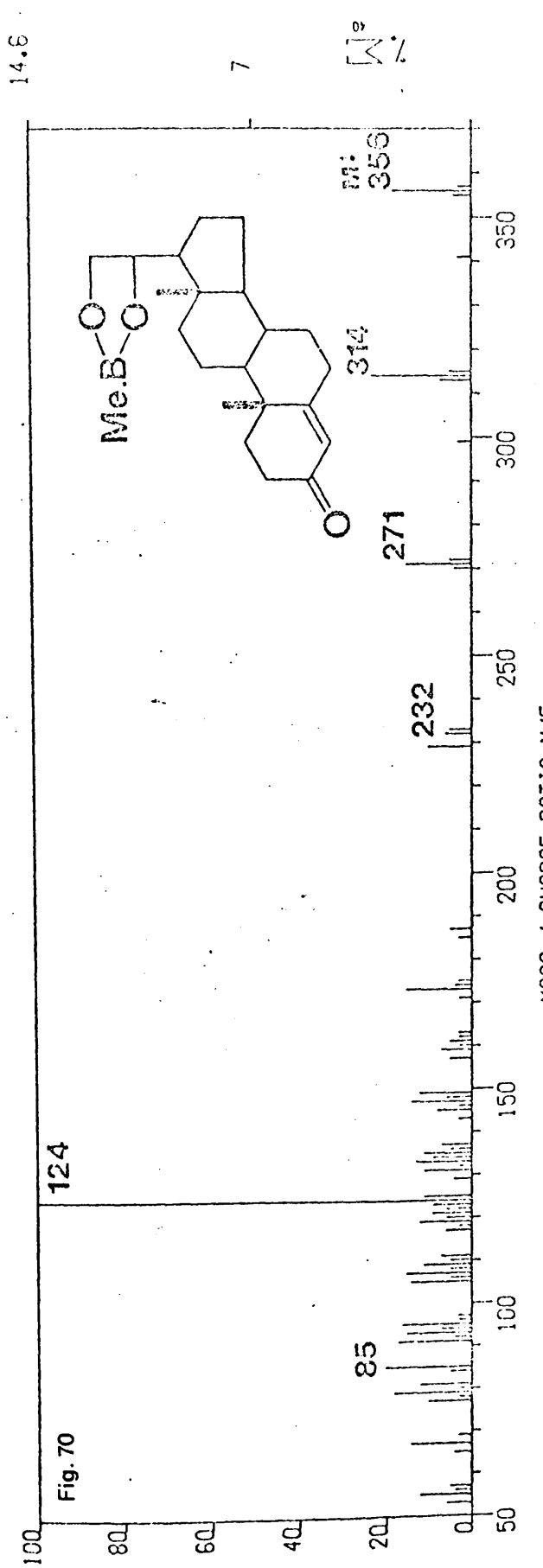


Fig. 69
M: 402
384
215
217
167
166
153
O-B.Bu^t
HO
H
RELATIVE ABUNDANCE
MASS / CHARGE RATIO M/E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Me.B
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O
O
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232
124
85
100
80
60
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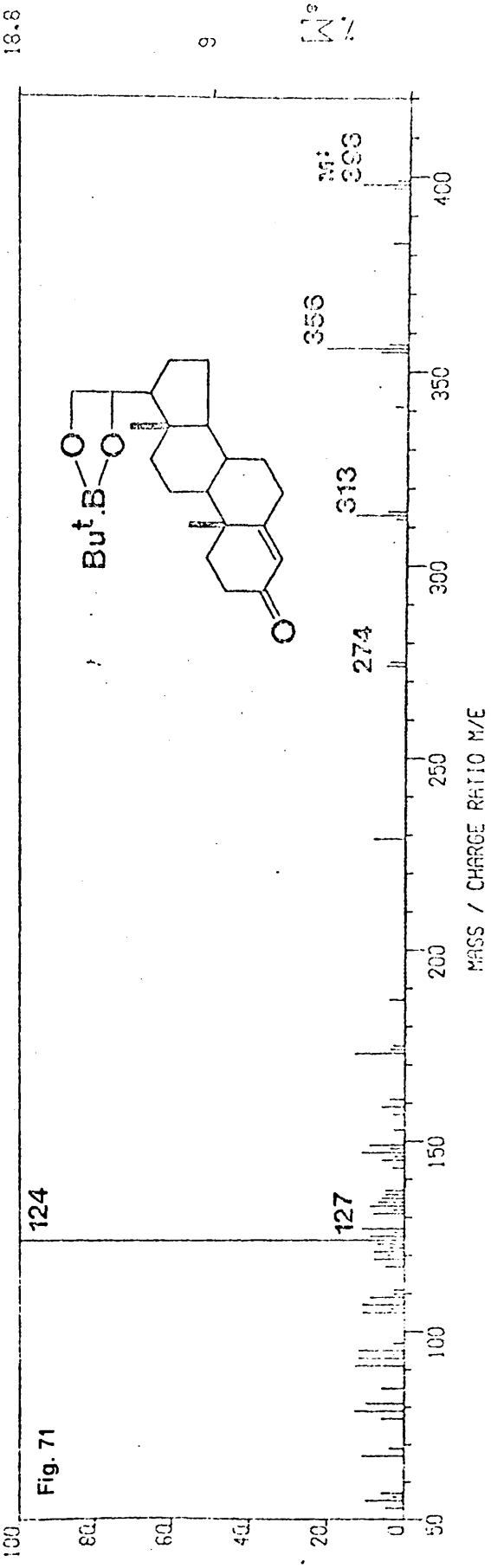
50
100
150
200
250
300
350
MASS / CHARGE RATIO M/E
RELATIVE ABUNDANCE

Fig. 70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M+ 398
356
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But.B
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O
O
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127
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100
150
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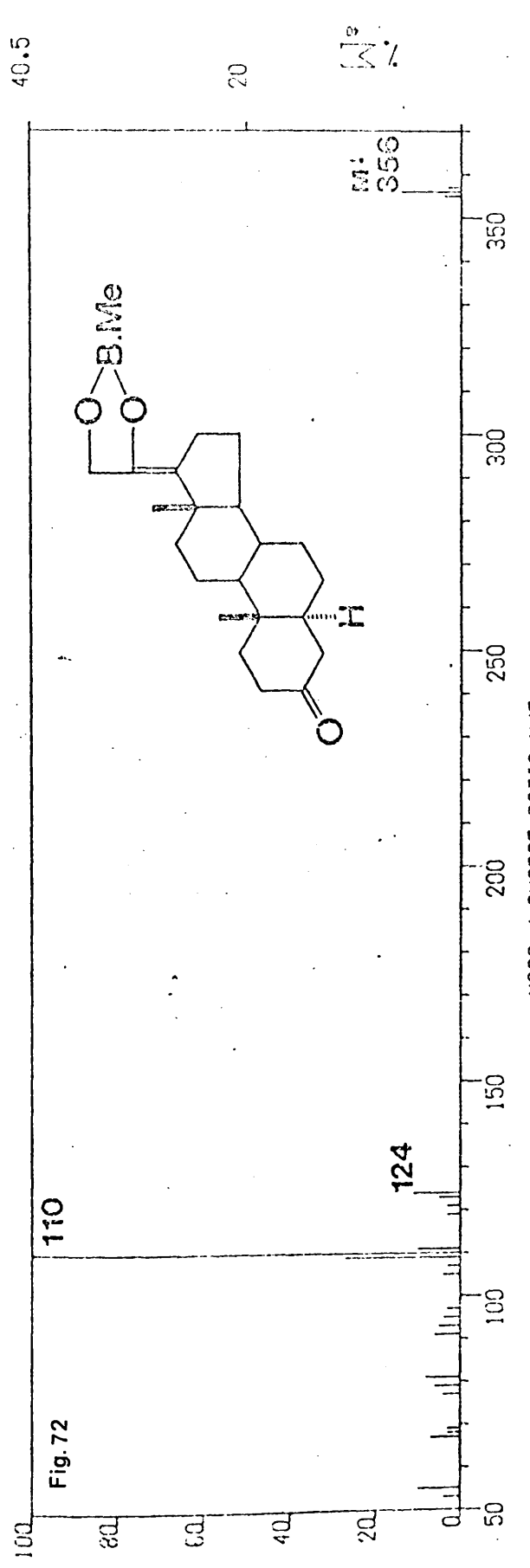
300
350
400
MASS / CHARGE RATIO M/E
RELATIVE ABUNDANCE

Fig. 71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100
80
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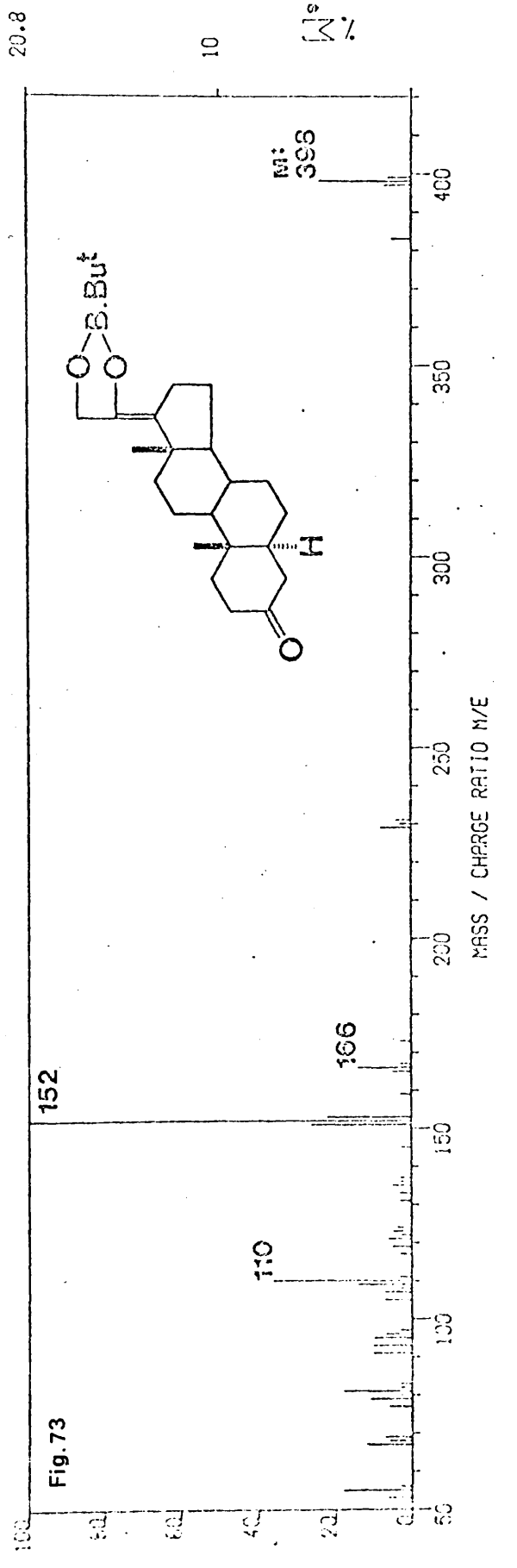
MASS / CHARGE RATIO M/E
RELATIVE ABUNDANCE

Fig.72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B.But
O
O
H
O
152
166
110
100
80
60
40
20
0
50
100
150
200
250
300
350
400
MASS / CHARGE RATIO M/E
RELATIVE ABUNDANCE

Fig.73

Table 11. Mass spectrometric data for boronates of 17α,21-dihydroxy-20-ketones

Boronate*	$M^{+\cdot}$	Ten most abundant fragment ions of m/e >100									
17α,21-Dihydroxypregn-4-ene-3,20-dione (Substance S, cortexolone)											
Ca	370	244	229	105	124	121	243	245	119	123	107
	73	100	49	32	28	24	23	21	21	20	20
Cb	412	244	229	124	105	121	243	119	107	413	147
	71	100	44	33	30	24	23	21	21	20	20
Cc	412	244	229	124	105	413	243	121	245	411	123
	74	100	39	29	26	23	22	22	21	19	18
Cd	432	244	105	124	229	119	121	107	123	136	122
	41	100	57	45	38	35	30	28	27	23	22
Ce	438	244	124	229	105	121	123	107	136	119	109
	34	100	47	40	33	28	26	26	22	22	22

Table 11 (cont.)

17α,21-Dihydroxypregn-4-ene-3,11,20-trione (cortisone)

CIa	384	258	257	135	122	121	123	197	136	259	385
	73	100	59	44	27	27	24	24	22	21	19
CIb	426	258	257	122	121	425	123	105	427	259	163
	74	100	55	25	24	20	20	20	19	19	18
CIc	426	258	257	135	122	121	123	105	259	163	161
	47	100	53	27	26	24	22	19	18	18	18
CId	446	258	257	105	121	122	123	119	136	117	163
	55	100	56	52	30	29	25	23	22	22	21
CIe	452	258	257	121	122	105	123	163	259	136	453
	50	100	57	27	26	22	21	19	18	18	16

17α,21-Dihydroxy-5β-pregnane-3,20-dione

CIIa	372	246	105	231	121	107	215	175	122	161	119
	45	100	40	38	37	36	25	25	25	24	24
CIIb	414	246	357	107	105	121	231	119	215	213	175
	25	100	64	43	43	40	36	29	28	28	25
CIIc	414	246	105	107	231	121	119	109	175	215	213
	46	100	43	38	36	31	28	28	25	24	24
CIId	434	246	105	231	107	121	119	161	213	135	109
	62	100	48	35	33	30	28	24	22	22	22
CIIe	440	246	231	107	105	121	357	215	213	247	175
	16	100	30	25	25	24	23	22	21	20	19

Table 11 (cont.)

17α,21-Dihydroxy-5β-pregnane-3,11,20-trione

CIIIa	386	260	259	121	135	109	122	136	123	105	107
	35	100	88	37	35	33	31	29	29	29	28
CIIIb	428	259	371	260	121	135	109	107	122	123	119
	18	100	84	70	38	35	35	33	32	28	23
CIIIc	428	259	260	246	121	107	135	109	105	136	245
	21	100	29	26	14	14	11	11	11	9	8
CIIId	448	259	260	246	105	109	121	107	123	135	449
	36	100	30	24	21	19	16	15	14	13	11
CIIIe	454	259	260	135	122	121	109	123	136	107	371
	10	100	90	43	43	43	41	37	35	35	30

11β,17α,21-Trihydroxypregn-4-ene-3,20-dione (Cortisol, hydrocortisone)

CIVa	386	242	227	119	105	260	123	368	121	107	124
	67	100	98	82	72	68	53	46	46	46	36
CIVb	428	242	227	119	260	105	121	123	124	107	122
	29	100	60	60	53	52	44	42	40	38	31
CIVc	428	242	227	260	119	410	121	105	123	395	124
	43	100	57	55	48	47	35	35	33	32	28

Table 11 (cont.)

11α,17α,21-Trihydroxypregn-4-ene-3,20-dione (11-Epicortisol)											
CVa	386	260	242	119	105	124	123	121	227	107	109
	67	100	87	75	67	57	52	52	50	50	43
CVb	428	260	242	119	124	105	123	121	227	107	122
	42	100	95	80	78	73	62	62	58	58	45

3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione (Tetrahydrocortisone)											
CVIa	388	243	370	105	107	121	147	244	261	135	108
	10	100	48	46	44	40	38	33	28	26	25
CVIb	430	243	412	105	107	121	244	147	261	135	119
	11	100	49	40	39	36	35	34	30	25	24
CVIc	430	243	147	412	107	105	121	135	109	261	244
	13	100	45	41	39	37	36	35	35	34	34

Table 11 (cont.)

3α,17α,21-Trihydroxy-5β-pregnan-20-one											
CVIIa	416	229	230	398	215	107	105	147	182	109	217
	8	100	45	39	37	35	32	30	25	25	24
CVIIb	374	229	230	215	107	105	147	356	140	121	217
	3	100	45	35	35	33	30	25	24	24	22
CVIIc	416	229	230	107	215	105	147	398	121	182	217
	4	100	47	35	34	33	28	25	25	24	23
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one											
CVIIIa	390	228	246	174	213	105	107	119	227	131	145
	1	100	100	59	56	55	46	39	37	34	32
CVIIIb	432	246	228	174	213	105	107	119	131	227	145
	1	100	84	40	39	37	34	27	24	23	23
CVIIIc	432	246	228	244	174	107	105	119	213	147	229
	5	100	60	54	41	38	35	30	29	25	23

Table 12 Mass spectrometric data for boronates of 17α,20,21-triols

Boronate	M^+	Ten most abundant fragment ions of m/e >100									
17α,20α,21-Trihydroxypregn-4-en-3-one											
CIXa	372	287	269	124	105	145	107	270	229	123	288
	30	100	100	35	34	31	28	25	24	23	22
CIXb	414	269	287	105	270	145	107	124	381	133	121
	23	100	73	26	25	24	21	20	19	19	19
CIXc	414	287	269	288	124	229	267	270	145	107	105
	35	100	46	29	27	25	23	21	21	21	21

Table 12 (cont.)

17α,20β,21-Trihydroxypregn-4-en-3-one

CXa	372	287	269	124	105	107	229	145	121	288	123
	26	100	47	34	32	29	28	26	24	23	23
CXb	414	287	269	105	107	124	145	229	121	119	123
	25	100	54	40	34	32	28	26	26	25	23
CXc	414	287	269	415	147	413	124	267	229	245	396
	100	46	39	33	30	29	29	26	25	25	23
CXd	434	105	147	124	159	107	121	119	173	123	109
	46	100	74	57	53	50	43	42	41	40	40
CXe	440	105	124	107	287	147	269	121	109	133	119
	87	100	99	90	83	77	75	62	61	58	58

3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one (cortolone)

CXIa	390	105	318	107	121	372	119	109	269	133	108
	30	100	91	89	71	68	52	52	51	49	49
CXIb	432	360	105	414	107	121	269	161	287	108	119
	30	100	84	80	77	61	53	50	48	44	43
CXIc	432	414	360	287	107	269	121	108	147	109	119
	95	100	79	57	54	51	44	37	35	35	30

Table 12 (cont.)

5α-Pregnane-3β,11β,17α,20β,21-pentol											
CXIIa	392	271	253	105	159	107	145	119	272	131	213
	1	100	49	43	37	30	27	25	22	22	20
CXIIb	434	271	105	253	107	159	119	145	131	215	246
	-	100	52	50	47	38	34	32	28	27	26

Table 13 Mass spectrometric data for boronates of 17α,20-diols

Boronate	M^+	Ten most abundant fragment ions of m/e >100									
5β-Pregnane-3α,17α,20α-triol											
CXIIIa	360 13	342 100	215 85	217 67	107 62	124 56	105 49	125 47	123 37	121 37	111 37
CXIIIb	402 18	384 100	215 76	107 63	217 62	167 55	105 48	166 45	121 40	135 36	109 35
CXIIIc	402 11	107 100	215 99	167 86	105 83	217 80	166 77	384 75	109 72	135 69	147 61
CXIIId	422 26	404 100	105 54	215 47	217 37	405 30	187 30	186 28	403 24	107 24	173 23
CXIIIe	428 11	410 100	215 51	217 38	193 38	107 38	411 32	105 30	192 28	109 27	409 25

Table 13 (cont.)

5β-Pregnane-3α,17α,20β-triol											
CXIVa	360	342	215	124	107	217	111	105	125	123	121
	10	100	77	68	61	60	52	51	49	35	34
CXIVb	402	384	215	107	217	166	167	105	121	385	109
	12	100	60	50	47	45	44	39	32	29	29
CXIVc	402	166	215	107	167	216	105	384	135	121	109
	8	100	95	93	78	75	72	70	57	56	50

5β-Pregnane-3α,11β,17α,20β-tetrol											
CXVa	376	124	125	111	105	107	178	213	358	215	228
	1	100	60	60	58	53	52	50	48	46	38
CXVb	418	166	167	105	107	215	400	213	119	246	228
	1	100	64	64	61	49	46	42	42	40	40
CXVc	418	166	400	167	105	107	246	215	213	119	109
	2	100	48	48	44	42	37	33	31	31	31

Table 14 Mass spectrometric data for boronates of 20,21-diols

Boronate	$M^{+\cdot}$	Ten most abundant fragment ions of m/e >100									
20β,21-Dihydroxypregn-4-en-3-one											
CXVIa	356	124	314	271	173	107	147	105	133	149	119
	18	100	23	15	15	15	14	14	13	12	12
CXVIb	398	124	356	173	313	147	127	107	105	229	149
	12	100	21	14	13	11	11	11	11	9.	9
CXVIc	398	124	107	127	105	173	356	125	109	313	145
	6	100	14	13	13	11	10	10	10	6	6
CXVId	418	124	147	105	107	173	376	149	133	125.	119
	10	100	33	24	15	12	10	10	10	10	10
CXVIe	424	124	382	341	107	383	381	173	147	153	105
	56	100	91	35	28	25	25	23	22	19	19

Table 14 (cont.)

5α-Pregnane-3α,11β,20α,21-tetrol											
CXVIIa	376	358	107	105	340	343	147	133	119	213	245
	6	100	94	79	77	73	61	61	60	58	51
CXVIIb	418	107	400	105	382	147	385	133	119	109	121
	6	100	84	76	70	70	64	49	47	47	45

5α-Pregnane-3α,11β,20β,21-tetrol											
CXVIIIa	376	107	105	358	119	147	325	133	340	106	131
	2	100	95	74	65	59	58	55	53	51	50
CXVIIIb	418	107	105	400	382	147	367	119	133	127	145
	1	100	92	72	64	63	61	61	52	51	48

Table 15 Mass spectrometric data for boronates of 20,21-ketols

Boronate	$M^{+\cdot}$	Ten most abundant fragment ions of m/e >100									
21-Hydroxy-5α-pregnane-3,20-dione											
CXIXa	356	110	109	124	111	123	355	105	357	121	119
	14	100	28	11	10	5	4	4	3	3	3
CXIXb	398	152	110	151	153	166	109	341	229	397	107
	24	100	36	26	22	14	14	12	8	7	7
CXIXc	398	152	151	153	383	399	166	397	147	107	105
	27	100	26	18	9	8	8	7	7	7	7
21-Hydroxypregn-4-ene-3,20-dione (Deoxycorticosterone, Cortexone)											
CXXa	354	110	109	111	124	105	355	353	123	119	107
	19	100	28	10	8	8	5	5	5	5	5
CXXb	396	152	339	110	105	338	151	340	109	153	191
	37	100	91	61	23	22	22	21	20	17	14

Table 15 (cont.)

3α,21-Dihydroxy-5α-pregnan-20-one											
CXXIa	358	110	109	124	107	215	111	105	135	121	123
	19	100	30	19	13	11	11	10	8	8	8
CXXIc	400	152	151	167	153	107	166	385	135	121	119
	24	100	25	20	13	13	9	8	7	7	7

3β,21-Dihydroxypregn-5-en-20-one											
CXXIIa	356	110	109	105	229	213	107	145	119	143	131
	25	100	33	25	19	19	18	17	15	14	13
CXXIIc	398	152	105	229	107	165	145	119	151	213	133
	66	100	46	43	34	31	30	28	27	25	25
CXXIId	418	172	105	171	173	145	107	229	143	119	400
	10	100	36	31	25	18	18	16	16	16	10

Table 16 Elemental compositions of some ions[a] in spectra of representative corticosteroid n-butyl boronates

Compound	Measured m/e	Elemental Comp.[b]	Error (p.p.m.)[c]
Cc	412.2784	$C_{25}H_{37}BO_4$	- 0.16
	397.2554	$C_{24}H_{34}BO_4$	+ 0.32
	244.1820	$C_{17}H_{24}O$	- 0.72
	229.1595	$C_{16}H_{21}O$	+ 0.23
	182.1129	$C_9H_{15}BO_3$	+ 1.45
	124.0897	$C_8H_{12}O$	+ 0.85
	123.1169	C_9H_{15} (72%)	+ 1.51
	123.0818	$C_8H_{11}O$ (28%)	+ 0.86
	121.1022	C_9H_{13} (61%)	+ 0.42
	121.0667	C_8H_9O (39%)	+ 1.34
CIXc	414.2904	$C_{25}H_{39}BO_4$	- 0.89
	287.1990	$C_{19}H_{27}O_2$	- 2.10
	269.1893	$C_{19}H_{25}O$	- 1.22
	229.1583	$C_{16}H_{21}O$	- 0.96
	183.1170	$C_9H_{16}BO_3$	- 2.27
	145.1010	$C_{11}H_{13}$	- 0.71
	127.0937	$C_6H_{12}BO_2$	+ 0.69
	124.0895	$C_8H_{12}O$	+ 0.68

Table 16 (cont.)

CXIIIc	384.3229	$C_{25} H_{41} BO_2$	+ 2.92
	217.1964	$C_{16} H_{25}$	+ 0.80
	215.1803	$C_{16} H_{23}$	+ 0.29
	167.1242	$C_9 H_{16} BO_2$	- 0.16
	166.1191	$C_9 H_{15} BO_2$	+ 2.61
CXVIc	398.2982	$C_{25} H_{39} BO_3$	- 1.20
	356.2889	$C_{23} H_{37} BO_2$	+ 0.04
	313.2341	$C_{20} H_{30} BO_2$	+ 0.13
	275.2173	$C_{17} H_{28} BO_2$	- 0.98
	147.1167	$C_{11} H_{15}$ (83%)	- 0.64
	147.0806	$C_{10} H_{11} O$ (17%)	- 0.35
	127.0946	$C_6 H_{12} BO_2$	+ 1.59
	124.0894	$C_8 H_{12} O$	+ 0.56
CXIXc	166.1176	$C_9 H_{15} BO_2$	+ 1.08
	152.1005	$C_8 H_{13} BO_2$	- 0.37

a $m/e > 120$.
b ions containing most abundant isotopes only listed.
c measured value minus calculated value.

comprising rings A-C and part of ring D.

The mass spectra (Figs. 64,65) of the methyl and t-butyl boronate derivatives of Substance S (C) are characteristic of boronates of steroidal 17α,21-dihydroxy-20-ketones ("dihydroxyacetones"). The molecular ions from compounds Ca-Ce* are fairly abundant (34-74%). All of these derivatives give rise to a base peak at m/e 244 evidently formed by scission of the C-13/17 and C-15/16 bonds (xxix). The majority of the ions in the lower mass region of the spectra appear to arise from fragmentation of this nuclear fragment. This is indicative of preferential charge residence on the steroid nucleus, which is consistent with the comparative paucity of characteristic boron-containing ions. There is an ion (xxx) at m/e 124 typical of the steroidal 4-en-3-one structure[189,194,195] but no ion at $[M-42]^{+\cdot}$ corresponding to the usual elimination of ketene from ring A of such steroids.[294-296]

The introduction of a further ketonic function, as in boronates of cortisone (CI), results in greater stability of the molecular ion and nuclear fragment. As expected[194,297] the presence of the 11-keto function prevents the formation of an ion of m/e 124. An interesting feature of the spectra of the cortisone boronates is the prominence of a nuclear fragment ion (m/e 257: possibly xxxi) containing one less hydrogen atom than xxix. The stabilisation of the even-electron ion

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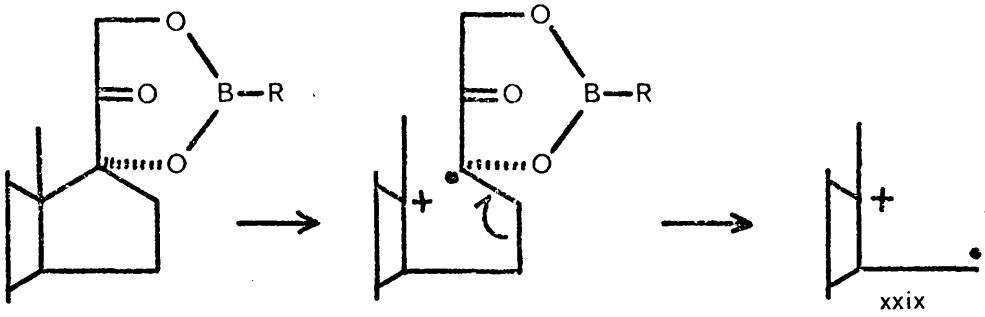
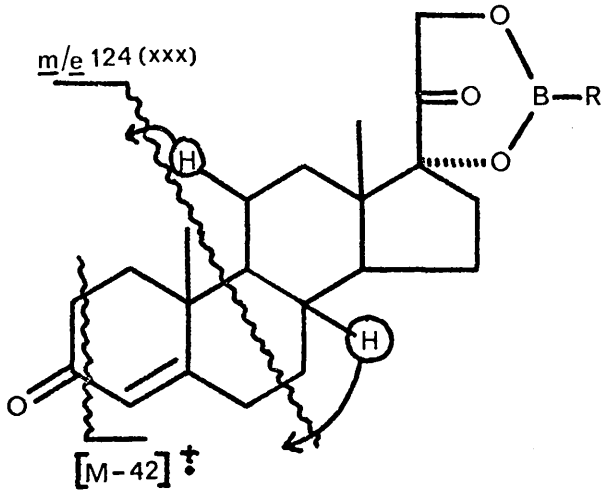
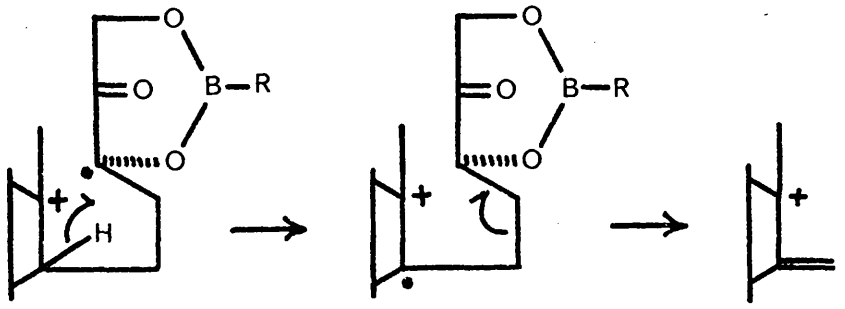
* Throughout this section the particular boronate types are indicated by suffixes a to c: a = methyl boronate; b = t-butyl boronate; c = n-butyl boronate; d = phenyl boronate; e = cyclohexyl boronate.



xxix





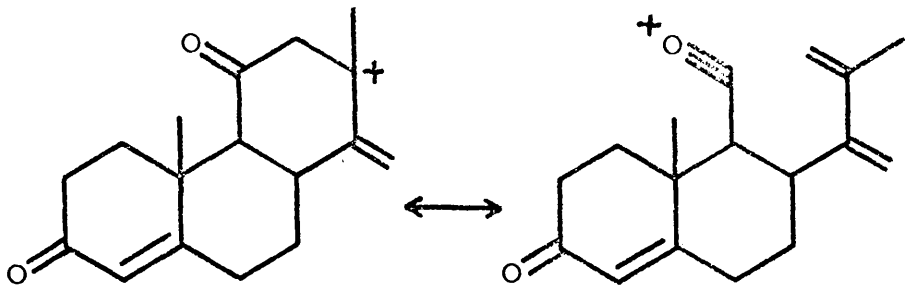
xxxi

by an 11-keto group may be rationalised on the basis of a structure such as xxxii. Ions at $[M-70]^{\ddagger}$ are formed by the usual ring A fragmentation.[298]

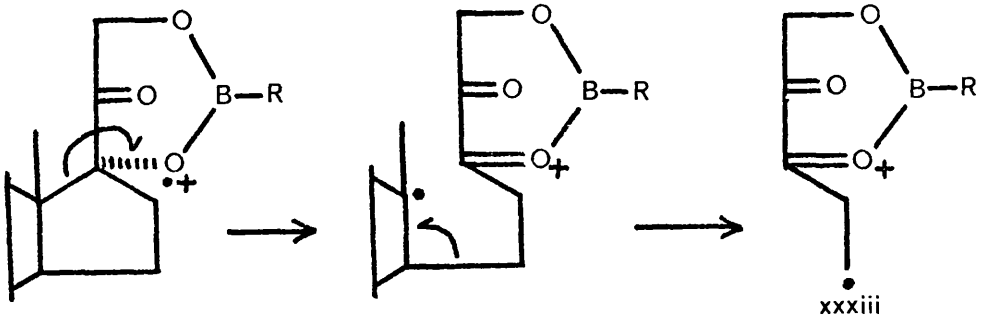
Molecular ions in the spectra of boronates of dihydro-S (CII) are less abundant than those of derivatives of C. The base peaks at m/e 246 are due to ions of type xxix. There are ions formed by loss of a t-butyl radical from CIIb (m/e 357, 64%) and a cyclohexyl radical from CIIe (m/e 357, 23%). Such ions are insignificant in the spectra of CIIa and CIId. The small degree of direct fragmentation of ring A of the molecular ion reflects the directing influence of the boronate function. Several ions can be discerned which show appropriate mass shifts with different substituents on the boron atom suggesting that they contain the boronate moiety: their relative abundances are given in Table 17. These ions appear at $[125+R]^{\ddagger}$, suggesting that they are formed by scission of the C-13/17 and C-14/15 bonds as in xxxiii.

The even-electron ion xxxiii is particularly abundant in the spectra of boronates of 5β-dihydrocortisone (CIII) (m/e 259,260: CIIIa,88,100%; CIIIb,100,70%; CIIIc,100,29%; CIIId,100,30%; CIIIe,100,90%).

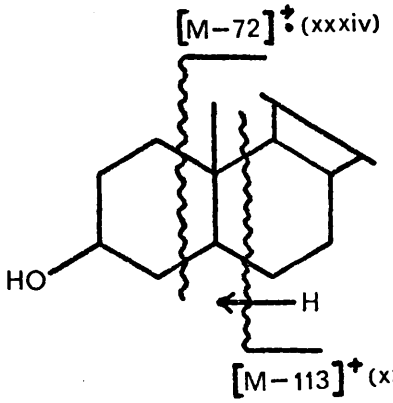
The ease of elimination of water from the molecular ions of boronates of cortisol (CIV) is illustrated by the low abundance of $[M-15]^{+}$ ions and high abundance of $[M-18]^{\ddagger}$ and $[M-18,15]^{\ddagger}$ ions. The base peak (m/e 242) corresponds to an ion of type xxix from which one molecule of water has been eliminated. The even-electron fragment ion

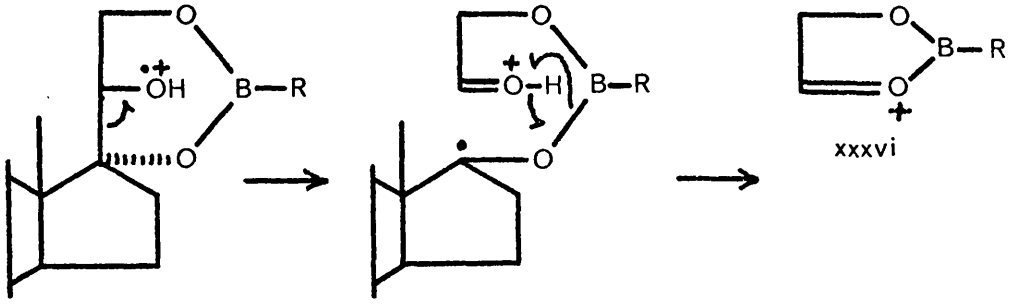


xxxii

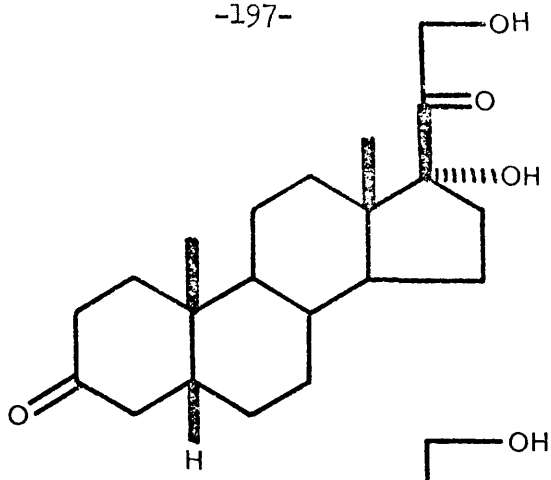


xxxiii





xxxvi



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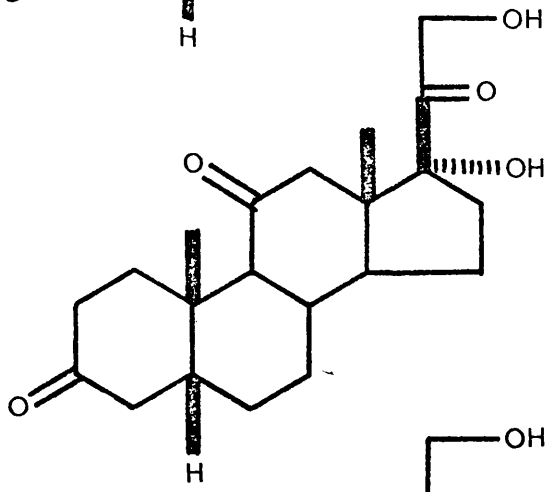

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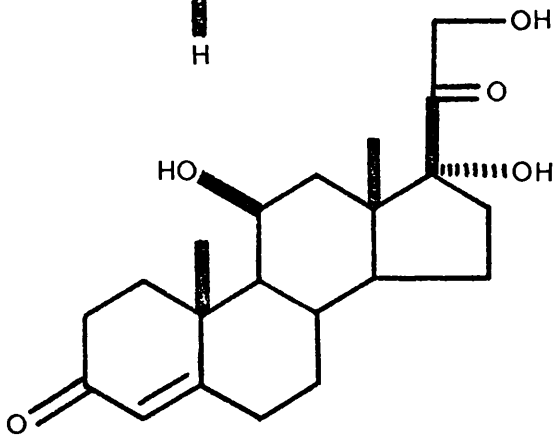
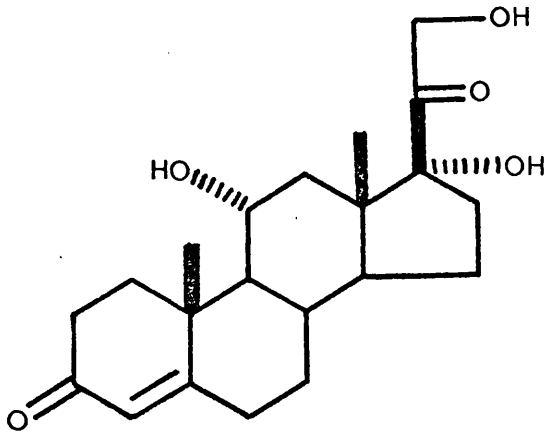


CIV



CV

Table 17 Relative abundances of boron-containing ions from boronates of CXII

m/e	140	182	202	208
Methyl	16[a]	0	3	0
t-Butyl	2	13[a]	1	0
n-Butyl	0	15[a]	0	0
Phenyl	0	0	15[a]	0
Cyclohexyl	0	2	0	12[a]

[a] $[125+R]^+$

Table 18 Relative abundances of boron-containing ions from boronates of CIX

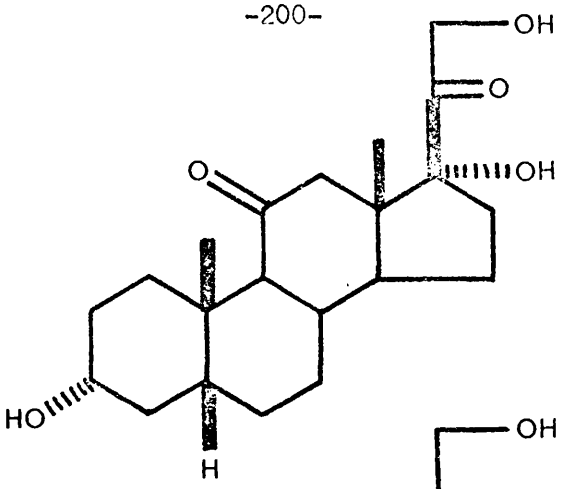
m/e	85	127
Methyl	24[a]	3
t-Butyl	6	12[a]
n-Butyl	6	11[a]

[a] $[70+R]^+$

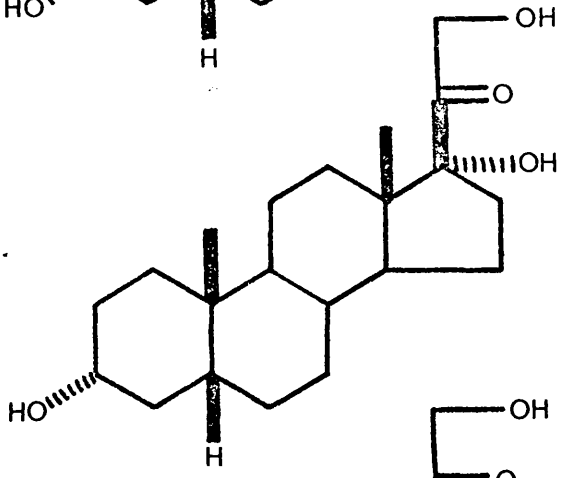
(m/e 241) is present only in low abundance. The boronates of cortisol (CIV) and its 11α-isomer 11-epicortisol (CV) show some significant differences. Ions corresponding to the elimination of the elements of water from the molecular ions, and from fragment ions of m/e 260, are more abundant in the spectra of CIVa (at 70 and 22.5 eV) than in the corresponding spectra of CVa. Also, the ion of m/e 124 (formed with transfer of a hydrogen atom from C-11) is less abundant in the spectra of the 11β-isomer.

Water is eliminated readily from the molecular ions of boronates of tetrahydrocortisone (CVI). As might be expected, the presence of the 11-keto function in the latter compound leads to predominant formation of even-electron nuclear fragment ions (xxxi, m/e 243,100%): ions of m/e 244 (xxix) are of relative intensity 33-35%, irrespective of the nature of the substituent on the boron atom. Ring A/B fragmentation gives rise to ions at $[M-72]^+$ (xxxiv) and $[M-113]^+$ (xxxv). Analogous ions are formed from the 3-trimethylsilyl ether of tetrahydrocortisone methyl boronate at $[M-144]^+$ and $[M-185]^+$. Ions xxxiv are well known[168,299] and those of type xxxv have been noted for 3-hydroxy steroids.[168]

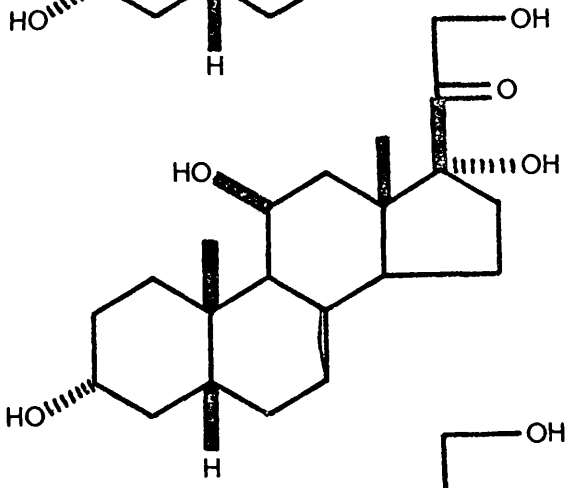
The base peak of the n-butyl boronate of tetrahydro-S (CVII) is due to the even-electron nuclear fragment (xxxi). The 3-substituent (keto- or 3α-hydroxy-) appears to exert a strong influence on the fragmentation of ring D. Long-range influences have previously been encountered in the study of steroid mass spectra[300] and



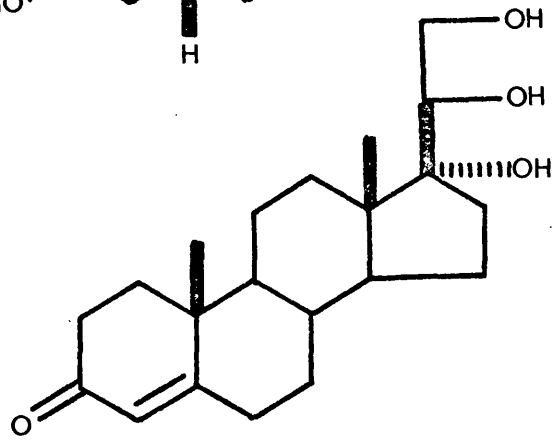
CVI



CVII



CVIII



CIX

intramolecular electrostatic interactions have been invoked to explain similar effects in steroid chemistry.[301]

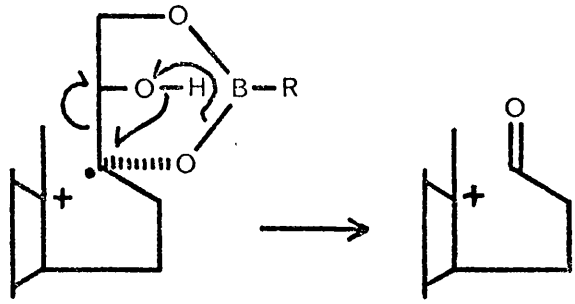
On the other hand, the base peaks of the boronates of tetrahydrocortisol (CVIII) are due to the odd-electron nuclear fragments (xxix): the 11β-hydroxy function appears to exert a greater influence on the fragmentation than the 11-keto moiety. The molecular ions of the tetrahydrocortisol boronates are weak, but there are fairly intense ions corresponding to successive eliminations of molecules of water and of methyl radicals.

Boronates of 17α,20,21-triols

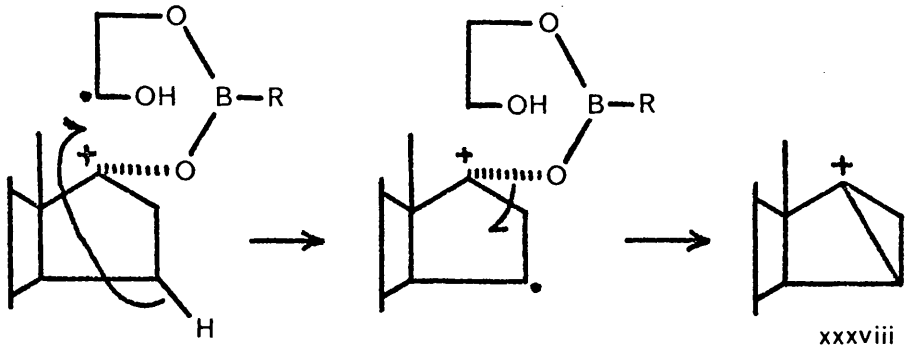
Present evidence suggests that these boronates are six-membered esters involving the 17- and 21-hydroxyl groups.[292] As already noted,[286] many of them give rise to abundant molecular ions.

The mass spectra of the methyl and t-butyl boronates of 17α,20α,21-trihydroxypregn-4-en-3-one (CIX) are shown in Figs. 66,67. It can be seen that fragmentation of the boronate group of the triols is more varied than that of the dihydroxyacetones. There are abundant ions at $[70+R]^+$ (Table 18) and m/e 267, probably as xxxvi and xxxvii, respectively. Prominent ions of m/e 269 are due to complete loss of the 17-substituent with hydrogen transfer from the steroid nucleus. A possible structure is xxxviii.

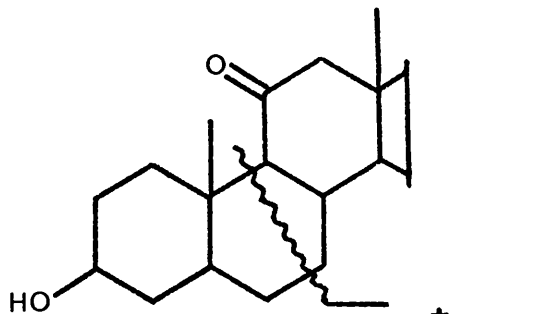
Boronates of the 20β-isomer (CX) yield spectra similar to those



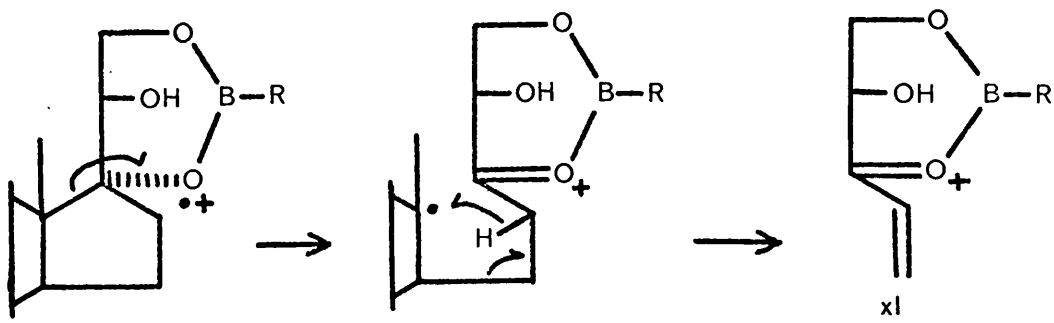
xxxvii



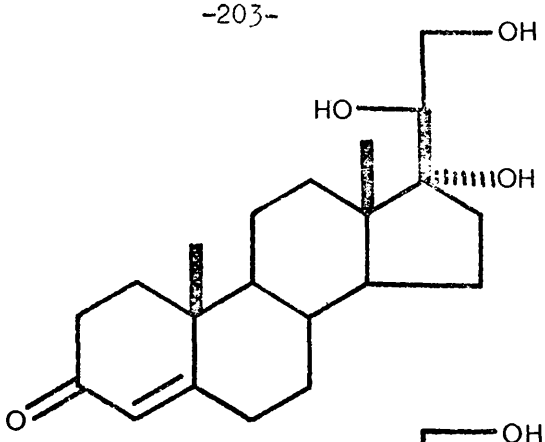
xxxviii



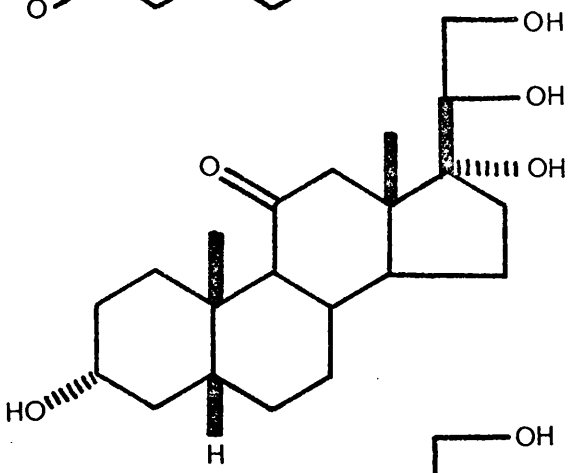
$[M-126]^{+\bullet}$ (xxxix)



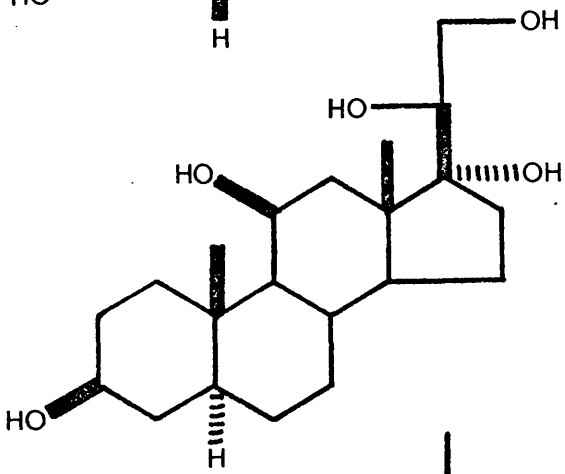
xl



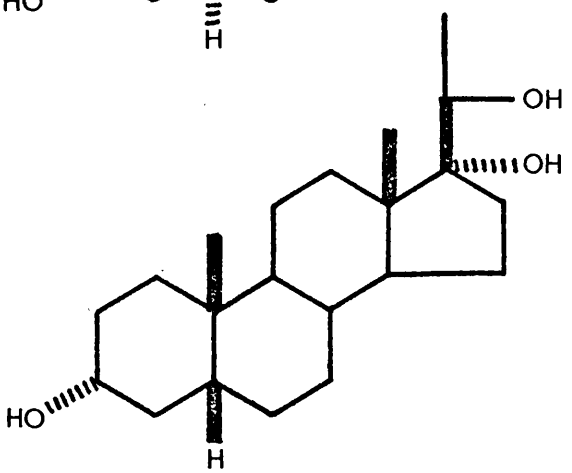
CX



CXI



CXII



CXIII

of CIX, although ions produced by elimination of water are formed in lower relative abundance.

The presence of the 11-keto group in the boronates of cortolone (CXI) appears to stabilise fragment ions arising from eliminations in rings A and D. There are ions containing boron at $[M-113]^+$ (xxxv) (CXIa: m/e 277, 19%; CXIb: m/e 319, 28%; CXIc: m/e 319, 24%) and at $[M-126]^+$ (CXIa: m/e 264, 10%; CXIb: m/e 306, 13%; CXIc: m/e 306, 10%) corresponding to fragmentation of ring B as in xxxix. Further boron-containing ions appear at $[126+R]^+$ (Table 19) and can be tentatively assigned structure xl.

The molecular ions of the boronates of 5α-pregnane-3β,11β,17α, 20β,21-pentol (CXII) are very weak, although there are well-defined ions arising from successive eliminations of water. The base peak (m/e 271) arises from a fragment of type xxxviii by elimination of a molecule of water. Further loss of water from this fragment ion is attested by the presence of a metastable ion (m/e 236.2; calc. for m/e 271→m/e 253: 236.19).

Boronates of 17α,20-diols

The principal fragmentations of several n-butyl boronates of this type have already been described.[286]

The mass spectra of methyl and t-butyl boronates of 5β-pregnane-3α,17α,20α-triol (CXIII) are shown in Figs. 68,69. There are intense ions ions at $[M-18]^+$ in the spectra of all the boronates of CXIII studied. Ring A fragmentation leads to the

Table 19 Relative abundances of boron-containing ions from boronates of CXI

m/e	141	183
Methyl	14[a]	5
t-Butyl	0	16[a]
n-Butyl	0	9[a]

[a] $[126+R]^+$

Table 20 Relative abundances of boron-containing ions from boronates of CXIII

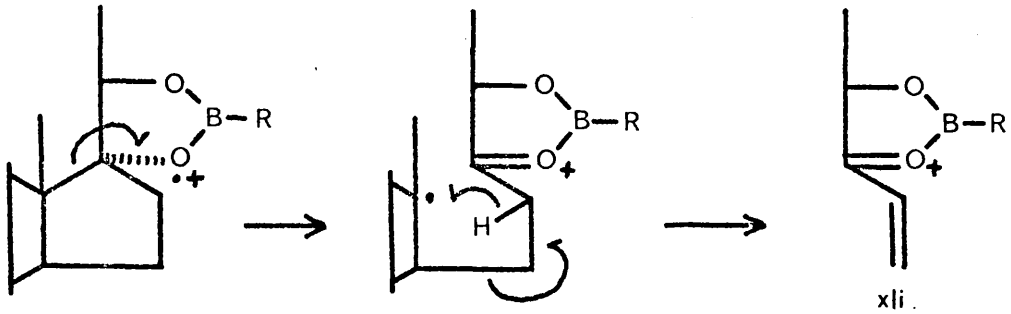
m/e	124	166	186	192	125	167	187	193	111	153	173	179
Methyl	56[a]	9	2	1	47[b]	7	9	0	37[c]	4	14	2
t-Butyl	11	45[a]	3	1	8	55[b]	11	0	14	24[c]	15	2
n-Butyl	13	55[a]	0	0	12	61[b]	9	0	23	28[c]	15	0
Phenyl	3	2	28[a]	0	1	0	30[b]	0	2	0	23[c]	0
Cyclohexyl	7	3	2	28[a]	3	2	5	38[b]	8	0	10	14[c]

[a] $[109+R]^+$ [b] $[110+R]^+$ [c] $[96+R]^+$

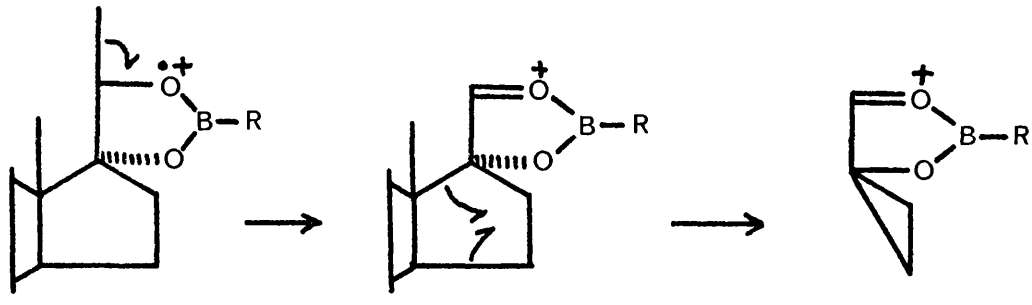
formation of ions at $[M-72]^{\ddagger}$ (xxxiv). Loss of the 17-substituents with hydrogen transfer as for the 17α,20,21-triols (xxxviii) and concomitant elimination of water gives rise to ions at m/e 255 (CXIIIa, 15%; CXIIIb, 21%; CXIIIc, 13%; CXIIId, 19%; CXIIIe, 13%). The presence of boron-containing ions at $[109+R]^{\ddagger}$ and $[110+R]^{+}$ (xli) has already been noted[286] in the spectra of n-butyl and phenyl boronates. These are also present in the spectra of methyl, t-butyl, and cyclohexyl boronates (Table 20). There is evidence (Table 20) for the formation of boron-containing ions (xlii) at $[96+R]^{+}$. These could arise by cleavage at C-13/17 and C-15/16 with hydrogen transfer to the nuclear fragment, but a more plausible mechanism is illustrated.

The spectra of boronates of the 3α,17α,20β-triol (CXIV) are closely similar to those of the 3α,17α,20α-triol (CXV). The slightly increased relative abundance of the $[M-18]^{\ddagger}$ ion observed for the 20β-isomer is an indication of the greater stability of the boronate moiety of that isomer. No molecular ion is observed in the spectrum of the 3-acetyl derivativesof the n-butyl boronate of the 20β-isomer, although there is an intense (69%) ion at $[M-60]^{\ddagger}$ due to loss of acetic acid.

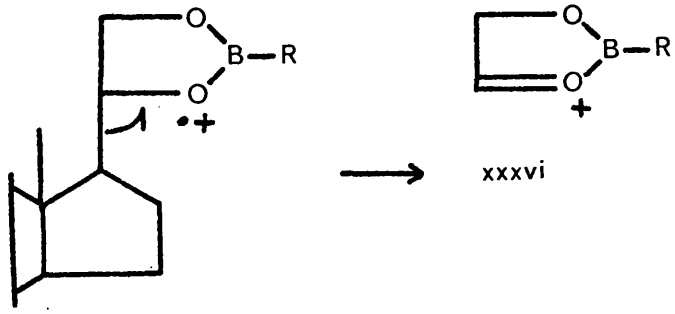
Boronates of 5β-pregnane-3α,11β,17α,20β-tetrol (CXV) give spectra analogous to those of the triol boronates. Additional ions arise from the possibility of elimination of two molecules of water.



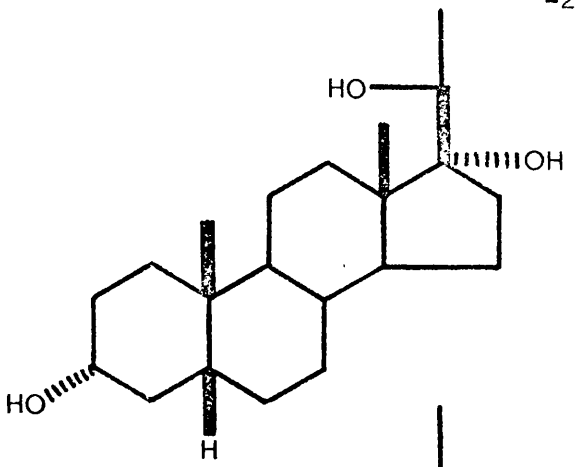
xli



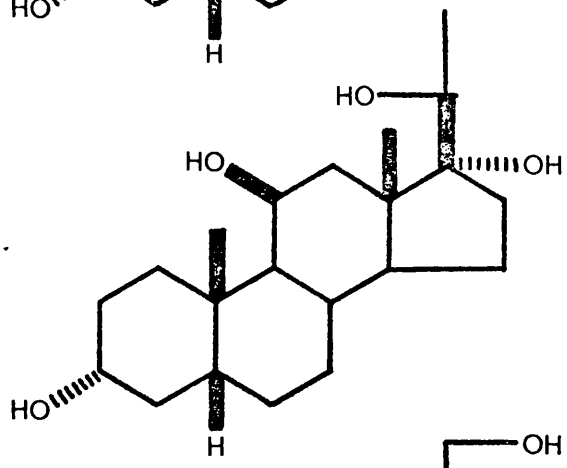
xlii



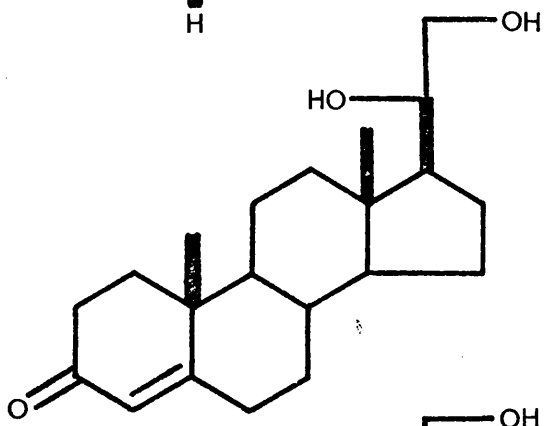
xxxvi



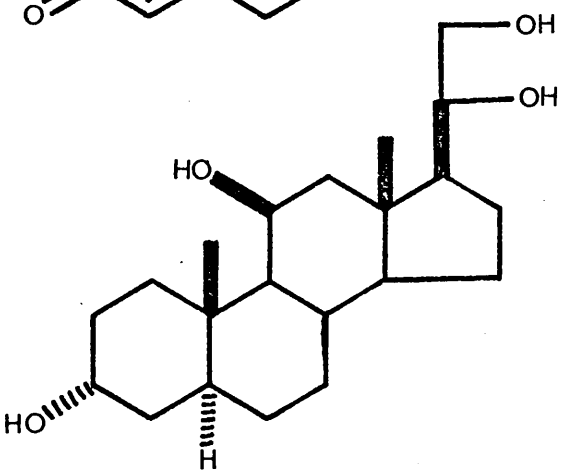
CXIV



CXV



CXVI



CXVII

Boronates of 20,21-diols

Boron-containing ions were not prominent in the spectra of the first compounds of this class that were studied.[286]

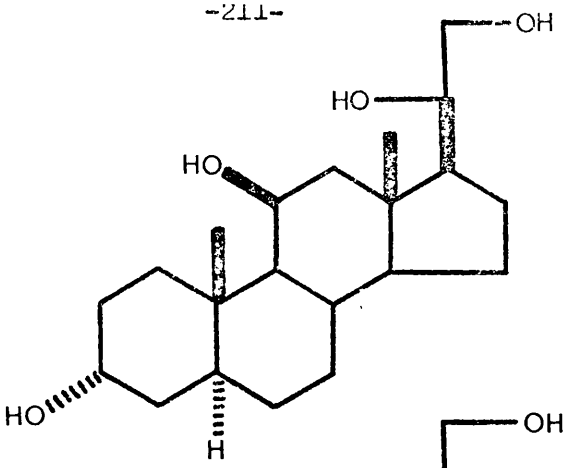
The mass spectra of methyl and t-butyl boronates of 20β,21-dihydroxypregn-4-en-3-one (CXVI) are shown in Figs. 70,71. Many of the fragmentations parallel those of androst-4-en-3-one and steroids of similar structure.[189,194,195,294-297] The base peaks appear at m/e 124 (xxx). Ions of low intensity at $[218+R]^+$ and $[217+R]^{\cdot+}$ (Table 21) probably arise from related fissions of ring B with the respective transfer of one or two hydrogen atoms, and charge retention on the larger fragment.[168] Elimination of ketene from ring A produces fairly abundant ions at $[M-42]^{\cdot+}$. A characteristic fission of the C-17/20 bond results in the formation of abundant ions (xxxvi) at $[70+R]^+$ (Table 21). An additional fragmentation mode is apparent in the mass spectrum of the cyclohexyl boronate. There is an ion at m/e 341 (35%) due to loss of the cyclohexyl radical, but the ion at m/e 342 (15%) appears to be too intense to represent ^{13}C isotope, and probably involves hydrogen transfer to the larger fragment, with loss of cyclohexene. Such a hydrogen transfer has been observed in the spectrum of tricyclohexylboroxine.[290]

The mass spectra of boronates of 5α-pregnane-3α,11β,20α,21-tetrol (CXVII) and 5α-pregnane-3α,11β,20β,21-tetrol (CXVIII) have been examined. In each case, there was no observed fragmentation of the boronate moiety, major fragment ions arising only from eliminations of

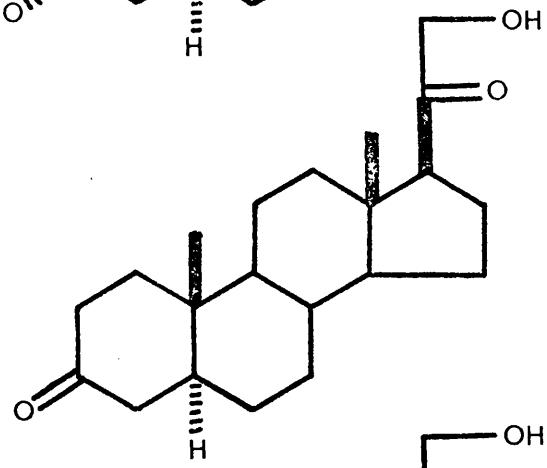
Table 21 Relative abundances of boron-containing ions from boronates of CXVI

m/e	232	274	294	300	233	275	295	301	85	127	147	153
Methyl	6[a]	0	0	1	5[b]	0	0	0	20[c]	2	14	0
t-Butyl	0	2[a]	1	1	0	4[b]	1	0	6	11[c]	11	3
n-Butyl	0	3[a]	0	0	0	3[b]	0	0	2	13[c]	10	3
Phenyl	0	0	3[a]	0	0	0	3[b]	0	0	0	34[c]	0
Cyclohexyl	0	0	1	15[a]	0	0	3	11[b]	0	0	22	19[c]

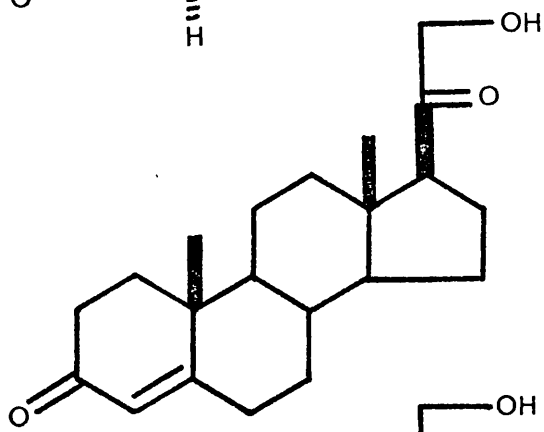
[a] $[217+R]^{+\cdot}$ [b] $[218+R]^{+}$ [c] $[70+R]^{+}$



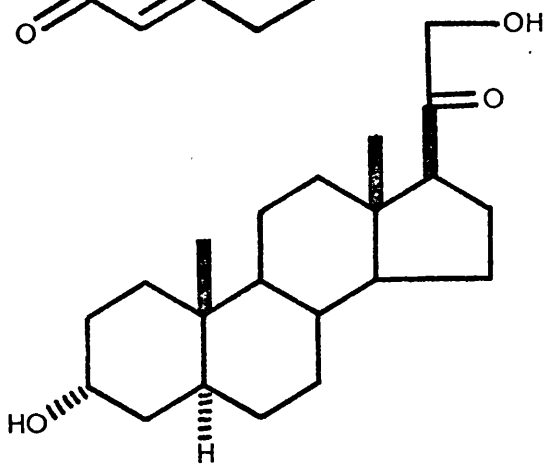
CXVIII



CXIX



CXX



CXXI

water and fragmentation of ring D. There is a marked tendency for more pronounced losses of water from the 20α-isomer.

Boronates of 20,21-ketols

It is now believed that these are formed with a Δ^{17}-20,21-boronate structure.[292]

The mass spectra of methyl and t-butyl derivatives of 21-hydroxy-5α-pregnane-3,20-dione (CXIX) are shown in Figs. 72,73. The spectra are dominated by ions at $[95+R]^{+}$ (Table 15, base peaks) formed by fission of the bonds C-13/17 and C-15/16. The presence of an abundant ion at m/e 110 in the t-butyl boronate spectrum was first noted during a series of measurements in which methyl boronates had been included. It seemed possible that this ion might have been due to methyl boronate formed by transesterification in the column. However, the ion was regularly observed in later experiments from which methyl boronates were excluded. Presumably it arises via rearrangement of the t-butyl group. The ions at $[109+R]^{+}$ are probably formed by fission of the bonds C-13/17 and C-14/15. There is little further fragmentation.

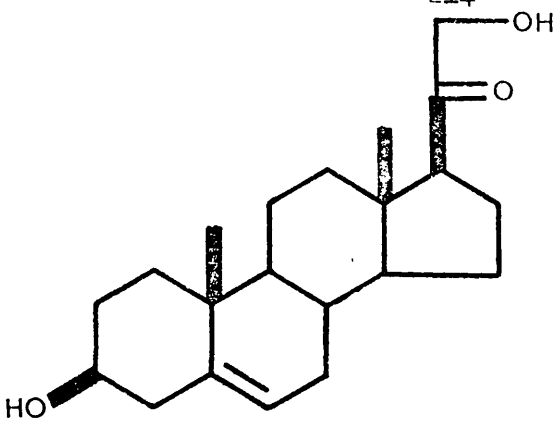
The methyl boronate of 21-hydroxypregn-4-ene-3,20-dione (CXX) gives a similar spectrum, whereas the t-butyl boronate undergoes much more fragmentation, producing an ion due to loss of the t-butyl radical at m/e 339 in high abundance (91%).

Fragmentation of ring A of the boronates of 3α,21-dihydroxy-

5α-pregnan-20-one (CXXI) produce ions of low abundance at $[M-72]^{\ddagger}$ (xxxiv) and $[M-73]^{+}$ which are completely absent from the spectra of boronates of 3β,21-dihydroxypregn5-en-20-one (CXXII).

The various boronates of each class of corticosteroid undergo characteristic fragmentations: the nature of the boron-substituent has little effect on these. Dihydroxyacetone derivatives generally give rise to stable molecular ions, together with intense nuclear fragments formed by elimination of C-16, C-17 and attached groups. Ther is little observed fragmentation of the boronate moiety. 20,21-ketol boronates produce characteristic ions at $[95+R]^{\ddagger}$. Derivatives of 17α,20,21-triols and 20,21-diols also, in most cases, give stable molecular ions and abundant nuclear fragments. The boronate moiety produces boron-containing ions at $[70+R]^{+}$. 17α,20-Diol derivatives undergo extensive fragmentation, producing many abundant ions of low mass. Additional hydroxyl groups undergo elimination, giving rise to ions at $[M-18]^{\ddagger}$, often as the base peaks. The quantitative differences between 20α- and 20β-hydroxy derivatives are insufficient for a priori identification of "unknown" substances.

The extensive data obtained in this investigation have fully substantiated earlier reports[286,292] on the characteristic features of the mass spectra of corticosteroid boronates. In particular, clear - and often intense - molecular ions are invariably observed, except for the single example of a pentol t-butyl boronate (CXIIb): in this case,



CXXII

well-defined ions at $[M-18]^{+\cdot}$ and $[M-36]^{+\cdot}$ are present. In addition to their effect in stabilising the molecular ions, boronate groupings direct further fragmentation, yielding ions characteristic of the parent corticosteroids. In most instances, the fragment produced from the steroid nucleus retains the positive charge. However, ions essentially comprising the boronate moieties are present in all the spectra: the individual m/e values are determined by the types of side-chain (and by the substituent on boron). In the spectra of the 20,21-ketol boronates, such ions form the base peaks.

THE MASS SPECTRA OF SOME 1,3,2-OXAZABOROLIDINES*

The behaviour of many five-membered heterocyclic ring systems upon electron impact is now well documented.[302-310] The mass spectra of several boroxines,[311-313] borazoles,[314] and diazaboretanes[315] have also been reported, but no detailed study of the mass spectra of oxazaborolidines had been published at the outset of this work. This section deals with the mass spectra of a series of alkylphenyl-1,3,2-oxazaborolidines prepared during a recent survey[287] of the use of cyclic boronate esters as derivatives for GLC and GC-MS. Representative spectra are shown in Figs. 74-82. The characteristic fragmentations are an aid to the elucidation of the identity of the substituents in the 1,3,2-oxazaborolidine ring.[289]

Use has been made of a "substituent shift" technique in the interpretation of the relevant ionic decompositions, and the elemental composition of several ions has been confirmed by high resolution mass measurement.** The principal features of the mass spectra are discussed in the following paragraphs.

Ions retaining the oxazaborolidine ring and 2-substituent

In each spectrum, there is a relatively intense molecular ion (xliii). This is a useful feature of these cyclic boronate derivatives, particularly for GC-MS, since it gives their molecular weights directly. The $[M-R]^+$

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* The synthetic work for this and the following section of the thesis was carried out by G.M. Anthony.

** Kindly carried out by Dr. A. McCormick, AWRE, Aldermaston, Berks.

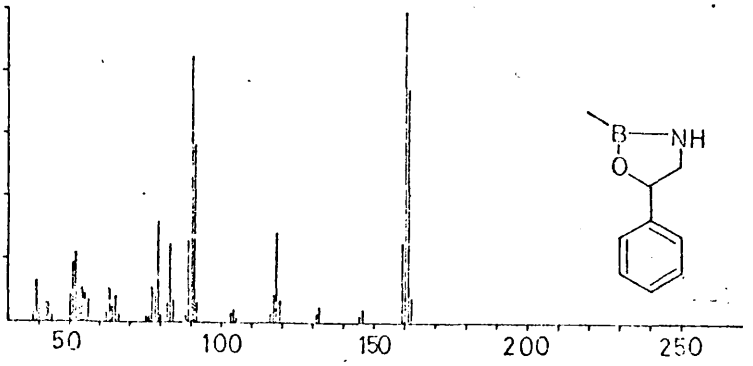


FIG. 74 Mass spectrum of 2-methyl-5-phenyl-1,3,2-oxazaborolidine.

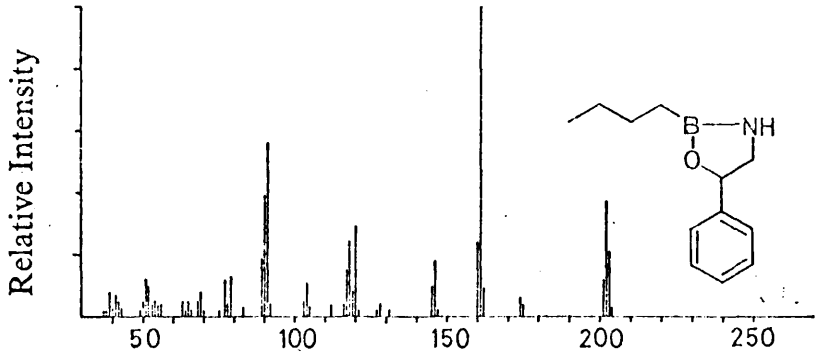


FIG. 75 Mass spectrum of 2-*n*-butyl-5-phenyl-1,3,2-oxazaborolidine.

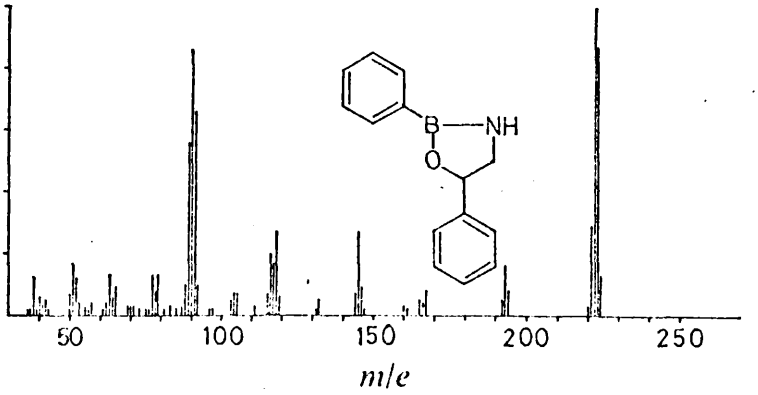


FIG. 76 Mass spectrum of 2,5-diphenyl-1,3,2-oxazaborolidine.

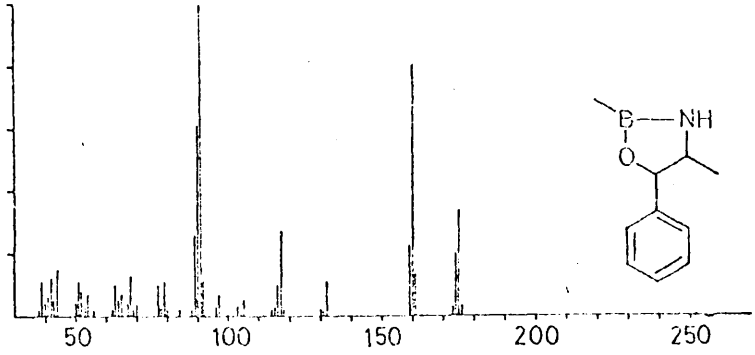


FIG. 77 Mass spectrum of 2,4-dimethyl-5-phenyl-1,3,2-oxazaborolidine.

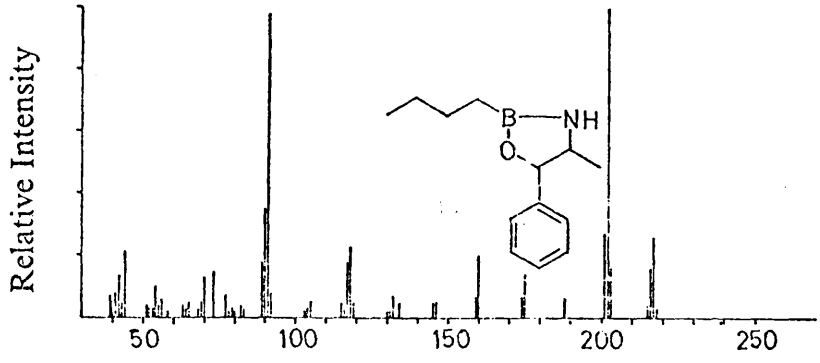


FIG. 78 Mass spectrum of 2-*n*-butyl-4-methyl-5-phenyl-1,3,2-oxazaborolidine.

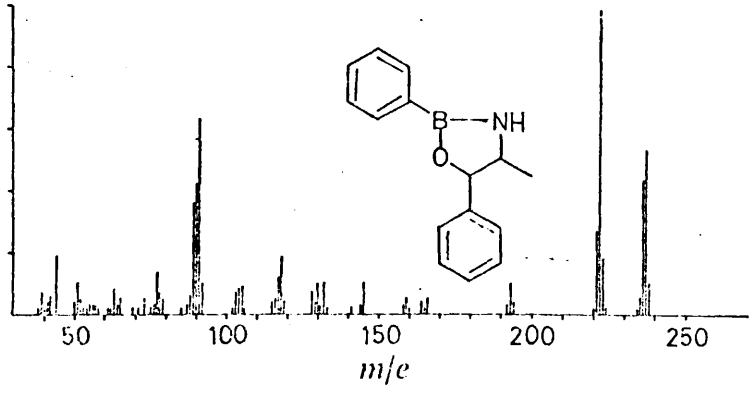


FIG. 79 Mass spectrum of 4-methyl-2,5-diphenyl-1,3,2-oxazaborolidine.

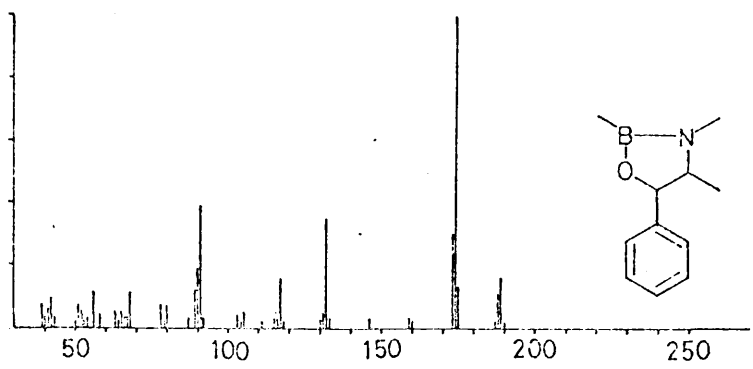


FIG. 80 Mass spectrum of 2,3,4-trimethyl-5-phenyl-1,3,2-oxazaborolidine.

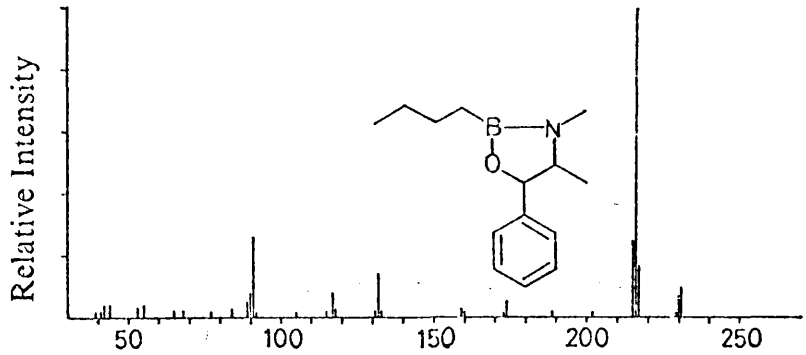


FIG. 81 Mass spectrum of 2-*n*-butyl-3,4-dimethyl-5-phenyl-1,3,2-oxazaborolidine.

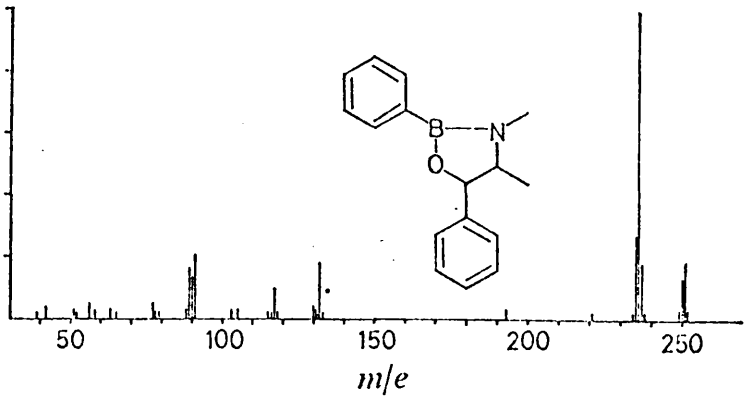
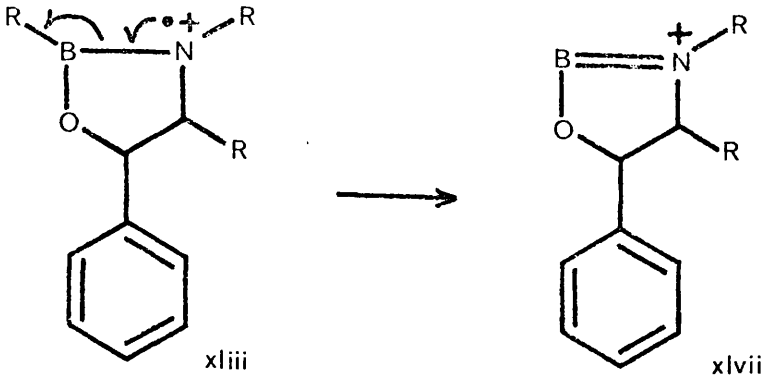
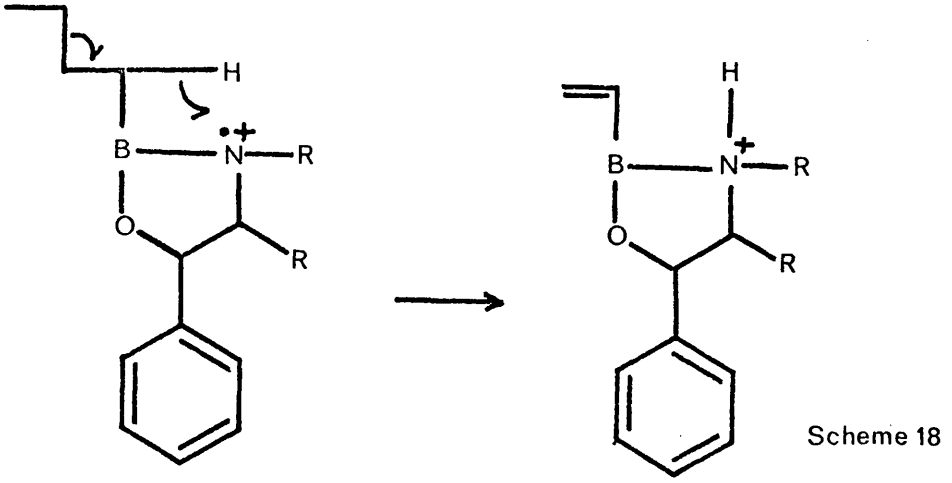


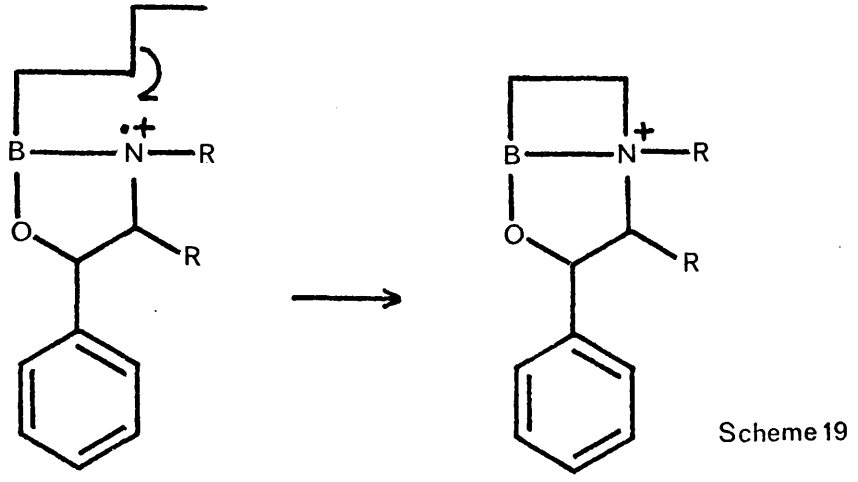
FIG. 82 Mass spectrum of 3,4-dimethyl-2,5-diphenyl-1,3,2-oxazaborolidine.



xliii xlvii



Scheme 18



Scheme 19

ion gives an intense peak, often the base peak, in the spectrum of each of the samples under investigation. Its origin can be inferred from a comparison of the spectra of differently substituted compounds: for example, 2,5-diphenyl-1,3,2-oxazaborolidine (CXXIII, Fig. 76) and 4-methyl-2,5-diphenyl-1,3,2-oxazaborolidine (norephedrine phenylboronate, CXXIV, Fig. 79, see Table 22). Its formation can be represented as in xliv → xlv. The alternative loss of a hydrogen atom gives rise to a moderately intense ion at $[M-1]^+$ (probably as xlvi) even when there is a methyl substituent in the 4-position.

Fragmentation involving loss or degradation of the 2-substituent

It can be seen from Figs. 74-82 and Table 22 that there is a tendency to lose the 2-substituent from the 1,3,2-oxazaborolidine ring (as in xliii → xlvii) although this is less pronounced than the formation of the $[M-R]^+$ ion. There is also evidence for the fragmentation of the 2-butyl substituent.

The peak at m/e 146 in the spectrum (Fig. 74) of 2-methyl-5-phenyl-1,3,2-oxazaborolidine (CXXV) is presumably formed from the molecular ion by loss of the substituent methyl radical. This fragmentation is obscured in the spectra (Figs. 77 and 80) of 2,4-dimethyl-5-phenyl-1,3,2-oxazaborolidine (norephedrine methylboronate, CXXVI) and 2,3,4-trimethyl-5-phenyl-1,3,2-oxazaborolidine (ephedrine methylboronate, CXXVII) by the predominant loss of the 4-methyl substituents.

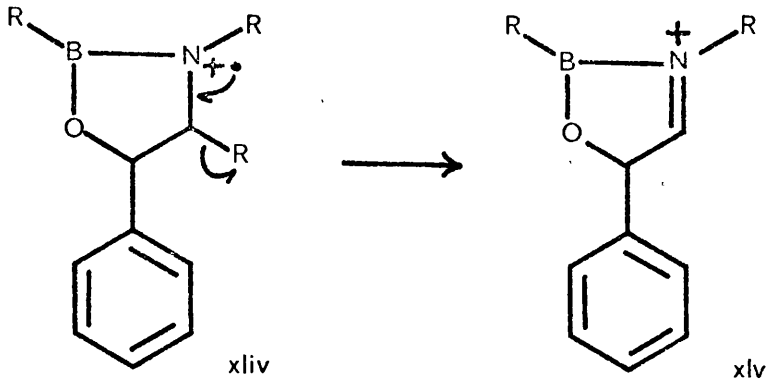
Peaks due to the $[M-57]^+$ ions in the spectra of 2-n-butyl-5-phenyl-1,3,2-oxazaborolidine (CXXVIII, Fig. 75), 2-n-butyl-4-methyl-

Table 22 Substituent shift correlations for the spectra represented in Figs. 74-82.

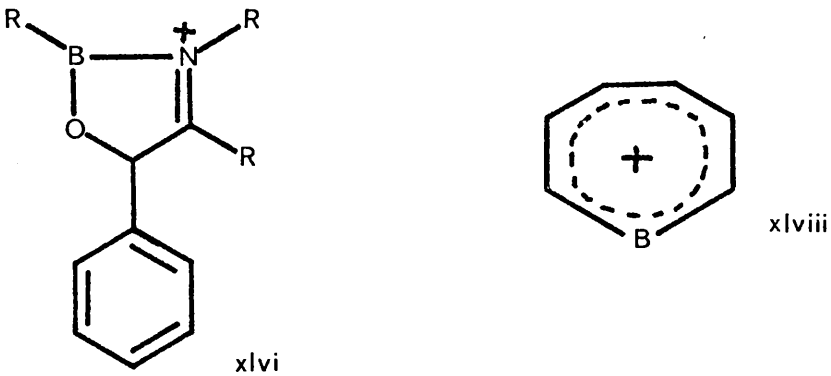
Fig.	Sample	$M^{+\cdot}$	$[M-1]^+$	$[M-R]^+$	$[M-R'']^+$	$[117+R']^+$	$[117+R'']^+$
74	CXXV	161	160	160	146	118	132
75	CXXVIII	203	202	202	146	118	174
76	CXXIII	223	222	222	146	118	194
77	CXXVI	175	174	160	160	118	132
78	CXXIX	217	216	202	160	118	174
79	CXXIV	237	236	222	160	118	194
80	CXXVII	189	188	174	174	132	132
81	CXXX	231	230	216	174	132	174
82	CXXXI	251	250	236	174	132	194

Table 23 Accurate mass measurements

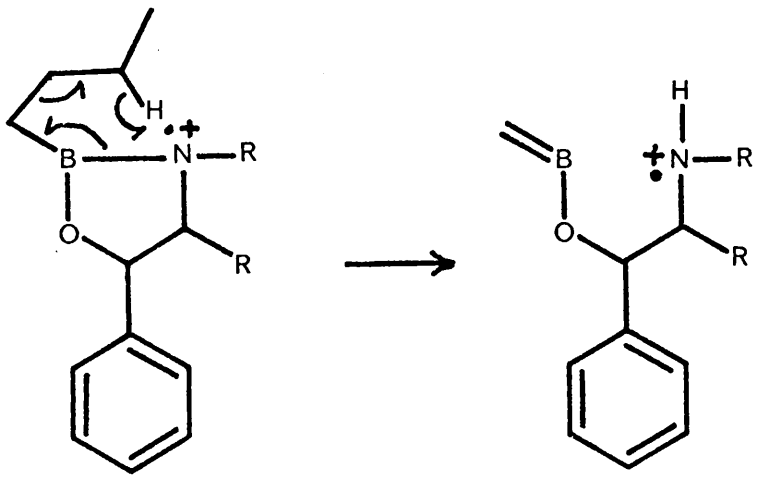
Sample	peak	measured mass	possible formula	calculated mass	intensity ratio
CXXX	174	174.1090	$C_{10}H_{13}BNO^+$	174.1090	singlet
	117	117.0704	$C_9H_9^+$	117.0704	2
		117.0577	$C_8H_7N^{+\cdot}$	117.0578	2.9
		117.0511	$C_7H_6BO^+$	117.0512	1
CXXXI	117	117.0704	$C_9H_9^+$	117.0704	1
		117.0577	$C_8H_7N^{+\cdot}$	117.0578	4
CXXIII	90	90.0471	$C_7H_6^{+\cdot}$	90.0470	singlet
	89	89.0392	$C_7H_5^+$	89.0391	2
		89.0560	$C_6H_6B^+$	89.0563	1



xliv xlv



xlvi xlviii



Scheme 20

5-phenyl-1,3,2-oxazaborolidine (norephedrine n-butylboronate, CXXIX, Fig. 78) and 2-n-butyl-3,4-dimethyl-5-phenyl-1,3,2-oxazaborolidine (ephedrine n-butylboronate, CXXX, Fig. 81) are present at m/e 146, 160, and 174, respectively. In the last instance (m/e 174), accurate mass measurement confirmed the elemental composition expected for structure xlvii (Table 23). The corresponding peaks in the spectra of the 2-phenyl derivatives are less intense, presumably because of the increased stability afforded by the charge delocalisation over an extra aromatic ring.

The 2-n-butyl derivatives apparently fragment to form ions $[M-29]^+$ and $[M-42]^{+\cdot}$. For example, there are peaks at m/e 188 and 175 in the spectrum of CXXIX, but no corresponding peaks in the spectra of CXXVI (at m/e 146 and 133) and CXXIV (at m/e 208 and 195). Two simple routes, both involving 4-membered cyclic intermediates, can be envisaged for the formation of $[M-29]^+$ ions (Schemes 18,19). The $[M-42]^{+\cdot}$ ion, on the other hand, is more likely to be formed via a six membered cyclic intermediate, with the elimination of a neutral propylene molecule (Scheme 20).

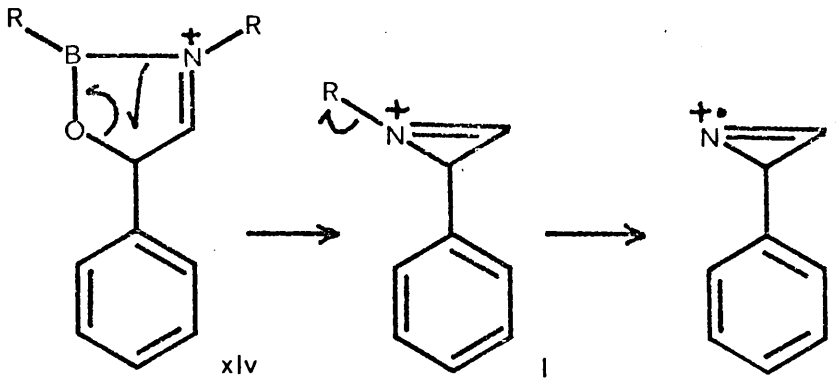
The 2-phenyl substituent does not appear to undergo extensive fragmentation, but it is of interest to note a possible incorporation of the boron atom into a tropylium-like ion (xlviii). High-resolution mass measurement has shown (Table 23) that the peak in the spectrum of CXXIII at m/e 89 is due, in part, to the ion $C_6H_6B^+$. Kotz et al. have

suggested[316] that such ions may have a linear structure.

Fragmentations of the 1,3,2-oxazaborolidine ring

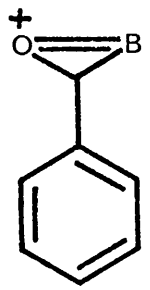
A study of the observed substituent shifts indicates that the ring undergoes extensive fragmentation, directed to some extent by the nature of the substituents. One of the most significant peaks in the low-resolution spectra is that at m/e 117: this was further investigated by high-resolution mass measurement (Table 23). For CXXX it was found that the peak at m/e 117 was atriplet, the components of which were shown unequivocally to have the elemental compositions $C_9H_9^+$, $C_8H_7N^{+\cdot}$, and $C_7H_6BO^+$. The nitrogen-containing species may be of the phenylazirine type (xlix) and is likely to be produced via the ions xlv and l (Scheme 21). Ions corresponding to l were observed, at m/e 118 and 132, for compounds with 3-substituent H and Me, respectively (Table 22). Ion xlix is similar to the thiiren species postulated in the spectrum of thiophene.[317,318] The ion $C_7H_6BO^+$ is probably formed in a similar way, and may be tentatively assigned the phenyloxaboriren structure (li). Ions retaining the 2-substituent and ascribable to the species lii (117+R", Table 22) are prominent in the spectra (Figs. 74 to 82).

Alternatively, acyclic structures for both nitrogen- and boron-containing species may be postulated. Similarly, the $C_9H_9^+$ ion can be satisfactorily rationalised from the 4-methyl-1,3,2-oxazaborolidines, as either a phenylcyclopropanyl or a phenylpropenyl species.

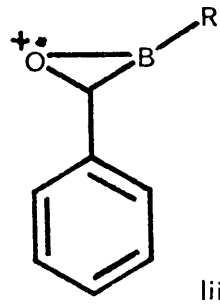


xlv l xlix

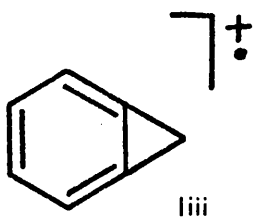
Scheme 21



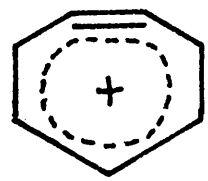
li



lii



liii



liv

Hydrocarbon fragments

The peak at m/e 91 in all of the spectra is ascribed mainly to the tropylium ion formed by incorporation of the adjacent carbon atom into the 5-phenyl substituent, with hydrogen transfer probably from C-4. Similar ions have been observed in the spectra of phenylboronates[319] and a variety of compounds of the type $\overbrace{(CH_2)_2.Y.BPh.X}$ where X and Y are O or S.[320]

The abundant fragments at m/e 89 and 90 have been shown by high-resolution mass measurement to be mainly of the hydrocarbon type (Table 23). Similar peaks are not observed in the spectrum[321] of toluene, so it can be assumed that they do not arise from fragmentation of the tropylium ion, but rather from further breakdown of other fragment ions. Structures liii and liv have been postulated for ions of m/e 90 and 89 observed, for example, in the spectra of benzofuran derivatives[322], coumarin,[323] and furanocoumarins.[324,325]

Metastable peaks

Metastable transitions were observed for all of the fragmentations proposed in this section, either for the 5-phenyl derivatives described here, or for other corresponding 5-aryl derivatives.[291]

THE USE OF n-BUTYL BORONATE DERIVATIVES IN THE CHARACTERISATION OF CATECHOLAMINES AND RELATED β-HYDROXYAMINES BY GC-MS

Difficulties are encountered in the gas chromatographic analysis of biological amines because of their low thermal stability. Satisfactory results are obtainable for certain amines by coating the support with potassium hydroxide[326-329] or by using high percentages of stationary phase.[330] Particular difficulty is, however, attached to the analysis of β-hydroxy-β-phenylethylamines, both with and without nuclear hydroxyl groups. In such cases, it is necessary to employ suitable derivatives.[331] Advocated procedures include Schiff's base formation,[326,329,332] trimethylsilylation of hydroxyl groups with conversion of the amine to a Schiff's base or oxazolidine,[333-335] trimethylsilylation of hydroxyl groups and primary amino groups,[336,337] trimethylsilylation of hydroxyl groups with acetylation on the nitrogen atom,[338] and acetylation of hydroxyl and amino groups.[331] Detection of catecholamines in very small quantities by electron-capture GLC is feasible if they are converted to trifluoroacetates[339] or if hydroxyl groups are trimethylsilylated and N-heptafluorobutyryl derivatives formed.[338] Moffat and Horning have recently reported[340] satisfactory results obtained using N-pentafluorobenzylidene-O-TMS derivatives.

The mass spectra of various phenylethylamines have been studied,[341,342] but they are of limited analytical utility because the molecular ions are, in general, of low abundance. In the present work, various cyclic boronates were evaluated for use as derivatives for GLC and GC-MS.[291]

The n-butylboronates were prepared (by G.M. Anthony) by treatment of the β-hydroxyamine (1 mg), in the form of its free base, hydrochloride, sulphate, or tartrate, with n-butylboronic acid (1-1.5 molar equivalents) in pyridine (1 ml) which had been dried and distilled over sodium hydroxide. The free base could be conveniently prepared from the hydrochloride by exposing the pyridine solution of the hydroxyamine salt to ammonia vapour and separating the precipitated ammonium chloride before derivative formation. For hydroxyamines, such as isoprenaline sulphate, which were not sufficiently soluble in pyridine, a suitable reaction solvent was dimethylformamide which had been dried by azeotropic distillation with benzene and further distilled over anhydrous sodium sulphate.

In most cases, aliquots of the reaction mixture were injected directly onto the GLC column. In the reactions involving octopamine and 4-deoxynoradrenaline, cyclic derivatives appeared to be formed in low yield, and vacuum sublimation at 250°/0.01 mm Hg was used to separate the derivative (in its free-base form) from non-volatile material.

The GLC properties of the boronates of β-hydroxyamines,[287] 1,2- and 1,3-diols[286] and a variety of other compounds[293] have been examined previously in the Chemistry Department of Glasgow Unviersity. In the series of β-hydroxy-β-arylethylamines studied, satisfactory peaks were generally obtained except for derivatives containing free phenolic groups. Retention indices are listed in Table 24.

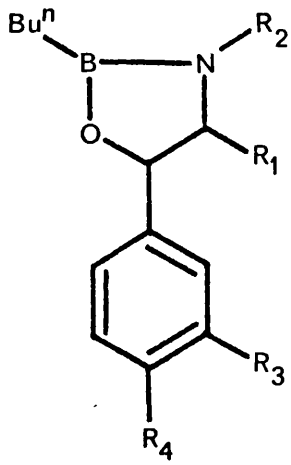
Table 24 Retention indices of n-butylboronates of β-hydroxy-β-arylethylamines on 1% OV-17

compound	temp. (°C)	I
CXXVIII	140	1800
CXXIX	140	1775
CXXXII	140	1775
CXXXIII	140	1780
CXXX	140	1795
CXXXIV	170	2220
CXXXV	170	2200
CXXXVI	170	2185
CXXXVII	170	2170
CXXXVIII	190	2315
CXXXIX	190	2270
CXL	190	2480
CXLI	190	2440
CXLII	190	2450
CXLIII	190	2510

Table 25 The effect of different groups on the boron atom in resolving the diastereoisomers ephedrine and ψ-ephedrine as their boronate derivatives by GLC

	temp. (°C)	retention index (I) ephedrine	ψ-ephedrine	I
Methyl	90	1515	1510	5
n-Butyl	140	1795	1780	15
t-Butyl	130	1680	1670	10
Cyclohexyl	150	2080	2065	15
Phenyl	170	2260	2240	20

	R_1	R_2	R_3	R_4	
β-Hydroxyphenylethylamine	H	H	H	H	CXXVIII
Norpseudoephedrine	Me	H	H	H	CXXIX
Phenylpropanolamine	Me	H	H	H	CXXXII
Pseudoephedrine	Me	Me	H	H	CXXXIII
Ephedrine	Me	Me	H	H	CXXX
Octopamine	H	H	H	OH	CXXXIV
4-Deoxynoradrenaline	H	H	OH	H	CXXXV
Synephrine	H	Me	H	OH	CXXXVI
Phenylephrine	H	Me	OH	H	CXXXVII
Normetanephrine	H	H	OH	OH	CXXXVIII
Metanephrine	H	Me	OMe	OH	CXXXIX
Noradrenaline	H	H	$Bu^n.BO_2$		CXL
Adrenaline	H	Me	"		CXLI
Isoprenaline	H	Pr^i	"		CXLIII
3,4-Dihydroxynorephedrine	Me	H	"		CXLII



Previously reported methods for distinguishing between diastereoisomers of (-)-ephedrine (1R,2S configuration) and (+)-ψ-ephedrine (1S,2S configuration) have been based on chemical conversion of the isomers, by reaction with acetone, to the corresponding oxazolidines. Although these two hydroxyamines, as their boronates, cannot be distinguished by mass spectrometry, we have obtained separation of the n-butylboronates by GLC with a moderately polar column. The difference in retention behaviour was enhanced by using boronates with substituents bulkier than n-Bu on the boron atom. This is illustrated in table 25.

The mass spectra of a series of 1,3,2-oxazaborolidines have been discussed in the previous section. Although the relative intensities of some fragment ions are influenced by the nature of the substituent on the boron atom, the fragmentations of the methyl-, n-butyl-, cyclohexyl-, and phenylboronates studied are, in general, rather similar. Proposed fragmentations characteristic of n-butyl boronates are shown in Fig 83, and relative abundances of ions are given in Table 26. It can be seen that molecular ions are relatively abundant and, therefore, molecular weights can be determined with ease. The masses of the substituents at C-4 can be inferred from the m/e value of fragment ions of type lvii. The $^{10}B/^{11}B$ isotope ratios for these ions indicate the numbers of boronate groups incorporated in the molecule and hence the number of suitable receptor moieties in the parent molecules. The formation of pyroboronates would, of course,

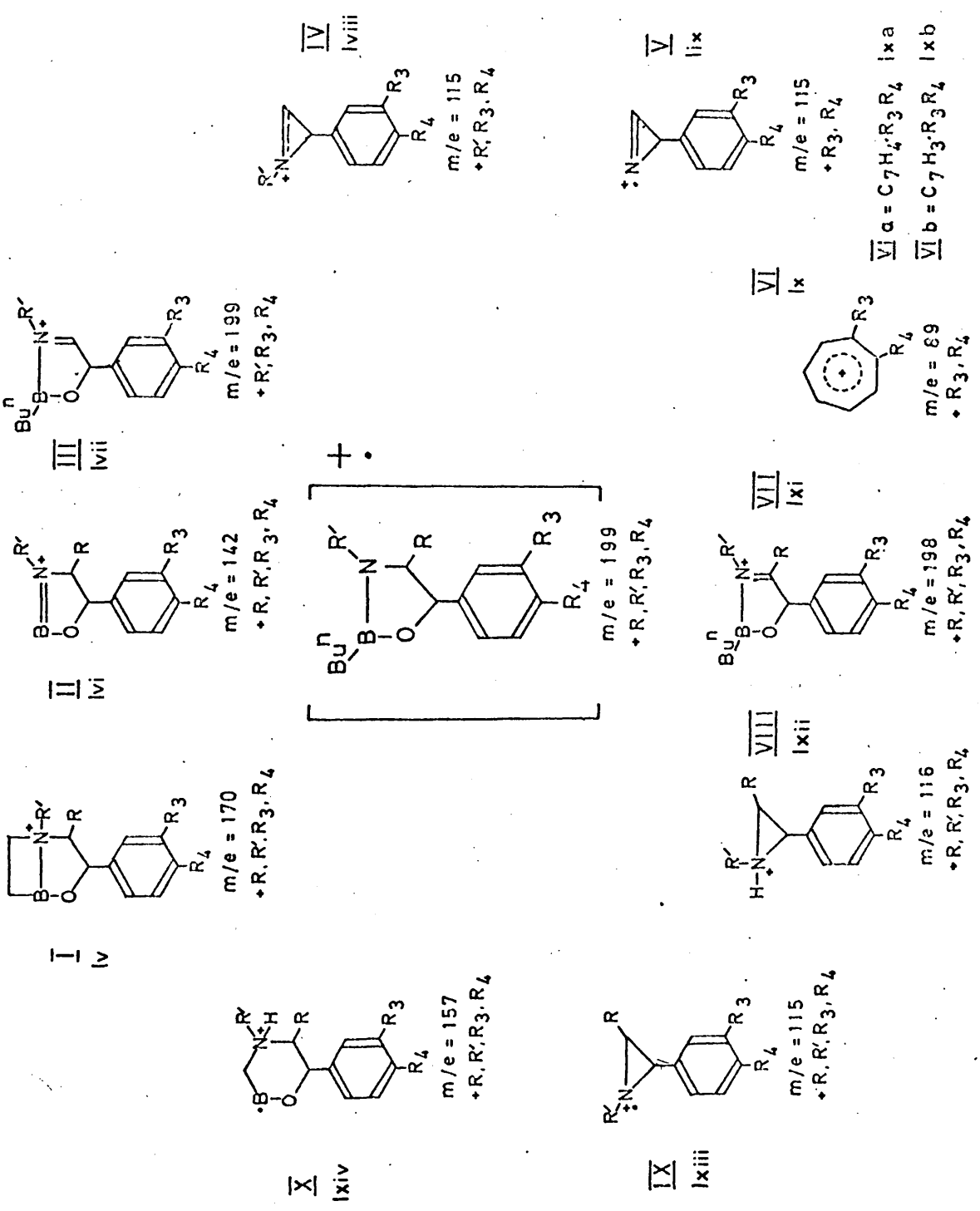

I lv
m/e = 170
+R, R', R3, R4
II lvi
m/e = 142
+R, R', R3, R4
III lvii
m/e = 199
+R', R3, R4
IV lviii
m/e = 115
+R', R3, R4
V lix
m/e = 115
+R3, R4
VI lx
m/e = 89
+R3, R4
VI a = C7H4R3R4 lxa
VI b = C7H3R3R4 lxb
VII lxi
m/e = 198
+R, R', R3, R4
VIII lxii
m/e = 116
+R, R', R3, R4
IX lxiii
m/e = 115
+R, R', R3, R4
X lxiv
m/e = 157
+R, R', R3, R4

Fig. 83

Table 26 Mass spectral breakdown of n-butylboronates of β-hydroxy-β-arylethylamines

compound	$M^{+\cdot}$	m/e of major fragment ions[a]									
CXXVIII	203 (21%)	161	91	90	202	120	118	89	146	117	104
CXXIX	217 (33%)	202	91	90	160	118	216	117	175	89	188
CXXXII	217 (26%)	202	91	90	118	160	117	89	216	175	132
CXXXIII	231 (9%)	216	91	132	117	90	89	230	118	105	174
CXXX	231 (10%)	216	91	132	117	90	230	174	89	159	118
CXXXIV	219 (100%)	107	218	134	177	133	105	162	136	106	135
CXXXV	219 (100%)	107	218	134	177	133	105	162	136	106	135
CXXXVI	233 (74%)	191	232	107	150	148	106	133	120	105	176
CXXVII	233 (84%)	191	232	107	149	148	120	133	150	134	105
CXXVIII	249 (100%)	137	248	219	218	163	232	135	166	136	150
CXXIX	263 (100%)	262	137	180	179	221	163	164	246	146	136
CXL	301 (84%)	300	189	217	216	218	215	188	244	259	272
CXLI	315 (71%)	273	314	231	232	189	230	188	258	202	215
CXLII	315 (46%)	300	189	314	188	216	215	230	273	258	231

[a] in order of relative abundance.

complicate matters but these can be readily detected by their gas chromatographic behaviour and mass spectra. The mass of the N-substituent is indicated by the difference in m/e of ions lviii and lix, although it should be noted (see below) that the N-isopropyl derivative gives rise to a special fragmentation.

In the compounds studied, the substituents on the benzene ring are retained in fragments of type lix, where the hydroxyamine side-chains are reduced to a common moiety (C_2H_2N), and of types lv, lva, and lvb, which are hydrocarbon fragments. These relatively prominent ions readily indicate the combined molecular weights of the substituents on the benzene ring (cf. Reisch et al.[342]). Certain other fragments arise from the breakdown of hydroxyl and methoxyl substituents on the benzene ring. Thus synephrine gives an ion at m/e 216 due to loss of $OH^{\bullet}$. Metanephrine gives a similar ion at m/e 246 and also one at m/e 232 due to loss of $OMe^{\bullet}$.

As noted above, the spectra of n-butylboronates contain, sometimes as major ions, fragments dependent on the presence of the n-butyl substituent. Thus, the ion of type lxiv is the base peak in the spectra of β-hydroxyphenylethlamine n-butylboronate, synephrine n-butylboronate, and adrenaline bis-n-butylboronate. When this fragment is predominant, the two daughter ions lxii and lxiii can also be observed. Fragment ion lv appears to arise by loss of an ethyl radical frpm the butyl side-chain.

Representative results are depicted in Fig. 84, in which the mass spectra of 3,4-dihydroxynorephedrine bis-n-butylboronate and

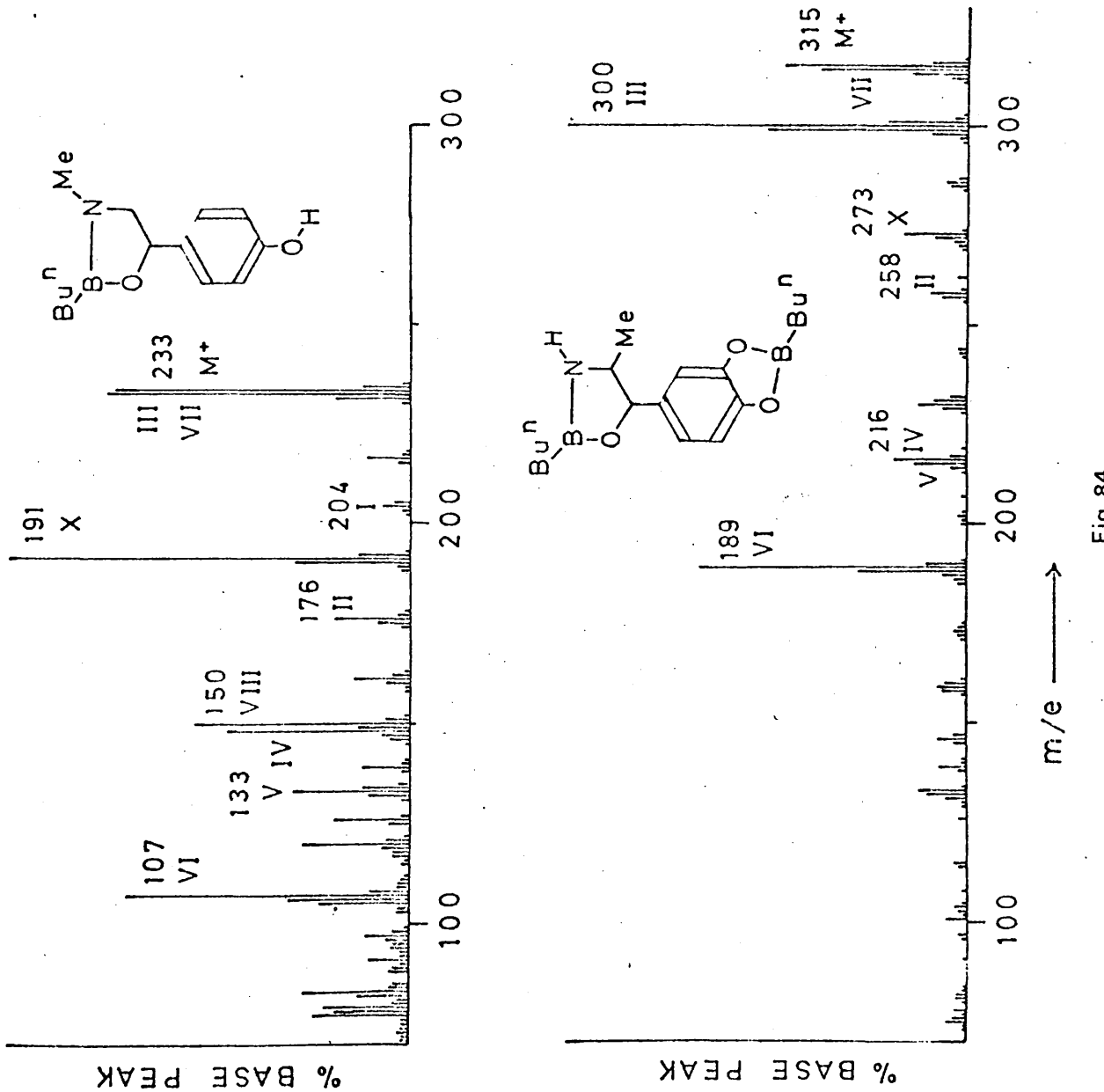

% BASE PEAK
100
200
300
m/e
315
M+
VII
300
III
273
X
258
II
216
IV
V
189
VI
233
M+
VII
III
204
I
191
X
176
II
150
VIII
133
V
IV
107
VI
Me
H
Bu^n
B
O
N

Fig. 84

synephrine n-butylboronate are given and the fragment types indicated.

Isoprenaline n-butylboronate gave only two major fragments. The first (m/e 328) is presumably due to loss of Me$^{\bullet}$ from the isopropyl group on the nitrogen atom. The other predominant peak (m/e 244) is most likely due to further loss of n-BuBO (Fig. 85). This transition is verified by a metastable peak at m/e 181.8.

Within the group of compounds studied, substituents in the benzene ring appear to have little effect on fragmentation, which is accordingly insensitive to positional isomerism in the ring. Consequently, n-butylboronates of octopamine and 4-deoxynoradrenaline, which have a free phenolic group at the para- and meta-position respectively, cannot be effectively distinguished by their mass spectra. Their retention times are, however, different (Table 24).

It may be concluded that qualitative analysis of catecholamines and related β-hydroxyamines after reaction with n-butylboronic acid is possible by the combined GC-MS technique. The boronic acid reacts under mild conditions both with the β-hydroxyamine group to form a 1,3,2-oxazaborolidine ring and with the catechol grouping to form a 1,3,2-dioxaborole ring. Mass spectrometry gives the molecular weight, indicates the mass of substituents at positions 2 and 4 of the oxazaborolidine ring, and gives the combined molecular weights of substituents on the benzene ring. Diastereoisomers on the oxazaborolidine ring and positional isomers on the benzene ring can be distinguished by GLC by use of a moderately polar stationary phase.

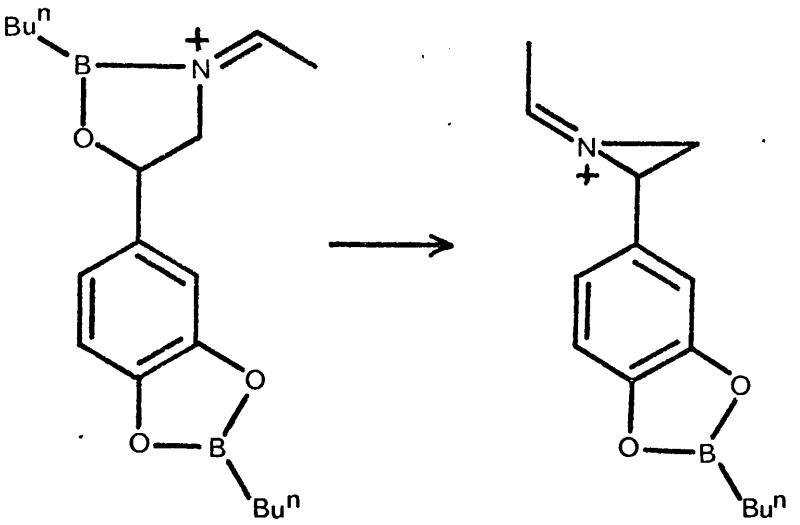


Fig. 85

The reaction of n-butylboronic acid with β-hydroxyamines as described above is not complete, but occurs without a catalyst. The selectivity of the reagent affords a clear distinction by GLC between catecholamines and their methylated analogues (eg. adrenaline and metanephrine), and between compounds with and without a β-hydroxyamine grouping.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF SOME BOROXINES*

The mass spectra of boroxine,[343] fluoroboroxine,[343] difluoroboroxine,[343,344] and trimethylboroxine[311] have been reported. Gas chromatographic and mass spectrometric data are described below for triphenylboroxine (CXLIV), tricyclohexylboroxine (CXLV), tri-t-butylboroxine (CXLVI), and tri-n-butylboroxine (CXLVII).[290] These compounds are observed as by-products when an excess of the boronic acid is used in the preparation of cyclic boronates of such compounds as diols[285] and hydroxyamines.[287,289] The value of such compounds as derivatives for the characterisation of steroidal diols and related compounds by gas chromatography and GC-MS[285-289] has been indicated in earlier sections (pp. 171-239).

The substituted boroxines were produced by thermal dehydration and trimerisation of the corresponding boronic acids.** This was conveniently carried out in a stream of nitrogen or helium in the "flash heater" of a gas chromatograph. The products were studied directly by GLC or GC-MS. Commercial triphenylboroxine gave GLC and MS behaviour identical to that of the dehydration product of

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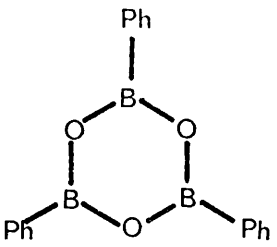
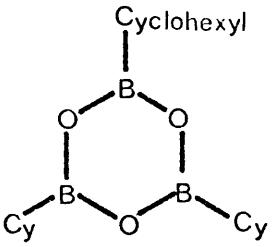
* Preliminary gas chromatography was carried out by Dr. D.J. Harvey.

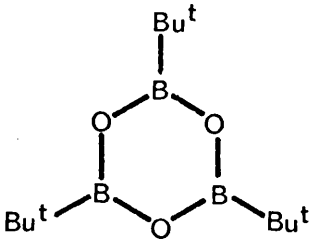
** t-Butylboronic acid was prepared by a variation of the method of Snyder *et al.*[345] in which a fractionating column was used (*cf.* McCusker *et al.*[346]) to reduce losses of product during evaporation of the ether extract. n-Butyl- and cyclohexylboronic acid were obtained from Alfa Inorganic Inc., Beverly, Mass., and phenylboronic acid from Aldrich Chemical Co. Inc., Milwaukee, Wis. Triphenylboroxine was obtained from K & K Laboratories, Inc., Plainview, N.Y.



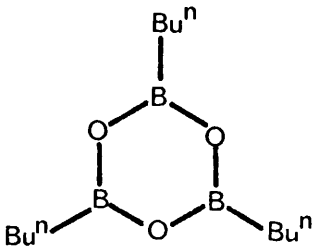
CXLIV



CXLV



CXLVI



CXLVII

phenylboronic acid. GLC data are shown in Table 27, and mass spectral line diagrams in Fig. 86.

Fragmentations of the boroxine ring

A number of mass spectral fragmentations are common to all the substituted boroxines studied.

The intensity of the molecular ion in each case mainly reflects the stability (towards fragmentation) of the substituent on the boron atom: triphenylboroxine gives the molecular ions as the base peak, whereas tri-t-butylboroxine gives the molecular ion of only 0.4% of the intensity of the base peak. In the latter case, the base peak arises from a fragmentation directed by a t-butyl substituent. It could be considered that the stability of the molecular ion of triphenyl-boroxine might reflect some degree of conjugation between the phenyl and boroxine rings. Evidence from calculations of electronic structures and from spectroscopic measurements,[347] however, indicates that phenyl substituents have little effect on electron distribution in unionised boroxine.

There is direct evidence for the loss of one substituent in each case, with the formation of an ion as illustrated in scheme 22. This ion may undergo an electron rearrangement analogous to a "retro-Diels-Alder" fragmentation to give an acyclic ion. Similar fragmentations are observed for triflouroboroxine[343,344] and trimethylboroxine.[311]

Skeletal rearrangements of the molecular ion, with the possible

Table 27 Gas chromatographic behaviour of boroxines.

	OV-1		OV-17	
	I	Temp.	I	Temp.
Triphenylboroxine	2410	180	2755	200
Tricyclohexylboroxine	2175	150	2295	150
Tri-t-butylboroxine	1140	50	1075	50
Tri-n-butylboroxine	1460	100	1485	85

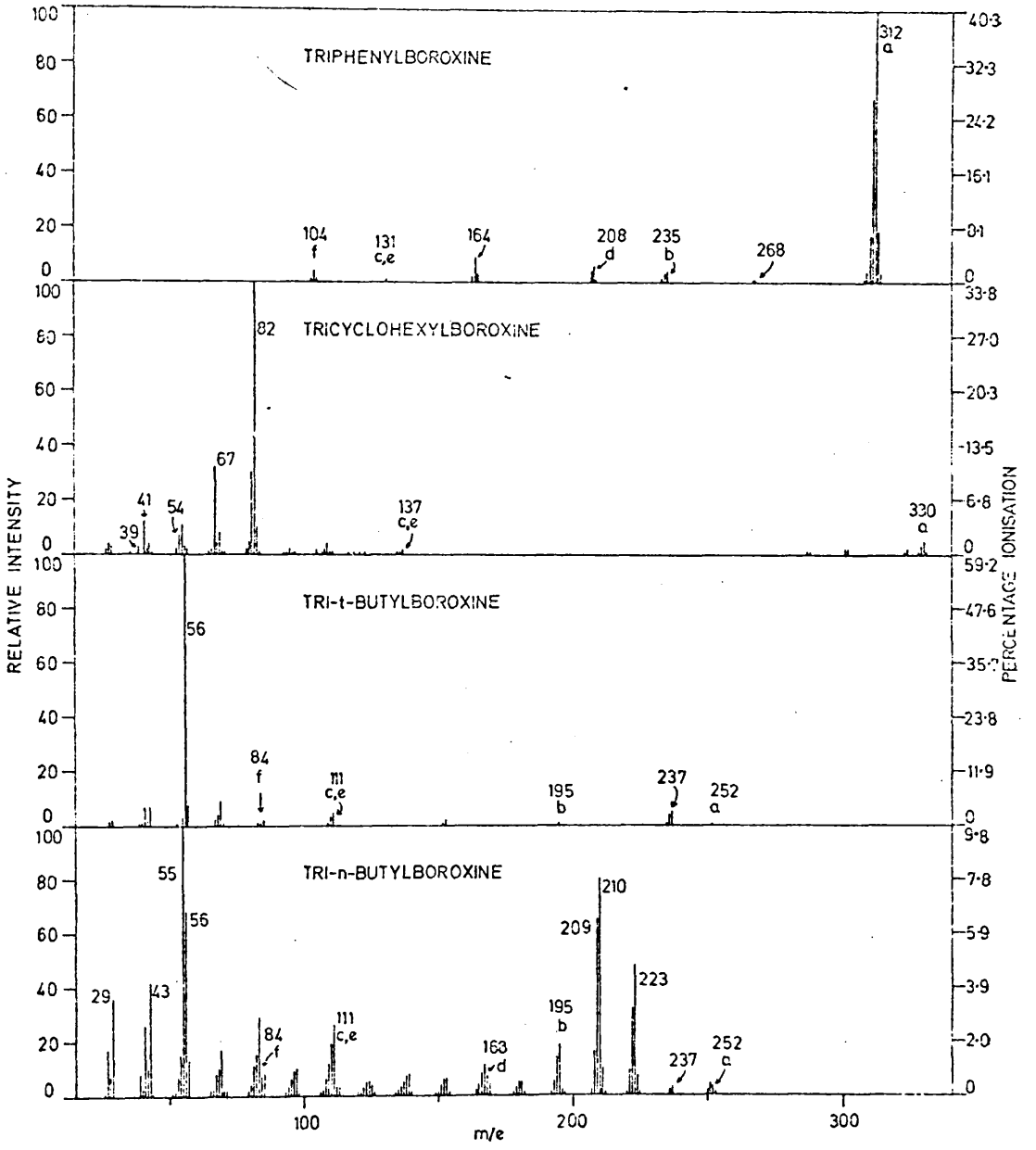
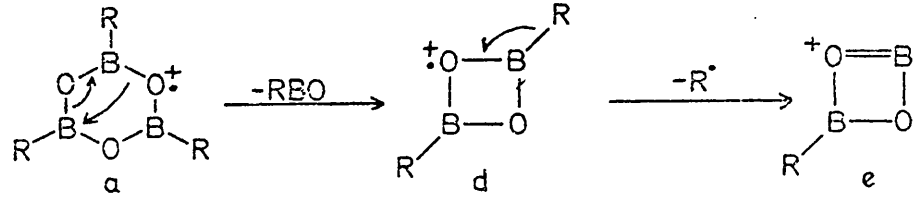
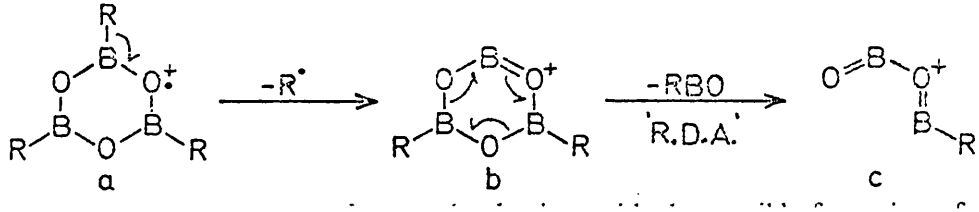

TRIPHENYLBOROXINE
312 a
104 f
131 c,e
164
208 d
235 b
268
TRICYCLOHEXYLBOROXINE
82
67
41
54
39
137 c,e
330 a
TRI-t-BUTYLBOROXINE
56
84 f
111 c,e
195 b
237
252 a
TRI-n-BUTYLBOROXINE
55
56
29
43
84 f
111 c,e
163 d
195 b
209
210
223
237
252 a
RELATIVE INTENSITY
PERCENTAGE IONISATION
m/e
100
200
300

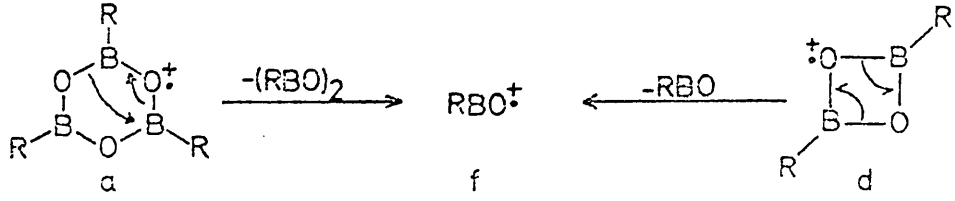
FIG. 86



Scheme 22



Scheme 23



Scheme 24

formation of a radical-ion containing a four-membered ring ia also appareht. This is analogous to the elimination of the RBO group from substituted oxazaborolidines.[287] A further substituent may be lost, as shown in Scheme 23.

Radical ions $RBO^{+\cdot}$ may be formed by two routes, dirctly from the molecular ion or via the dimeric radical ion(Scheme 24). Doubly-charged dimeric radical-ions could also give rise to peaks of similar m/e. Their formation is unlikely, however, in view of the relatively low pressure in the ion source; moreover, there was no indication of the presence of doubly-charged ions of odd mass.

The major ion from trifluoroboroxine corresponds to a loss of BO_2 from the molecular ion. This was postulated as arising from a ring opening and migration of a fluorine atom.[344] There is no evidence for any analogous fragmentations in the spectra of the boroxines described here, although ions are produced which could be attributed to losses of BO_2 or HBO_2 from certain fragment ions.

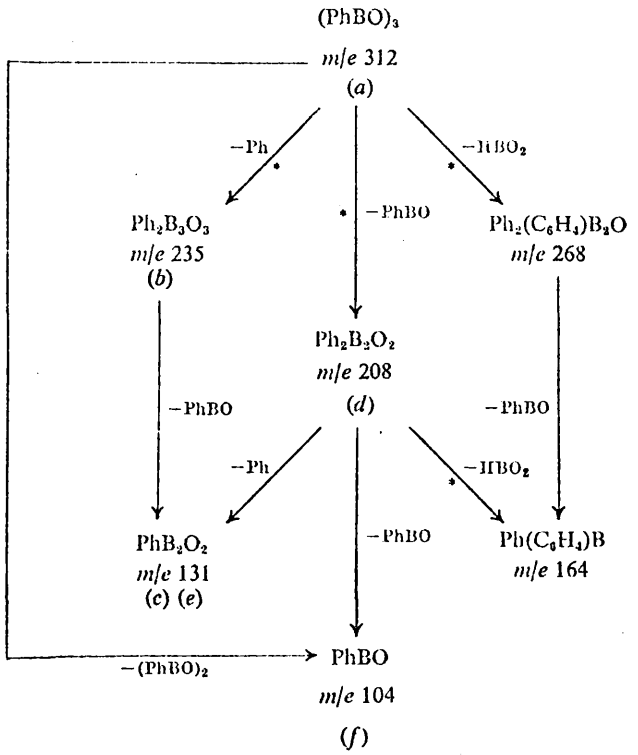
Other fragmentations

In contrast to the spectrum of triphenylboroxine, the typical ions arising from the fragmentation of the boroxine ring of tricyclohexyl-boroxine are present in low abundance. The base peak apparently results from the scission of a boron-carbon bond accompanied by hydrogen transfer - probably from the cyclohexyl ring to the boron atom - with the formation of a cyclohexenyl radical-ion (m/e 82). This is further confirmed by the striking similarity of the low mass portion of the mass

spectrum to that of cyclohexene itself,[348] with characteristic ions at m/e 67, 54, 41, and 39. Similar fragmentations are observed for tri-t-butylboroxine and tri-n-butylboroxine, giving intense peaks at m/e56. The base peak of the latter compound, at m/e 55, apparently ensues from a similar fission accompanied by elimination or transfer of two atoms of hydrogen. Isotope ratio measurements on ions of m/e 54 and 55 in this spectrum confirm the absnce of boron in the ion giving rise to the base peak.

Other ion characteristic of the substituents are observed, such as $[M-15]^+$ from the tributylboroxines and $[M-29]^+$, $[M-42]^+$, and $[M-43]^+$ from tri-n-butylboroxine. Scheme 25 summarises the principal fragmentations of triphenylboroxine.

Since completion and publication of the work described in this section, a further paper[349] containing details of the mass spectrum of triphenylboroxine has appeared. The results therein are similar to those shown in Scheme 22, but are given without mechanistic details. This paper also included discussion of the mass spectrum of triferrocenylboroxine.


(PhBO)3
m/e 312
(a)
−Ph
−HBO2
−PhBO
Ph2B3O3
m/e 235
(b)
Ph2(C6H4)B2O
m/e 268
Ph2B2O2
m/e 208
(d)
−PhBO
−PhBO
−Ph
−HBO2
−PhBO
PhB2O2
m/e 131
(c) (e)
Ph(C6H4)B
m/e 164
−(PhBO)2
PhBO
m/e 104
(f)

SCHEME 25

IV

ALIPHATIC COMPOUNDS

THE MASS SPECTRA OF O-METHYLOXIMES OF SOME ALIPHATIC ALDEHYDES AND KETONES.*

O-Methyloxime derivatives have been used (see below) as an aid to the identification, by GC-MS, of aliphatic aldehydes of cuticular leaf waxes[350] and the aldehydes and dialdehydes produced by cleavage of ozonides formed from aliphatic dienes extracted from the green alga Botryococcus braunii.[351] The realisation of the utility of these derivatives in the gas chromatographic and mass spectrometric characterisation of such compounds, and of steroid ketones,[111,120,124,352-353] terpenoid ketones,[354] and prostaglandins[355-356] prompted a closer examination of the mass spectra of O-methyloximes.

The mass spectral fragmentations of aliphatic ketones are fairly well understood,[357-358] whereas those of aliphatic aldehydes[359] appear to be rather more complex.[360-363] The mass spectra of several unsubstituted oximes have been discussed.[364-365] An attempt is now made to rationalise the mass spectral fragmentations of the O-methyloxime derivatives by comparison with these reports.

---

* Preparative work and preliminary gas chromatography was carried out by Dr. B.A. Knights (Department of Botany).

Line diagrams representing low-resolution mass spectra of O-methyloximes of various aldehydes and ketones (CXLVIII-CLXIII) are shown in Figures 87 to 101. A molecular ion is present in each case, although it is of lower abundance for the samples of higher molecular weight.

Ions arising from simple cleavage. Ions, the formation of which may be formally ascribed to simple cleavage with charge retention either on the nitrogen-containing fragment or on the hydrocarbon fragment, are listed in Table 28. α-Cleavage leads to the production of abundant ions only from molecular ions of relatively low mass, particularly from the branched-chain molecules. For example, α-cleavage of CLIII gives rise to ions of m/e 43 (100%) and 100 (32%), whereas α-cleavage of CLXIII produces no hydrocarbon fragment, and the ion (m/e 72, 1%) corresponding in mass to the nitrogen-containing species is of very low abundance. Similarly, β-cleavage produces abundant ions only from the branched-chain samples of low molecular weight. γ-Cleavage has been observed to account for ions of relatively high abundance in the mass spectra of aldoximes and ketoximes.[359] This process appears to be paralleled in the fragmentation of O-methyloximes, particularly for the production of nitrogen-containing ions. The mechanism of such fragmentations has been discussed[364] and, although no definite conclusions were reached, it seemed likely that a cyclic fragment ion was produced. The equivalent mechanism for γ-cleavage of the O-methyloximes is lxv→lxvi/lxvii (Scheme 26). An alternative mechanism involving reciprocal hydrogen transfer has been suggested[365] and substantiated by deuterium labelling for di-n-hexyl and di-n-heptyl ketoximes. However, it does not, for example, account for the $[M-15]^+$ ion from CXLVIII. Ions are produced, the formation of which may be formally ascribed to cleavages more remote from the O-methyloxime moiety, although there is no direct evidence for their formation from the molecuar ion.

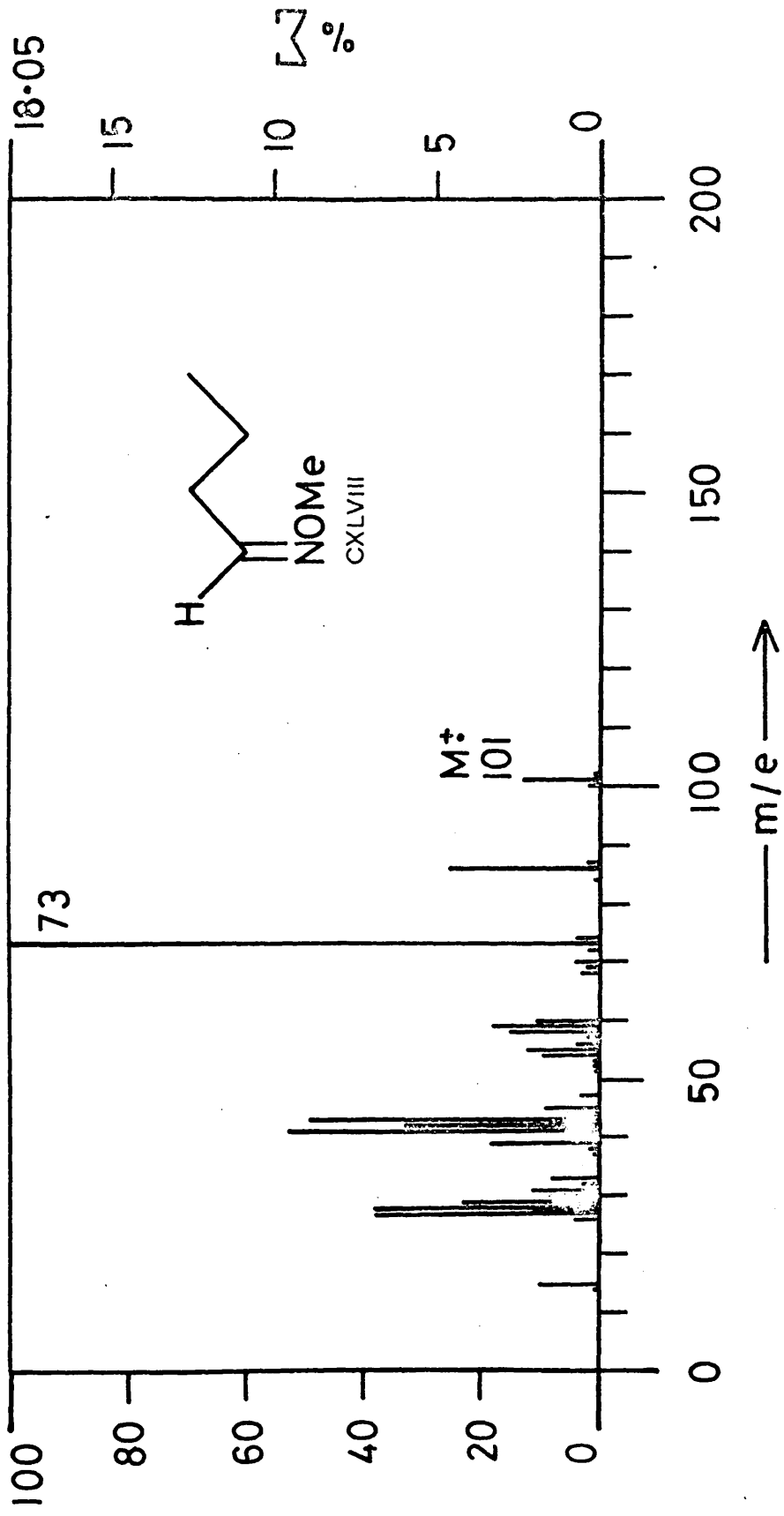

18·05
Σ %
15
10
5
0
NOMe
CXLVIII
H
73
M+
101
100
80
60
40
20
0
0
50
100
150
200
m/e

Fig. 87

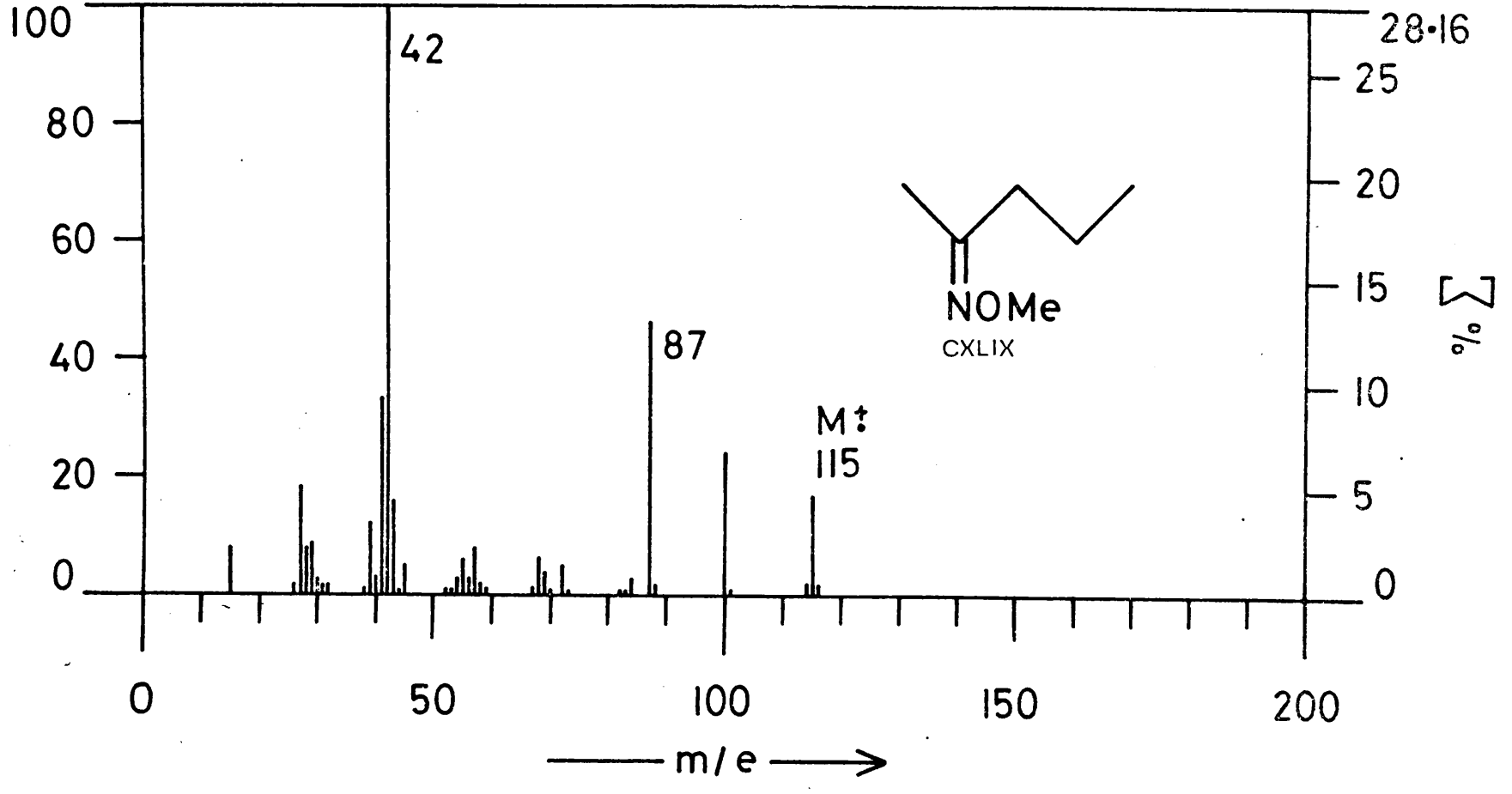

100
80
60
40
20
0
42
87
M+
115
NOMe
CXLIX
28·16
25
20
15
10
5
0
%
0
50
100
150
200
m/e

Fig. 88

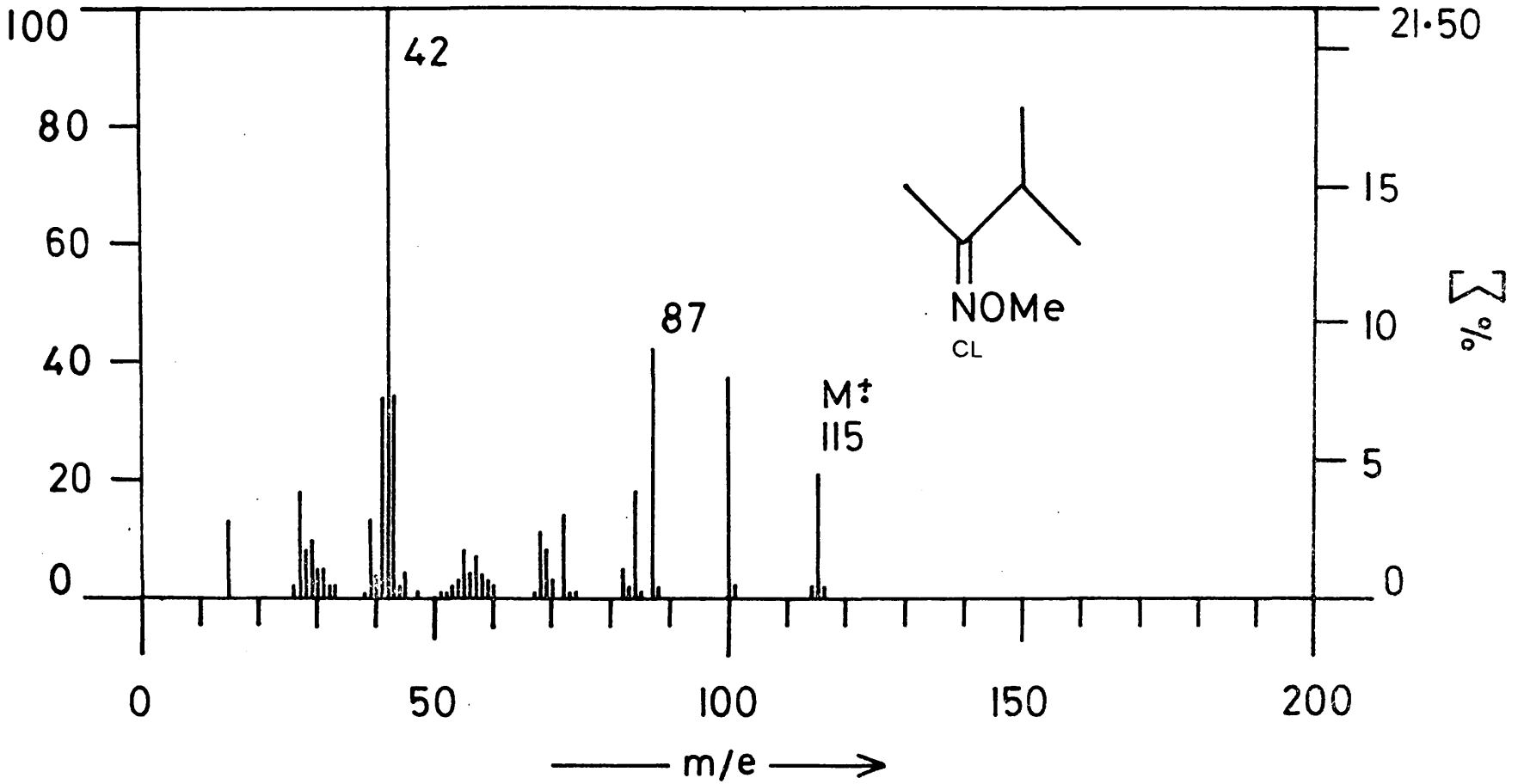

100
80
60
40
20
0
42
87
M+
115
NOMe
CL
21·50
15
10
5
0
%
0
50
100
150
200
m/e

Fig. 89

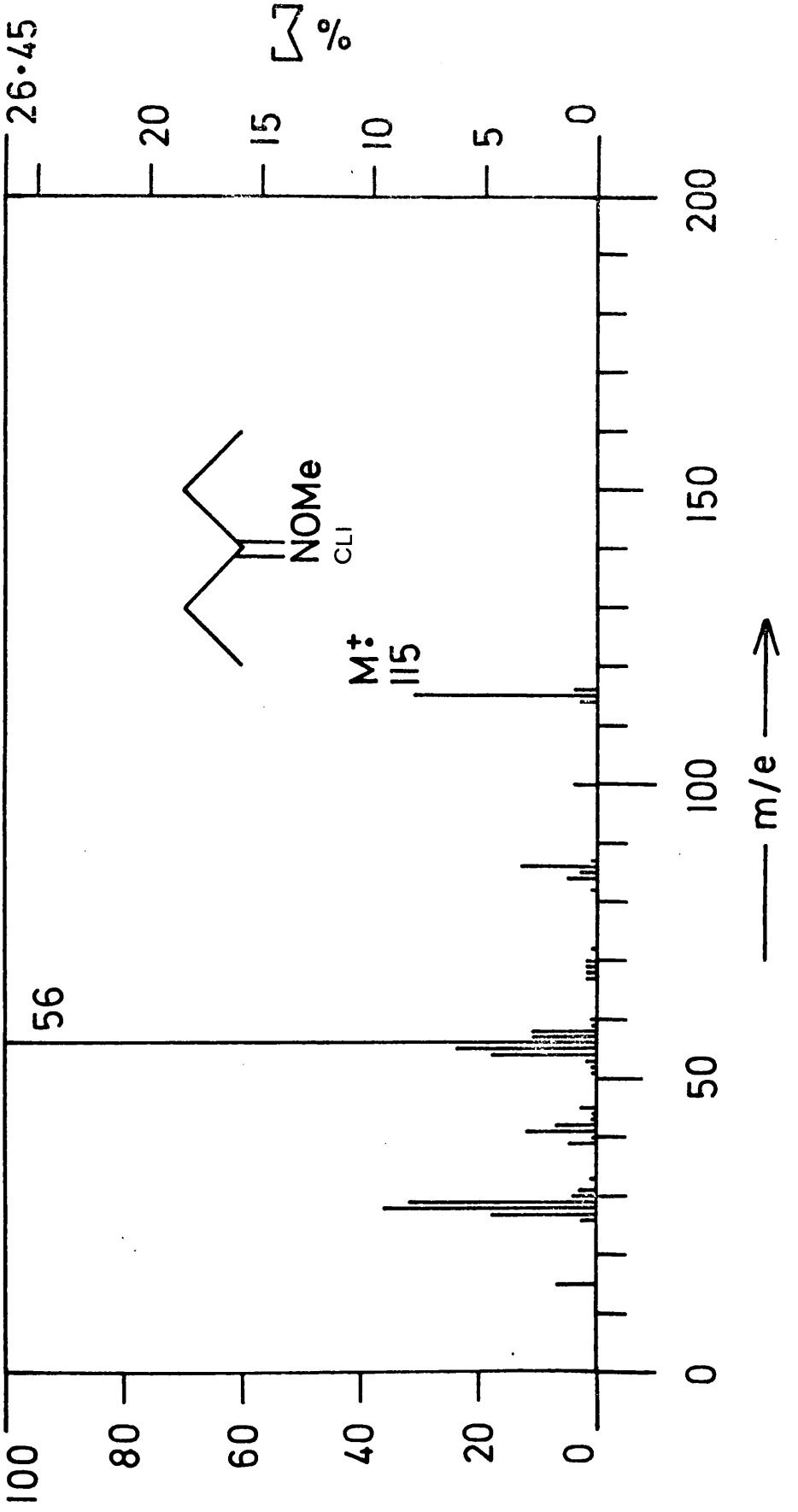

26·45
%
20
15
10
5
0
200
150
100
50
0
m/e
100
80
60
40
20
0
56
M+
115
NOMe
CLI

Fig. 90

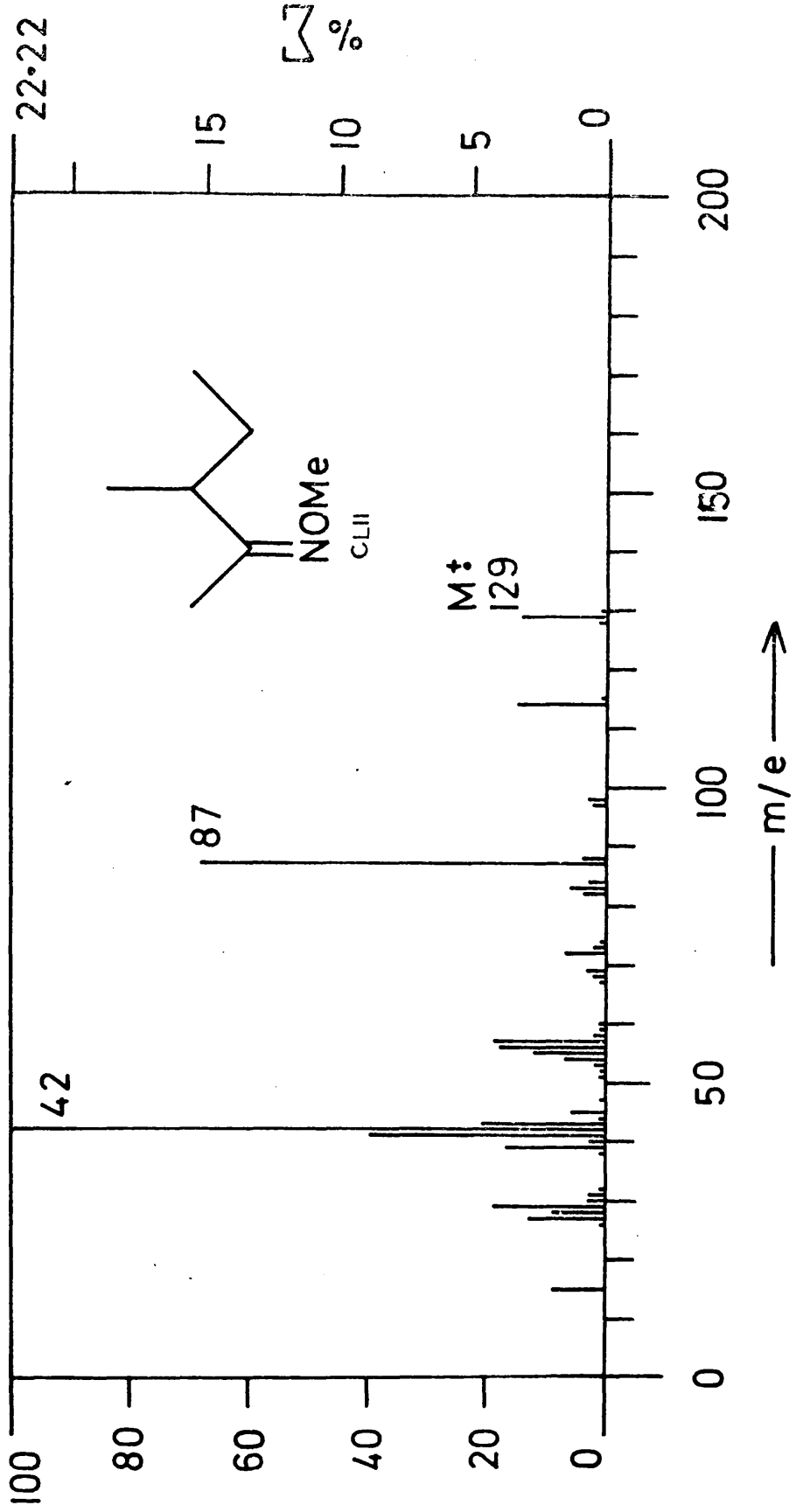

22·22
%
15
10
5
0
NOMe
CLII
M+
129
87
42
100
80
60
40
20
0
0
50
100
150
200
m/e

Fig 91

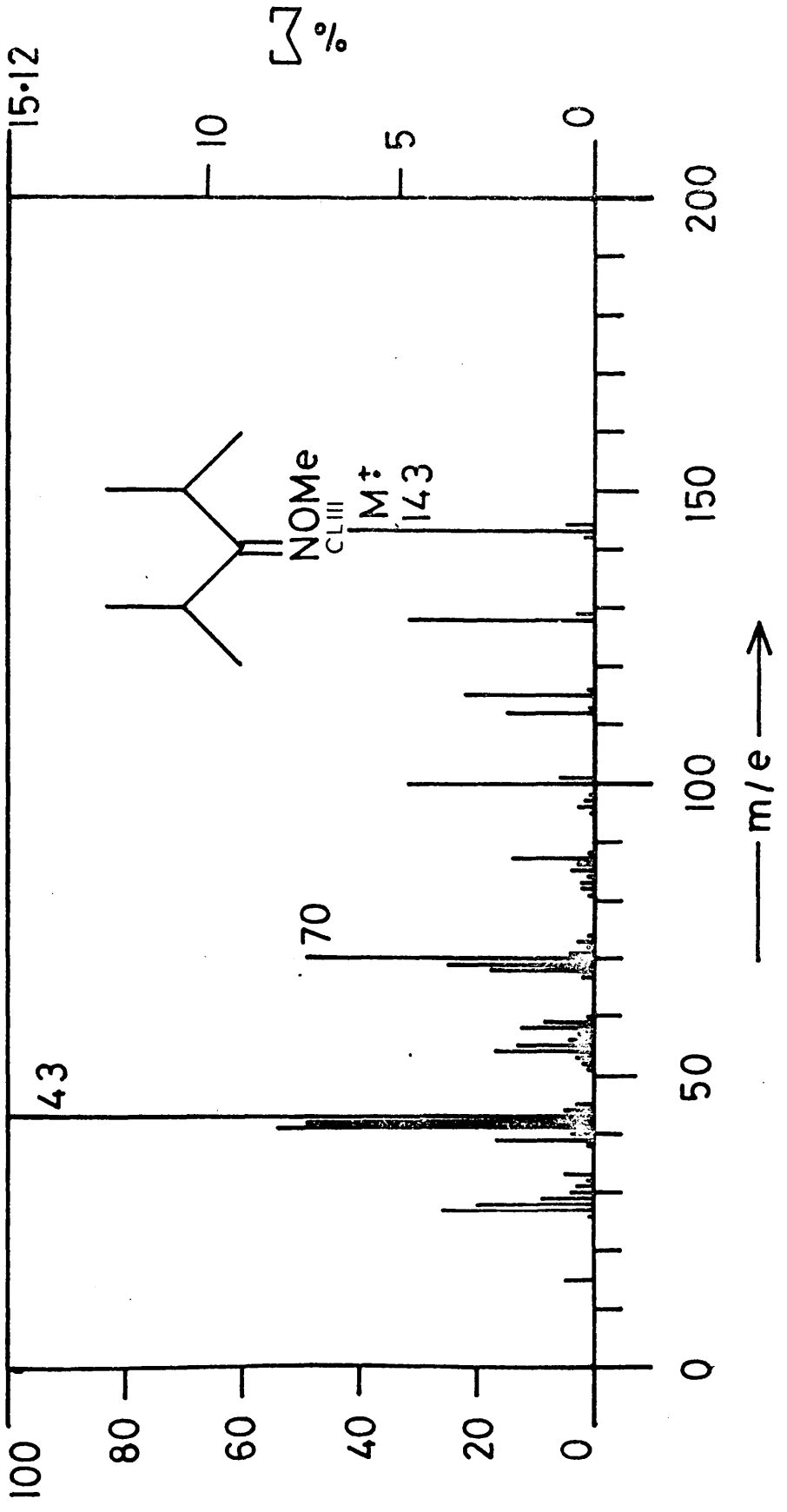

15·12
%
10
5
0
NOMe
CLIII
M+
143
43
70
100
80
60
40
20
0
0
50
100
150
200
m/e

Fig 92

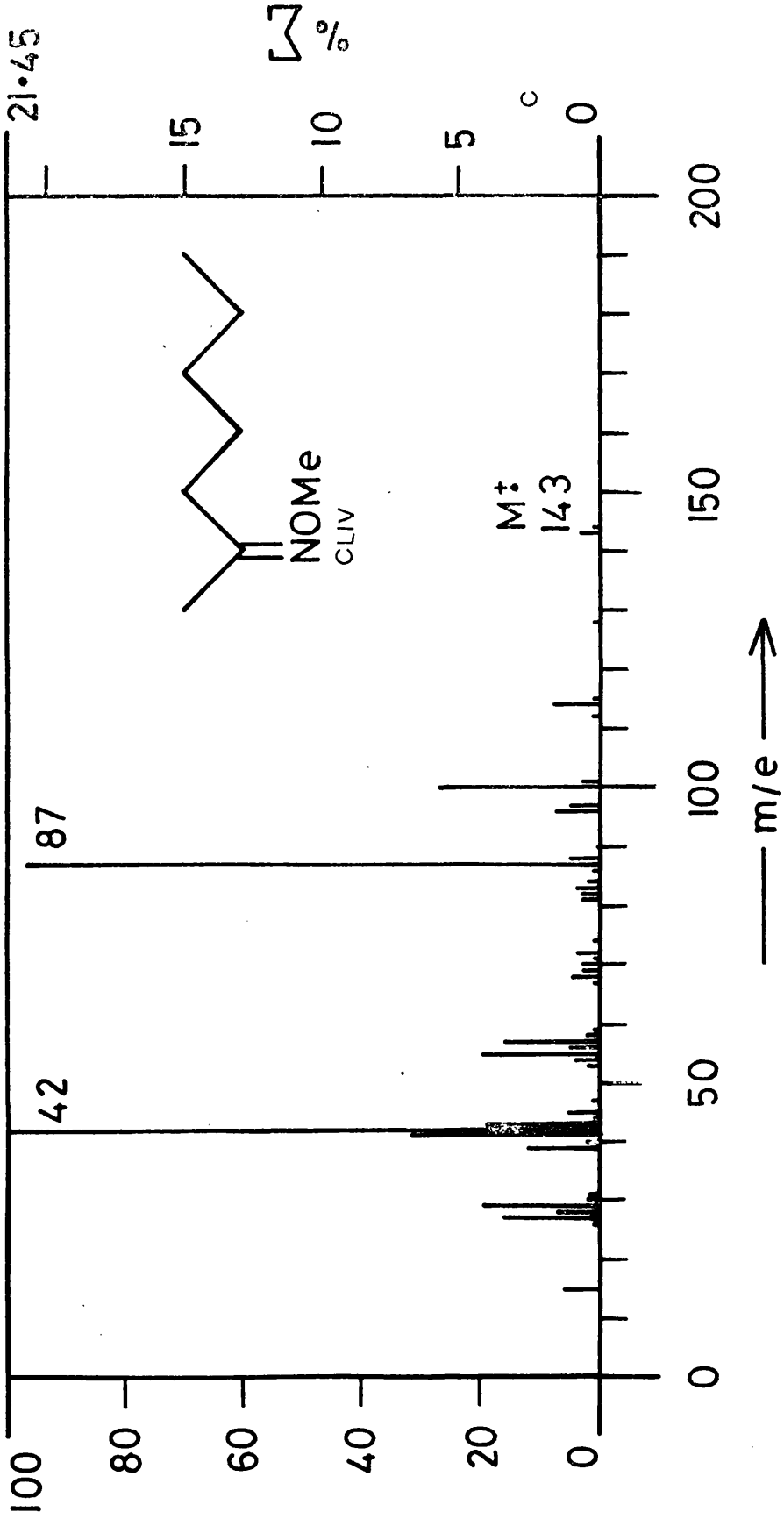

21·45
%
15
10
5
0
c
NOMe
CLIV
M+
143
87
42
100
80
60
40
20
0
0
50
100
150
200
m/e

Fig. 93

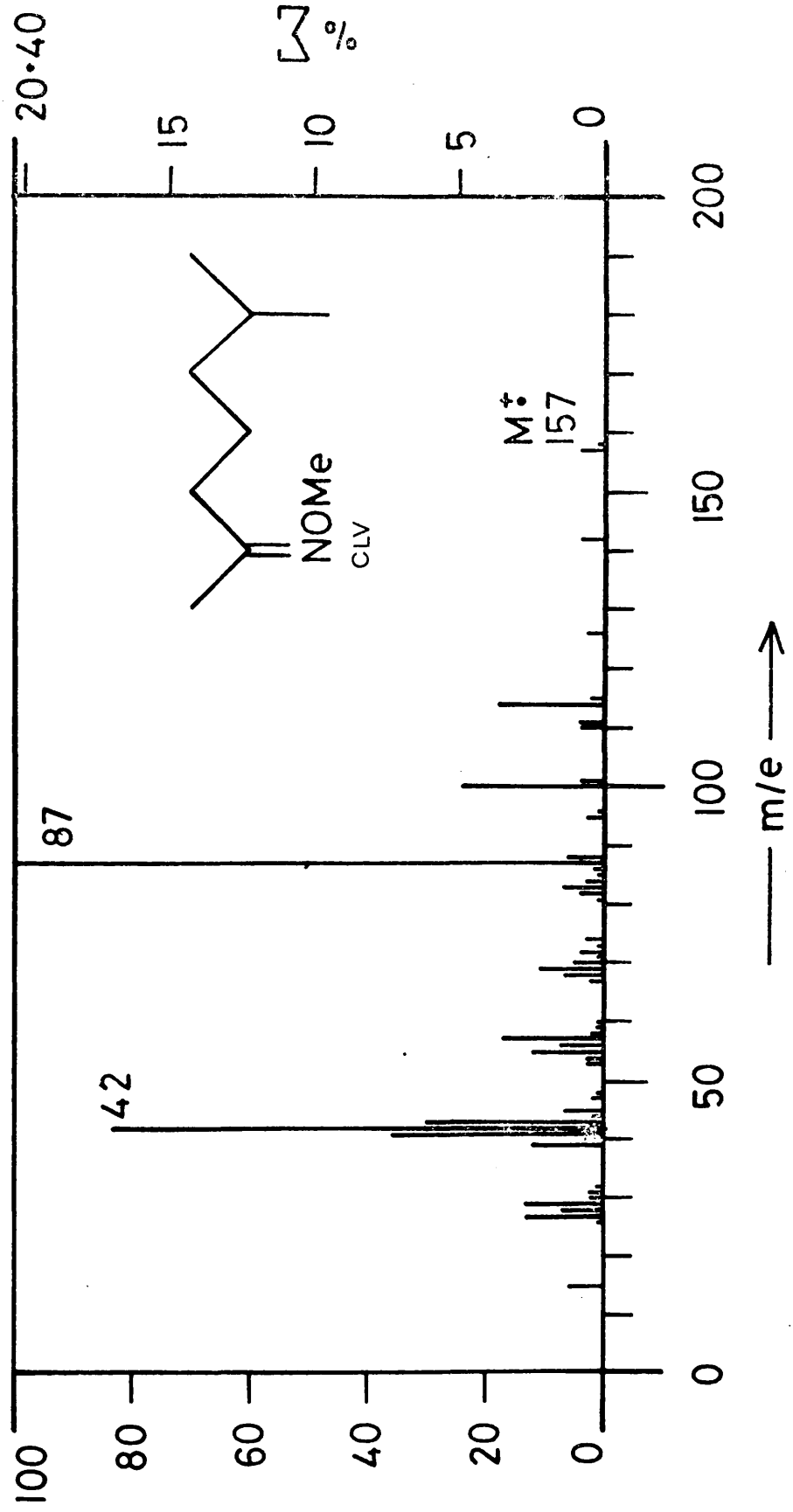

20·40
15
10
5
0
%
NOMe
CLV
M+·
157
87
42
100
80
60
40
20
0
0
50
100
150
200
m/e

Fig. 94

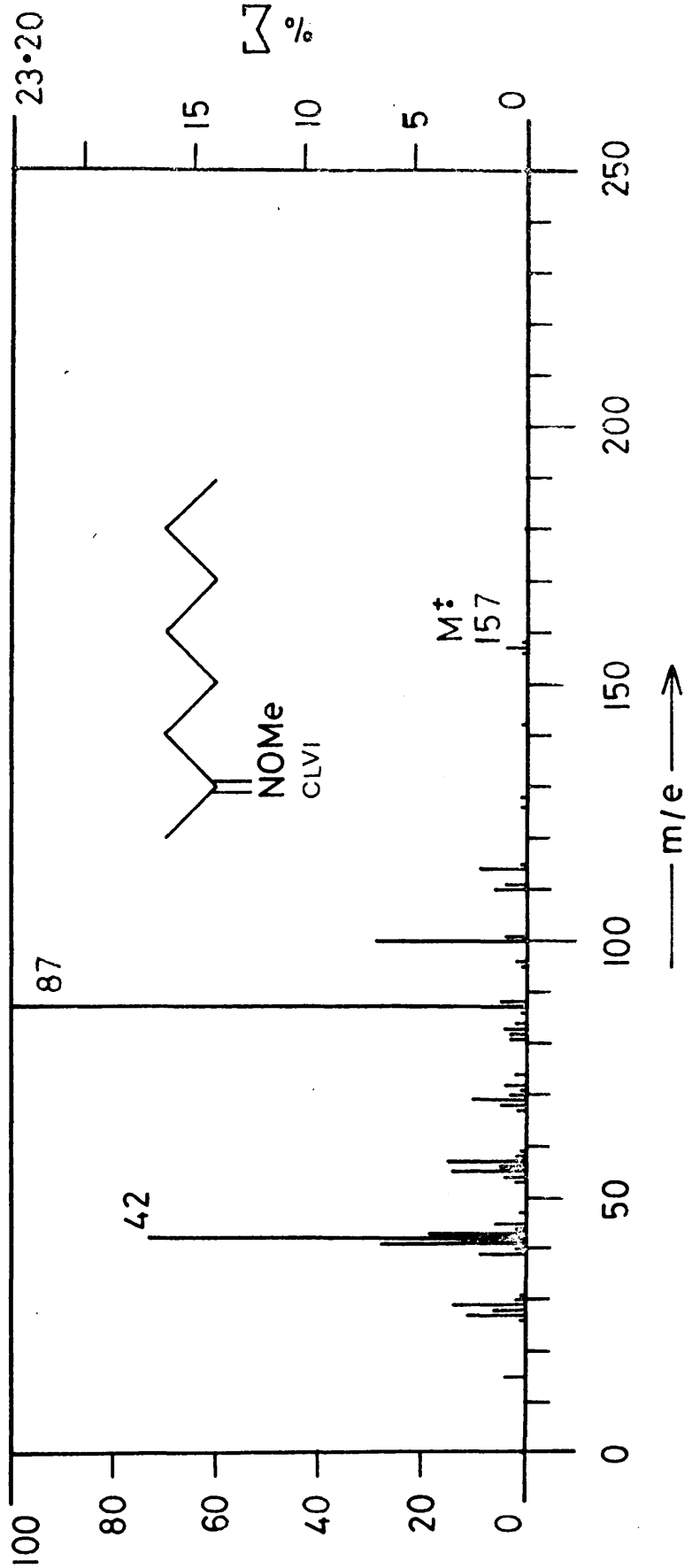

23·20
%
15
10
5
0
NOMe
CLVI
M+
157
87
42
100
80
60
40
20
0
0
50
100
150
200
250
m/e

Fig. 95

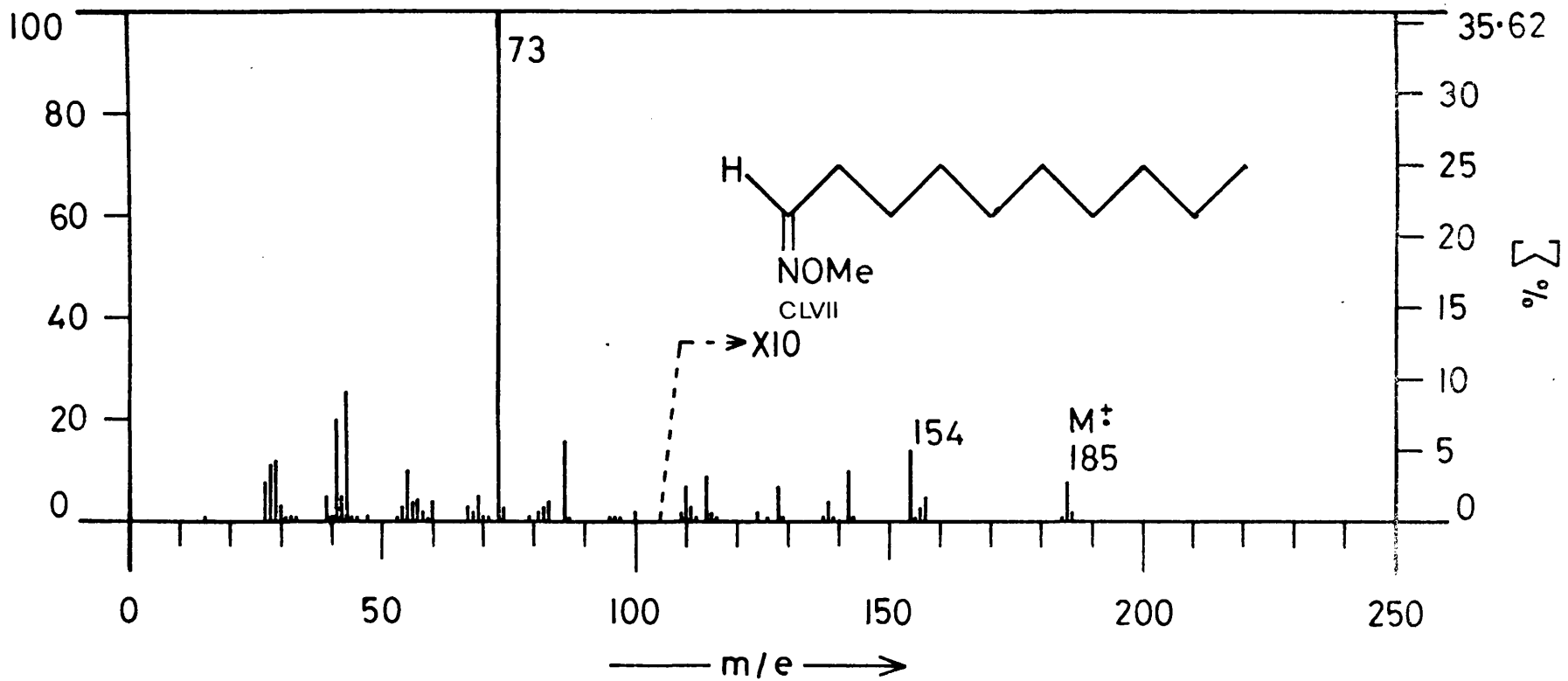

100
80
60
40
20
0
73
H
NOMe
CLVII
X10
154
M+
185
35·62
30
25
20
15
10
5
0
%
0
50
100
150
200
250
m/e

Fig. 96

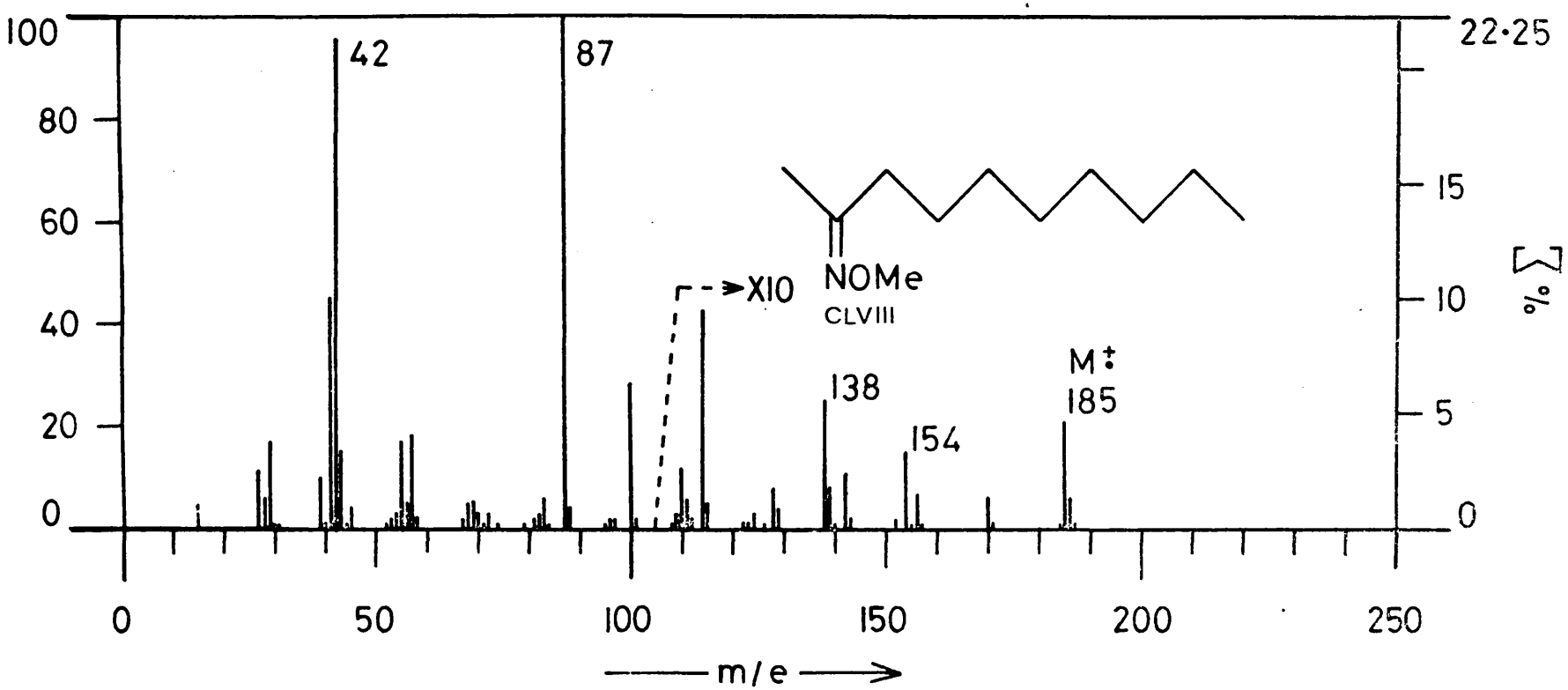

100
80
60
40
20
0
42
87
22·25
15
10
5
0
% Σ
X10
NOMe
CLVIII
138
154
M+
185
0
50
100
150
200
250
m/e

Fig.97

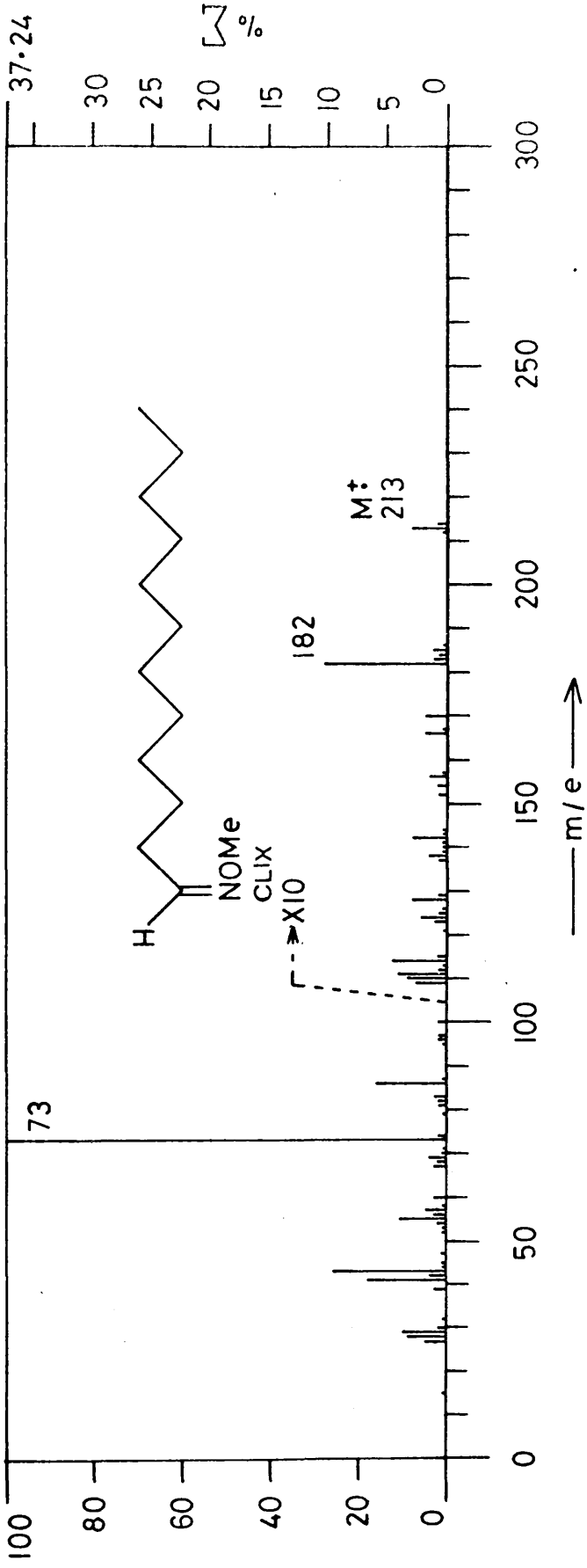

37·24
%
30
25
20
15
10
5
0
300
250
200
150
100
50
0
m/e
73
182
M+
213
NOMe
CLIX
X10
H
100
80
60
40
20
0

Fig. 98

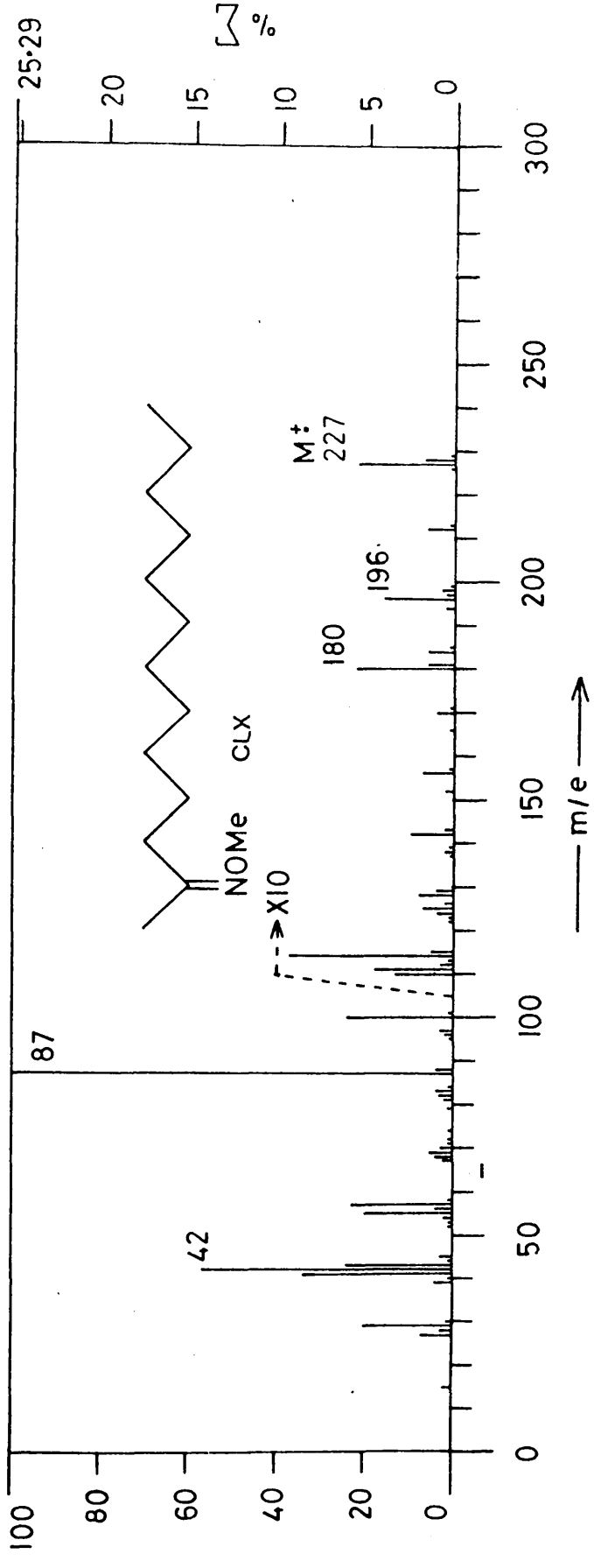

25·29
%
20
15
10
5
0
300
250
200
150
100
50
0
m/e
M+
227
196
180
87
42
NOMe
CLX
X10
100
80
60
40
20
0

Fig. 99

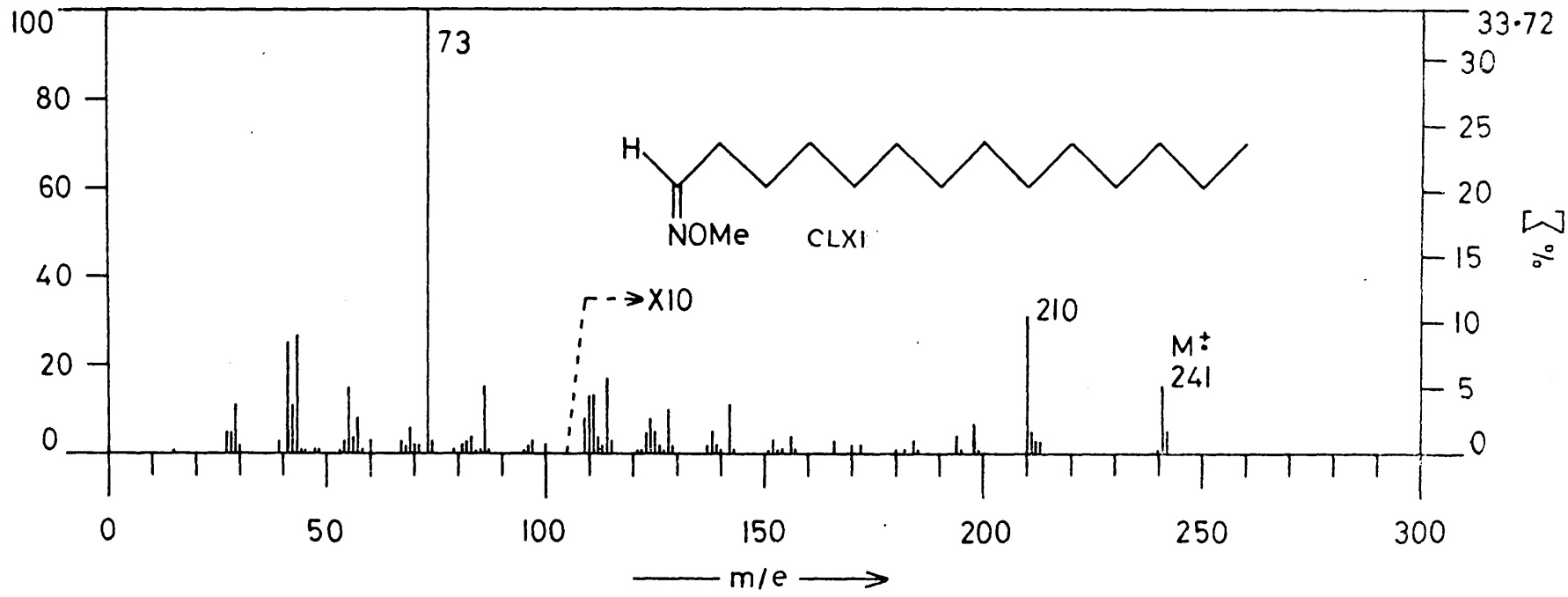

100
80
60
40
20
0
73
H
NOMe
CLXI
X10
210
M+
241
33·72
30
25
20
15
10
5
0
% Σ
0
50
100
150
200
250
300
m/e

Fig. 100

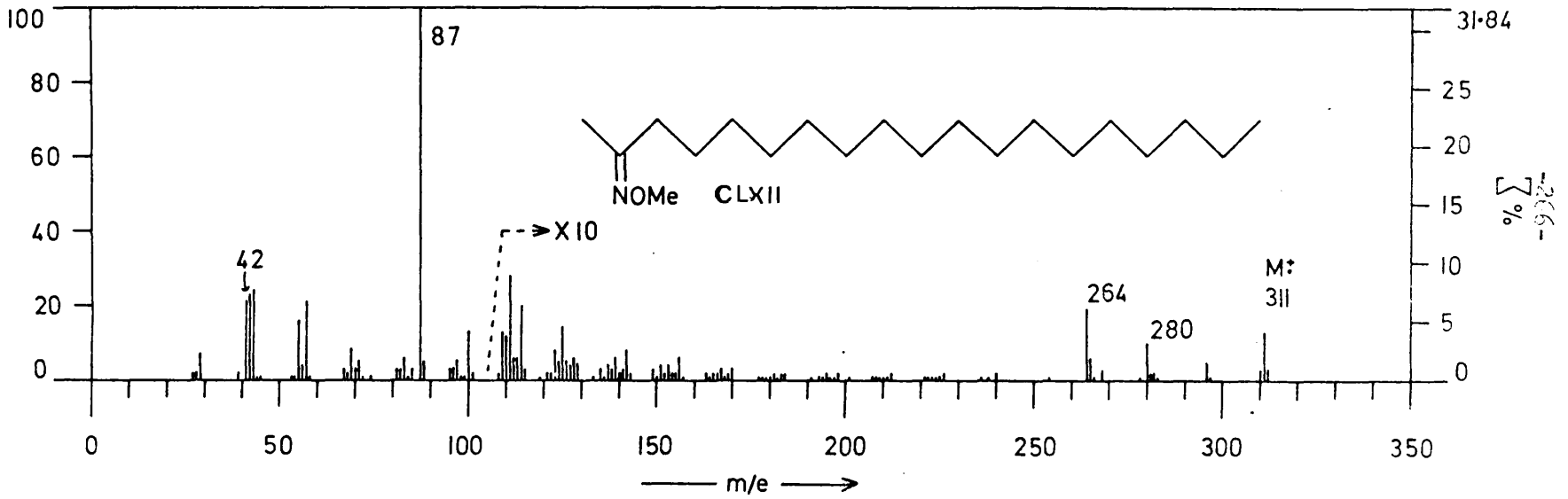
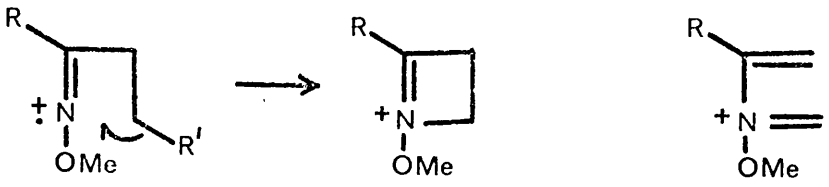

100
80
60
40
20
0
87
42
X10
NOMe
CLXII
264
280
M:
311
31·84
25
20
15
10
5
0
0
50
100
150
200
250
300
350
m/e

Fig. 101

Table. 28. Peaks corresponding to ions produced by simple cleavage (relative abundances in parenthesis). Nitrogen-containing fragment ions are of even mass.

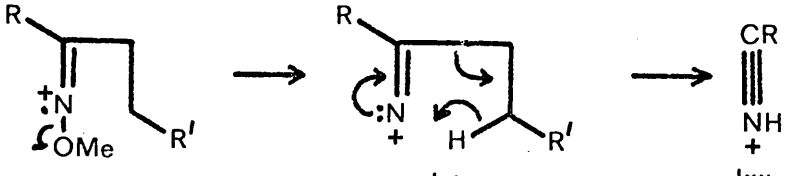
Sample	CXLVIII	CXLIX	CL	CLI	CLII	CLIII	CLIV	CLV
α-Fission	100 (2) 58(15) 43(33)	100(24) 72 (5) 43(16) 15 (8)	100(37) 72(14) 43(34) 15(15)	86(13) 29(32)	114(15) 72 (7) 57(19) 15 (9)	100(32) 43(100)	128 (1) 72 (4) 71 (1) 15 (6)	142 (4) 72 (4) 85 (1) 15 (6)
β-Fission	72 (2) 29(23)	114 (2) 86 (-) 29 (9)	114 (2) 100(37) 15(13)	100 (4) 15 (7)	128 (1) 114(15) 100 (-) 29(19) 15 (9)	128(32) 15 (5) 142 (1) 86 (1) 57(16)	142 (1) 86 (1) 57(16)	156 (1) 86 (2) 71 (1)
γ-Fission	85(25) 15(10)	100(24) 15 (8)	114 (2)	114 (3)	128 (1) 114(15) 15 (9)	142 (2)	100(27) 43(19)	100(24) 57(17)
δ-Fission	100 (2) 114 (2)		-	-	128 (1)	-	114 (8) 29(19)	114(18) 43(30)

Sample	CLVI	CLVII	CLVIII	CLIX	CLX	CLXI	CLXII
α-Fission	142 (1) 72 (4) 85 (-) 15 (4)	184(0.1) 58 (2) 127 (-)	170(0.6) 72 (3) 113 (-) 15 (5)	212(0.1) 58 (1) 155 (-)	212(0.6) 72 (1) 155 (-) 15 (2)	240(0.1) 58 (1) 183 (-)	296(0.5) 72 (1) 239 (-) 15 (-)
β-Fission	156 (1) 86 (1) 71 (1)	72 (-) 113 (-)	184(0.1) 86 (-) 99 (-)	72 (-) 141(0.1)	226(0.1) 86 (-) 141 (-)	72 (-) 169 (-)	310(0.3) 86 (-) 225(0.1)
γ-Fission	100(29) 57(15)	86 (15) 99 (-)	100 (28) 85 (-)	86 (16) 127 (-)	100 (24) 127 (-)	86 (15) 155 (-)	100 (13) 211(0.1)
δ-Fission	114 (9) 43(19)	100 (2) 85 (-)	114(4.3) 71 (1)	100 (2) 113(0.1)	114(3.7) 113(0.1)	100 (2) 141 (-)	114 (2) 197(0.1)



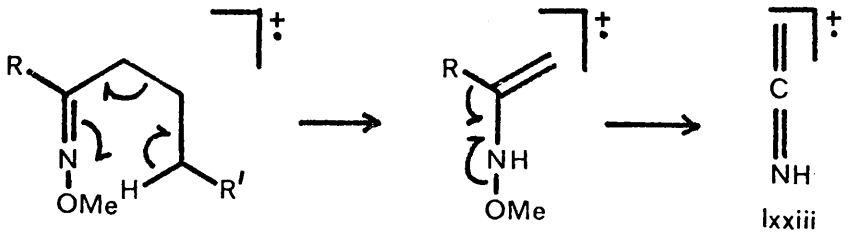
lxv lxvi OR lxvii

Scheme 26



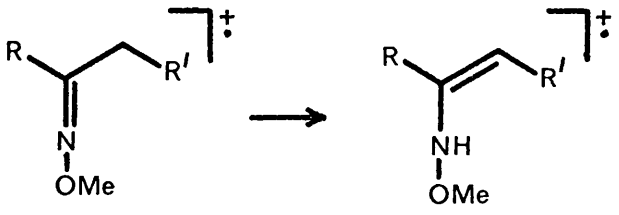
lxix lxx

Scheme 27



lxxi lxxii lxxiii

Scheme 28



lxxiv lxxv

Scheme 29

Cleavage of the nitrogen-oxygen bond gives rise to ions (Table 29) of m/e 31 (lxviii) and $[M-31]^+$ (lxix). It is possible that the latter ions undergo further degradation by fragmentation of their hydrocarbon substituents.

Ions arising from rearrangement. An abundant ion (lxx) in many of the spectra appears (Table 29) at $[27 + R]^+$, where R is the smaller substituent attached to the O-methyloxime moiety. The formation of a similar ion, observed in the spectra of ketoximes,[364] has been ascribed to further degradation of lxix. The corresponding mechanism for O-methyloximes is lxix → lxx (Scheme 27). An ion (lxxi) analogous to the McLafferty rearrangement product provides the base peak in many of the spectra, particularly those of longer chain length (Table 29). A smilar fragment ion was observed in the spectra of aldoximes and ketoximes[364] and it was postulated that it underwent further degradation to form an ion of m/e 41. A similar mechanism (lxxi → lxxii, Scheme 28) appears to operate in the case of the O-methyloximes (Table 29).

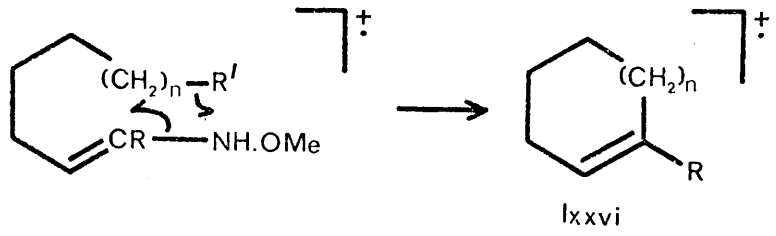
Fragmentation mechanisms invoking tautomerisation. Tautomerisation has been suggested as a factor contributing to the formation of several fragment ions of aldehydes,[362] and has been discussed in connection with the fragmentation of aldoximes and ketoximes.[364] It is possible that it is implicated in the fragmentations of O-methyloximes. The ion $[M-46]^+$ would arise from simple cleavage of the enamine tautomer (lxxv , Scheme 29). A similar fragmentation, with hydrogen transfer to the nitrogen-containing moiety, gives rise to an ion $[M-47]^+$.

A further mode of tautomer fragmentation would produce ions (lxxvi) $[67 + R + 14n]^+$. This type of mechanism (Scheme 30) accounts for many abundant fragment ions of aldehydes,[362] but does not appear to be of importance in the fragmentation of O-methyloximes.

Table 29. Peaks corresponding to selected fragment ions (relative abundances in parenthesis)

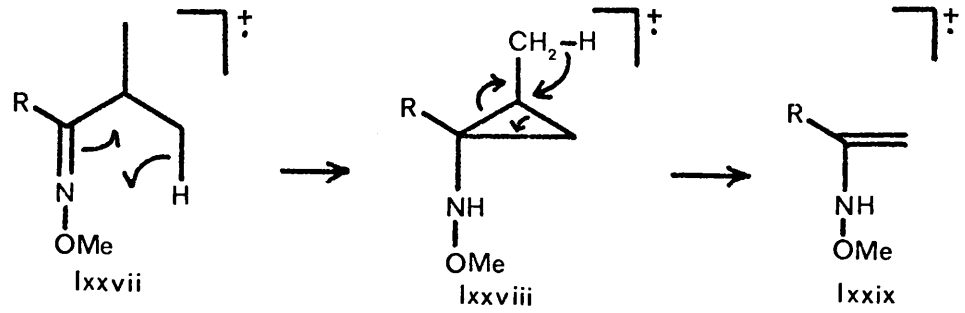
Sample	CXLVIII	CXLIX	CL	CLI	CLII	CLIII	CLIV	CLV
lxviii	31(11)	31 (2)	31 (5)	31 (3)	31 (3)	31 (3)	31 (2)	31 (2)
lxix	70 (4)	84 (3)	84(18)	84 (5)	98 (3)	112(15)	112 (1)	126 (3)
lxx	28(38)	42(100)	42(100)	56(100)	42(100)	70(49)	42(100)	42(83)
lxxii	73(100)	87(46)	87(41)*	101 (-)*	87(68)	115(22)*	87(97)	87(100)
lxxiii	41(52)	41(33)	41(34)	41(12)	41(40)	41(54)	41(32)	41(36)
lxxv	55(12)	69 (4)	69 (8)	69 (2)	83 (6)	97 (2)	97 (5)	111 (4)

Sample	CLV	CLVI	CLVII	CLVIII	CLIX	CLX	CLXI	CLXII
lxviii	31 (2)	31 (1)	31 (1)	31 (1)	31 (-)	31 (-)	31 (-)	31 (-)
lxix	126 (3)	126 (1)	154(1.4)	154(1.5)	182(2.8)	196(1.6)	210(3.1)	280(1.0)
lxx	42(83)	42(73)	28(11)	42(95)	28 (9)	42(57)	28 (5)	42(23)
lxxii	87(100)	87(100)	73(100)	87(100)	73(100)	87(100)	73(100)	87(100)
lxxiii	41(36)	41(28)	41(20)	41(45)	41(18)	41(34)	41(11)	41(21)
lxxv	111 (4)	111(4)	139(0.1)	139(0.8)	167(0.1)	181(0.6)	195(0.1)	265(0.6)



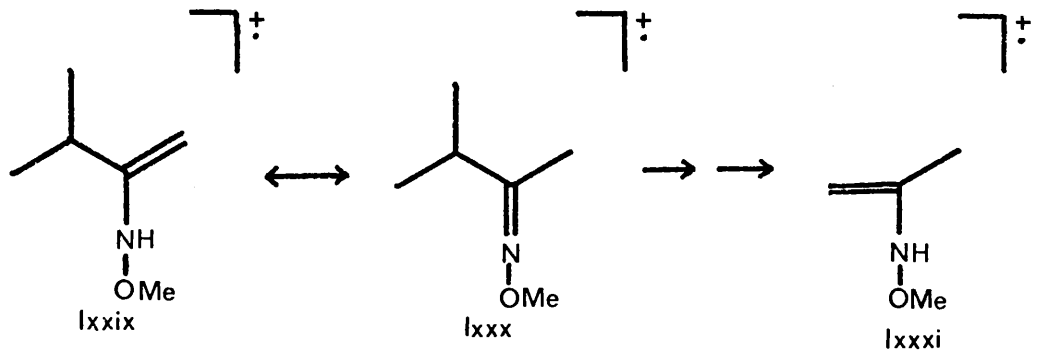
lxxvi

Scheme 30



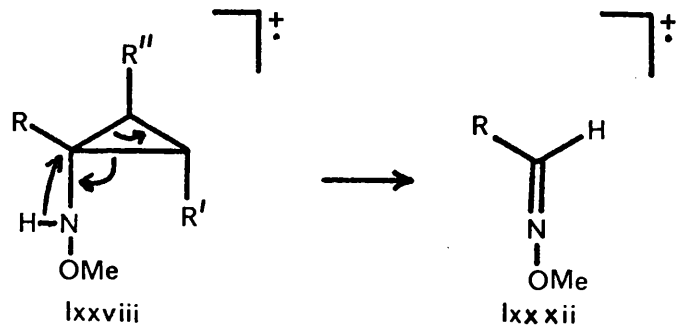
lxxvii lxxviii lxxix

Scheme 31



lxxix lxxx lxxxi

Scheme 32



lxxviii lxxxii

Scheme 33

Fragmentation mechanisms entailing cyclic intermediates. The formation of most of the fragment ions has been accounted for on the basis of previously proposed fragmentation pathways. Many of these have been substantiated by high-resolution and isotopic-labelling studies and agree well with our present knowledge of mass spectral fragmentation. The mechanisms requiring tautomerisation of the molecular ion, however, involve cleavage of a single bond adjacent to a double bond, whereas allylic cleavage is normally favoured. Also, it is observed that CL eliminates a fragment equal in mass to that produced by McLafferty rearrangement of CXLIX: CLIII eliminates two such fragments. Neither CL nor CLIII contains the requisite γ-hydrogen atom for the McLafferty rearrangement and so an alternative or additional fragmentation mode may be operative. The fragment of 28 mass units eliminated from III could be C_2H_4, CO or CNH_2. Of these, C_2H_4 seems to be the most likely (this is to be checked by high-resolution mass measurement), particularly since the spectra of CXLIX and CL are so similar and CLIII appears to lose two such fragments. A possible mechanism for this fragmentation is lxxvii⟶lxxviii⟶lxxix (Scheme 31). The second such elimination from CLIII can be represented as lxxix⟶lxxx⟶lxxxi (Scheme 32). The postulated fragmentation of the cyclopropane ring is analogous to that proposed by Weinberg et al.[366] An alternative mode of fragmentation of lxxviii may produce an ion $[58 + R]^+$ (lxxxii), Scheme 33. Such an ion is observed in moderate abundance in the spectra of CXLVIII and CLIII, but is almost or completely absent from the other spectra.
It is also possible that ions arising from scission of the carbon-nitrogen bond may be formed via mechanisms involving intermediate ring formation rather than oxime-enamine tautomerisation. Deuterium labelling studies on di-n-hexyl and di-n-heptyl ketoximes,[365] however, suggest that this mechanism cannot be extended to these compounds unless specific reciprocal hydrogen transfer takes place. This is not

impossible,[364-365] but the fact that ions of type lxxix are only observed in the spectra of CXLVIII-CLIII indicates that this type of mechanism may be restricted to compounds of relatively low molecular weight. In any case, it is not suggested that this mechanism should supplant that of the "conventional" McLafferty rearrangement or those postulating the existence of an ionised cyclobutane intermediate.[360,361,367]

HYDROCARBONS FROM THE GREEN FORM OF THE FRESHWATER ALGA BOTRYOCOCCUS BRAUNII*

Botryococcus braunii (Kutz.) is a freshwater green colonial alga of widespread occurrence, which is known to occur in at least two physiologically distinct forms. The first of these is a green exponentially growing stage of limited abundance and the second is a brown resting stage which often arises as massive rust-coloured algal blooms on the surface of lakes.[378] From paleobotanical studies it has been suggested[379] that B. braunii may be the causal organism of the boghead coals (e.g. Torbanite), Coorongite, and also oil shales of the tertiary period[380] and a number of investigations of these theories have been undertaken (for brief reviews see Refs. 381-382). It has been shown in the brown resting stage that 70 per cent of the dry weight of B. braunii may be accounted for by two isomeric hydrocarbons, botryococcene and isobotryococcene, which occur in a 9:1 ratio.[382] In the green exponential form, however, it has been found that only about 20 per cent of the dry weight of the alga could be accounted for as hydrocarbons[383-384] and also that less than 5 per cent of these hydrocarbons was botryococcene or its isomer. In fact, three homologous series of hydrocarbons were demonstrated by GLC and the dominant "A" series with five members was found by mass spectrometry to have the general formula C_nH_{2n-2}. The next most-abundant series with four members was shown to have the formula C_nH_{2n-4}. These results were similar to those found for what was described as the "golden brown alga B. braunii",[380] when six compounds of the general formula C_nH_{2n-2} and one of the formula C_nH_{2n-4} were described.

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* Growth of the alga, extraction of, and chemical transformation of, the hydrocarbons, and preliminary gas chromatography were carried out by Dr. A.C. Brown, Dr. E. Conway and Dr. B.A. Knights (Dept. of Botany).

In the present work,[351] locations of the positions of the two double bonds of the "A" series hydrocarbons of the green stage of B. braunii have been determined. Hydrocarbons were isolated by acetone extraction of the dried alga and separated by chromatography on alumina.[383] I.r. spectroscopy of these hydrocarbons indicated the presence of a vinyl group (1638, 990 and 908 cm^{-1}) and a cis disubstituted double bond (720 cm^{-1}). Gas-liquid chromatography (GLC) indicated that the mixture contained three components (>90 per cent of the total fraction) and, by inspection of the data (Table 33), these were found to be members of the previously decribed[383] "A" series of hydrocarbons, and to correspond to those compounds which had been shown by GC-MS to have the formulae $C_{27}H_{52}$ (peak 1), $C_{29}H_{56}$ (peak 2) and $C_{31}H_{60}$ (peak 3).

Ozonolysis of the hydrocarbon fraction was attempted using a Supelco microozonizer[385] and the method described by Beroza and Bierl.[386] Under the prescribed conditions, no reaction products could be detected using GLC, in spite of a positive reaction to ozone from the indicator solution. In addition, it was found that triphenylphosphine and triphenylphosphine oxide could be detected in GLC traces and it was thought that in this case these compounds might interfere with the analysis by GLC of possible products of ozonolysis. Ozonolysis was therefore attempted by adapting the method of Munavalli and Ourisson[387] for use with the microozonizer. Using a flame ionization detector, tetracyanoethylene, which was incorporated into the reaction mixture to decompose ozonides, could not be detected under the conditions for GLC used in this work. Reaction with ozone was continued until hydrocarbons could no longer be detected by GLC. The aldehyde fraction so formed showed carbonyl absorption (1720 cm^{-1}) but no double bond absorption in the i.r. GLC (Table 33) indicated three products derived from the three corresponding hydrocarbons.

TABLE 33. GLC data for the ozonolysis products from hydrocarbons of B. braunii

Fraction	OV-17 Peak 1	%	OV-17 Peak 2	%	OV-17 Peak 3	%	SE-30 Peak 1	%	SE-30 Peak 2	%	SE-30 Peak 3	%
Hydrocarbon*	2705	12	2915	42	3115	46	2705	-	2905	-	3100	-
Aldehyde†	2355	8	2560	46	2760	46	2115	7	2330	46	2530	47
O-Methyloxime†	2525	9	2730	42	2930	49	2310	13	2520	42	2720	45
Methyl ester†	2550	19	2750	36	2955	45	2320	15	2525	40	2725	45

*244°.
†OV-17, 223°; SE-30, 230°.

.....

TABLE 34. Partial ozonolysis products

Fraction		OV-17 Mobile Compound	OV-17 Peak 1	OV-17 Peak 2	OV-17 Peak 3	SE-30 Mobile Compound	SE-30 Peak 1	SE-30 Peak 2	SE-30 Peak 3
Aldehyde	*	-	2135	2340	2545	-	2030	2225	2420
	†	85	193	211	229	-	-	-	-
O-Methyloxime	*	-	2230	2435	2630	-	2130	2325	2520
	†	95	201	219	237	105	212	231	249

*205°.
†Temperature of emergence (programme rate 2°/min from 50°).

Using GC-MS, mass spectra (the most significant and the eight most abundant ions in these spectra are listed in Table 35) were obtained for peaks 2 and 3 and it was clear from inspection of these data that these two compounds were homologous, differing in mass by 28 units (i.e. C_2H_4). Fragmentations for the loss of water, $[M-18]^+$, $[M-36]^+$, ethylene, $[M-28]^+$, and ethylene plus water, $[M-46]^+$, from these compounds were observed, similar to those observed by Gilpin and McLafferty[359] for mass spectra of aldehydes. The ion arising by loss of hydrogen, $[M-1]^+$, formed via α-cleavage was not significant in the present work, and the corresponding ion at m/e 29 was only of medium intensity. The ions for $[M-43]^+$ and $[M-44]^+$ probably arise by β-fission processes.[359,388]

The aldehyde fraction was converted to the corresponding O-methyloxime derivatives[124] and were thought to be derived by loss of fragments including methoxyl and methyl radicals, and methanol. The base peak of these spectra at m/e 73 and the ion observed at $[M-72]^+$ for each compound were probably formed by β-cleavage reactions. The ion at m/e 73 from O-methyloximes appears to be equivalent to the ion found by Goldsmith et al.[364] to occur at m/e 59 for the oxime derivatives of butyraldehyde and valeraldehyde. The same group also described an ion at m/e 72 from these compounds and an equivalent ion at m/e 86 was noted for the O-methyloximes in the present work.

Oxidation of the aldehyde fraction produced an acid fraction from which methyl esters were prepared. Analysis by GLC (Table 33) demonstrated the presence of three compounds in this fraction. GC-MS analysis indicated the presence of two carboxylic acid methyl ester groups and confirmed that the original fraction was composed of dialdehydes. The McLafferty rearrangements[360] produced the ion at m/e 74, in agreement with previous work on the mass spectra of methyl esters.[389] The spectra were similar to those reported in the literature for α,ω-dicarboxylic acid methyl esters.[381,390]

Aldehyde fraction

	M	M-18	M-28	M-36	M-43	M-44	M-46	Eight most abundant ions							
Peak 2 Mass	296	278	268	260	253	252	250	55	41	43	57	69	95	67	81
Abundance	8	13	4	3	11	11	12	1000	900	850	820	530	500	420	390
Peak 3 Mass	324	306	296	288	281	280	278	55	41	43	57	69	82	81	67
Abundance	14	14	5	5	10	10	11	1000	860	800	800	550	550	500	460

O-Methyloxime fraction

	M	M-31	M-46	M-63	M-71	M-72	M-78	M-88	M-104	Eight most abundant ions							
Peak 1 Mass	326	295	280	263	255	254	248	238	222	43	73	55	41	57	69	71	83
Abundance	45	72	11	40	25	150	11	12	12	1000	940	740	630	610	450	280	280
Peak 2 Mass	354	323	308	291	283	282	276	266	250	73	43	41	55	86	57	69	56
Abundance	6	98	20	45	35	160	13	12	11	1000	750	500	500	320	260	260	190
Peak 3 Mass	382	351	336	319	311	310	304	294	278	73	43	55	41	57	86	69	83
Abundance	5	94	22	48	35	150	13	11	11	1000	660	450	410	310	290	290	200

Methyl ester fraction

	M	M-31	M-64	M-73	M-92	M-105	M-106	M-123	M-146	Eight most abundant ions							
Peak 1 Mass	328	297	264	255	236	223	222	205	182	43	55	57	41	69	74	71	67
Abundance	4	34	11	29	-	21	13	17	13	1000	880	780	770	580	450	400	240
Peak 2 Mass	356	325	292	283	264	251	250	233	210	55	43	41	57	69	74	98	83
Abundance	4	73	20	58	13	35	18	15	20	1000	950	750	650	650	650	600	370
Peak 3 Mass	384	353	320	311	292	279	278	261	238	55	43	41	57	98	74	69	83
Abundance	5	77	24	55	14	38	24	17	10	1000	900	700	670	660	630	620	370

TABLE 35

Mass spectral data for the three main compounds produced upon complete ozonolysis of hydrocarbons of *B. braunii*

A second ozonolysis experiment using a shorter reaction time was carried out. GLC analysis indicated the presence of unreacted hydrocarbons together with three main aldehyde products. Retention data for these aldehydes are listed in Table 34. In addition, a single, low molecular weight aldehyde was detected when using temperature programmed GLC. This compound was not detected in the previously described ozonolysis experiment.

Analysis by GC-MS (see Table 36) of the aldehyde and O-methyloxime fractions indicated that the three main components of the partial ozonolysis experiment were monoaldehydes containing one double bond. The molecular weights and GLC data, when compared with the dialdehyde series, were consistent with the double bond present in the monoaldehydes being the vinyl group. The low molecular weight aldehyde afforded a mass spectrum similar to that recorded by Gilpin and McLafferty for n-nonanal.[359] The data for the corresponding O-methyloxime were also in agreement with this compound's being n-nonanal, the mass spectrum being similar to that obtained from the O-methyloxime of an authentic sample of n-decanal.

B 36.

ss spectral data for partial ozonolysis products from hydrocarbons of B. braunii

Aldehyde fraction

		M	M-18	M-29	M-43	M-44	Eight most abundant ions							
k 1	Ion	266	248	237	223	222	55	41	43	57	69	83	29	67
	Abundance	11	9	7	10	9	1000	810	800	710	680	500	460	460
k 2	Ion	294	276	265	251	250	55	41	43	69	57	83	81	67
	Abundance	18	12	7	8	7	1000	760	610	560	530	430	370	340
k 3	Ion	322	304	293	279	278	55	41	43	69	57	83	29	67
	Abundance	14	8	4	6	5	1000	800	640	540	500	390	320	310

O-Methyloxime fraction

		M	M-15	M-31	M-41	M-43	Eight most abundant ions							
k 1	Ion	295	280	264	254	252	73	55	43	41	59	69	83	86
	Abundance	15	4	60	4	6	1000	390	360	340	280	240	200	150
k 2	Ion	323	308	292	282	280	73	55	41	43	86	69	57	29
	Abundance	34	7	103	8	7	1000	380	370	335	210	180	140	130
k 3	Ion	351	336	320	310	308	73	55	43	41	69	86	57	83
	Abundance	26	6	86	5	6	1000	350	300	290	170	160	140	115

Lower molecular weight compound detected by temperature programmed GLC

Aldehyde

	M	M-1	M-18	M-28	M-44	Eight most abundant ions							
n	142	141	124	114	98	57	41	43	29	44	56	55	27
ndance	2	4	40	50	270	1000	1000	840	770	660	600	570	550
erature value[359]	5	4	70	90	400	1000	700	680	360	560	630	500	260

O-Methyloxime

	M	M-29	M-31	M-43	Eight most abundant ions							
n	171	142	140	128	73	43	41	86	29	28	27	55
ndance	10	8	13	12	1000	330	320	200	190	180	140	140

Previously, it had been shown[383] that the principal series of hydrocarbons from the green exponentially growing stage of Botryococcus braunii had the empirical formula C_nH_{2n-2}. Further, for the three main members of this series, it was found that n was 27, 29 and 31 with the order of relative abundance being $C_{29}H_{56} > C_{31}H_{60} > C_{27}H_{52}$. Since the i.r. data showed the presence of a vinyl group and a cis-disubstituted double bond, it is possible to write a general formula (i) for these three hydrocarbons, as shown in Scheme 34. From the complete

$$CH_2{=}CH{-}(CH_2)_x{-}CH{=}CH{-}(CH_2)_y{-}CH_3 \qquad \text{(i)}$$

GLC: Peak 1 M.W. 376: x + y = 22
Peak 2 M.W. 404: x + y = 24
Peak 3 M.W. 432: x + y = 26

Ozonolysis

$$[CH_2{=}O]^* + O{=}CH{-}(CH_2)_x{-}CH{=}O + [O{=}CH{-}(CH_2)_y{-}CH_3]^* \qquad \text{(ii)}$$

Peak 1 [M.W. 268]: x = 15
Peak 2 M.W. 296 : x = 17
Peak 3 M.W. 324 : x = 19

Partial ozonolysis

$$CH_2{=}CH{-}(CH_2)_x{-}CH{=}O + O{=}CH{-}(CH_2)_y{-}CH_3 \qquad \text{(iii)}$$

Peak 1 M.W. 266: x = 15 M.W. 142: y = 7
Peak 2 M.W. 294: x = 17
Peak 3 M.W. 322: x = 19

$$CH_2{=}CH{-}(CH_2)_{15}{-}CH{=}CH{-}(CH_2)_7{-}CH_3 \qquad \text{(iv)}$$
$$CH_2{=}CH{-}(CH_2)_{17}{-}CH{=}CH{-}(CH_2)_7{-}CH_3$$
$$CH_2{=}CH{-}(CH_2)_{19}{-}CH{=}CH{-}(CH_2)_7{-}CH_3$$

*Not observed.

SCHEME 34

ozonolysis experiment, three dialdehydes were produced in the same relative proportions as were found for the three parent hydrocarbons. The mass spectral data in Table 35 showed that the formulae for these three aldehydes may be expressed as indicated (ii) and that X = 15, 17 and 19 for GLC peaks (1), (2) and (3) respectively. Thus, it would be expected that Y = 7 for all three hydrocarbons, although the presumed aldehyde n-nonanal could not be detected in this reaction mixture using GLC. Partial ozonolysis was found to produce four monoaldehydes, together with small amounts of the dialdehydes and some unreacted hydrocarbon. Three of these monoaldehydes were closely related to the dialdehydes (ii) and had the structures (iii). The fourth aldehyde, a more mobile substance on GLC, had the correct molecular weight (142 as the aldehyde and 171 as the O-methyloxime) for n-nonanal and thereby confirmed that y = 7. Thus the formulae for the three hydrocarbons represented by (i) are as shown in (iv). This method of analysis does not rigorously exclude the possibility of a branched-chain structure. Since fragmentation is most likely to occur at highly branched carbon atoms,[391-392] irregularities in the relative abundances of fragmentations, due to C-C fission of long-chain molecules, would not be expected to occur in unbranched molecules. No such irregularities were observed in the spectra recorded. Further, the similarity between the mass spectra from the methyl esters and those recorded by Ryhage and Stenhagen[390] for α,ω-dicarboxylic acid methyl esters lends additional support for the view that these hydrocarbons from B. braunii are the unbranched, diunsaturated compounds heptacosa-1,18-diene, nonacosa-1,20-diene and hentriaconta-1,22-diene.

The presence of a terminal double bond (i.e. a vinyl group) in long-chain hydrocarbons of freshwater green algae has been previously reported for unnamed species of Scenedesmus and Chlorella.[394] The

cis-disubstituted double bond in each of the hydrocarbons of *B. braunii* is located at the same position with respect to the terminal methyl group of the carbon chain as the double bond of oleic acid. This is consistent with the current theories on hydrocarbon biosynthesis via decarboxylation of the corresponding fatty acid[395-396] and suggests that, in *B. braunii* at least, decarboxylation of an α,β-unsaturated fatty acid may occur to produce the vinyl group. Oleic acid has been demonstrated as a major component of the fatty acid fraction of the green stage of *B. braunii*[384] but no evidence can be advanced to support the occurrence of long-chain α,β-unsaturated fatty acids in this organism, although such compounds have been isolated from pollen.[397]

CUTICULAR LEAF WAXES :

CHENOPODIUM ALBUM L. AND LOLIUM PERENNE L.*

Plant waxes have been the subject of increasing study in recent years.[368-369] Investigations have included the isolation of novel compounds[368-371] and detailed studies of biosynthesis.[372] A recent report[373] described the occurrence of long chain n-aldehydes in cabbage, apple and broccoli waxes and showed that reexamination of previously studied waxes can demonstrate the presence of unexpected lipid classes. As part of a study of the penetration of herbicides into leaves (by the Liverpool group*), the less familiar waxes of two plant species have been examined: the monocotyledon Lolium Perenne L. (perennial rye grass) and the di-cotyledon Chenopodium Album L. (fat hen).[350]

The separation of Lolium perenne L. wax by column chromatography was described in a preliminary communication by Hamilton and Power.[374] In the present work, better resolution of the more polar lipids was obtained by TLC, which resulted in separation of the wax into five discrete fractions (Table 30). Fractions I and II contained the hydrocarbons and esters, respectively. Fraction III, which was not separated from Fraction II by column chromatography, gave a yellow colour when treated with 2,4-dinitrophenyl hydrazine reagent. The composition of this fraction from L. perenne and C. album is described here.

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* Preliminary practical work was carried out by Dr. R.J. Hamilton, Miss J.E. Allebone and Mr. D.M. Power (Liverpool Regional College of Technology) and Dr. B.A. Knights.

TABLE 30

TLC separation of waxes

Fraction	Rf*	Weight (mg)		% of Total Wax	
		L. perenne	*C. album*	*L. perenne*	*C. album*
I	0.86–0.90	5.3	26.8	4.6	8.3
II	0.55–0.58	9.3	24.6	8.0	7.6
III	0.38–0.41	10.6	37.6	9.1	11.6
IV	0.18–0.20	0.7	235.0	0.6	72.5
V	0.0 –0.15	90.4		77.7	
		116.3	324.0	100.0	100.0

* Solvent system: petroleum ether, (b.p. 40–60°): ether 19:1.

TABLE 31

Retention indices (I) and mass spectral data (Molecular ion M^+) for Components of Fraction III (*L. perenne* L) and corresponding derivatives

Fraction III		Authentic aldehydes (from grape)		Fraction III O-Methyloximes		Reduced fraction III	Standard n-alcohols		Reduced fraction III acetates		Standard n-alcohol acetates		Reduced fraction III TMSi ethers	
(i)		(i)		(ii)		(iii)	(iii)		(iii)		(iii)		(ii)	
I	M	I	Chain length	I	M	I	I	Chain length	I	M	I	Chain length	I	M
							2580	nC_{22}			2730	nC_{22}		
							2780	nC_{24}			2930	nC_{24}		
2960	380	2960	nC_{26}	3015 3075	409	2980	2990	nC_{26}	3135	424	3140	nC_{26}	2935	454*
3160	408	3160	nC_{28}	3215 3280	437	3180	3190	nC_{28}	3335				3140	482*
3360	436	3360	nC_{30}	3420 3485	465	3380			3535				3340	510*
									3735				3540	

(i) 1.5 % OV-17 7 ft. column 270°C.
(ii) 3 % OV-17 9 ft. column 246°C.
(iii) 1.5 % OV-17 7 ft. column 250°C.
* Molecular ion not recorded. Ion of highest mass recorded was (M − 15).

Experimental

Fraction III: L. perenne

Fraction III was present to the extent of 9.1% by weight of the total wax. The presence of aldehydes was indicated by infrared spectroscopy (carbonyl absorption at 1725 cm^{-1}) and nuclear magnetic resonance (NMR) spectroscopy (triplet at 0.3 :CHO and doublet at 7.7 : CH_2CHO). The absence of ethylenic and acetylenic unsaturation was confirmed by NMR and silver nitrate TLC.

Analysis by GLC indicated the presence of three major and six minor components whose retention data (Table 31) corresponded to the values for authentic n-aldehydes, both synthetic[375] and extracted from grape fruit wax.[376] The components of Fraction III were reduced to the corresponding alcohols, which were analysed by GLC both free and as their acetate and trimethylsilyl ethers. These results (Table 31) confirmed the identity of the major components as n-C_{26}, n-C_{28} and n-C_{30} aldehydes with the minor components as homologues.

Fraction III wax material was treated in ethanol solution with excess sodium borohydride at 50° for 1 hour. After acidification (HCl) the mixture was extracted with ether, the ethereal solution dried ($MgSO_4$) and solvent removed under vacuum. The reduced material was purified by TLC.

The free aldehydes, their O-methyloximes, and the acetates and TMS ethers of the alcohols obtained by reduction of the aldehydes were examined by GLC and GC-MS. Results are summarised in Table 31. All of the mass spectra were consistent with the structures indicated.

Fraction III: C. album

Fraction III was present to the extent of 10.6% by weight of the total wax. Infrared and NMR spectroscopy gave similar results to those obtained for L. perenne, indicating the presence of aldehydes. However, absorption in the infrared at 1738 cm^{-1} and 1250 cm^{-1}

suggested the presence of an acetate group. The fraction from C. album was analysed by GLC and proved to be more complex than that from L. perenne. The retention data (Table 32) suggested that two homologous series of compounds were present. The first series (80.8% of the total) contained thirteen members (peaks 1, 2, 3, 6, 7, 9, 10, 12, 13, 15, 16, 18 and 20) with peak 15 predominating, and the second series consisted of six members (peaks 5, 8, 11, 14, 17 and 19). Members of the principal series had identical retention data to the aldehydes in the corresponding L. perenne fraction. GLC retention data for the other series corresponded with that obtained for authentic acetates of n-alcohols.

Spectra were obtained for peaks 11, 12, 14, 15, 17 and 18. Peaks 12, 15 and 18 gave spectra identical to those of n-hexacosanal, n-octacosanal and n-tricontanal. Peaks 11, 14 and 17 gave molecular ions at m/e 396, 424 and 452 and major fragment ions at m/e 336, 364 and 392, respectively. These data are consistent with the formulation of peaks 11, 14 and 17 as acetates.

The components of Fraction III, after treatment with O-methylhydroxylamine were re-examined by GC-MS. Peaks 11, 14 and 17 were unchanged, again giving typical acetate fragmentations. The aldehydes (peaks 12, 15 and 18) formed the expected O-methyloximes.

It has been shown that surface waxes of Lolium perenne L. contain a mixture of nine aldehydes with chain lengths n-C_{25}-n-C_{34} and that n-hexacosanal is the major component. Similarly, Chenopodium album L. wax contains thirteen aldehydes, with n-octacosanal as the principal component. Reports of the occurrence of n-aldehydes in plant waxes are becoming more frequent[373,376] and it has been suggested[376] that the use of alumina in the past, for the chromatographic separation of waxes, has led to destruction of the aldehydes. Kolattakudy[377] recently demonstrated that administration of ^{14}C-labelled precursors

TABLE 32

GLC data for *C. album* and *L. perenne* waxes

C. album fraction I alkanes (a)			*C. album* fraction III aldehydes and acetates (b)				*L. perenne* fraction III aldehydes (c)		
I	Chain length	Area %	Peak No.	I	Chain length	Area %	I	Chain length	Area %
2200	nC_{22}	0.6	1	2170	nC_{18}	0.3			
2300	nC_{23}	1.0	2	2280	nC_{19}	0.1			
2400	nC_{24}	0.5	3	2380	nC_{20}	0.1			
2500	nC_{25}	2.7	4	2500		0.1			
2600	nC_{26}	0.5	5	2540	nC_{20}	Trace			
2700	nC_{27}	14.2	6	2580	nC_{22}	0.4			
2800	nC_{28}	1.4	7	2680	nC_{23}	0.2			
2900	nC_{29}	64.6	8	2730	nC_{22}	0.1			
3000	nC_{30}	1.1	9	2760	nC_{24}	2.9			
3100	nC_{31}	12.8	10	2870	nC_{25}	0.6	2860	nC_{25}	1.6
			11	2930	nC_{24}	3.3			
			12	2960	nC_{26}	13.6	2960	nC_{26}	55.7
			13	3060	nC_{27}	1.2	3060	nC_{27}	1.2
			14	3120	nC_{26}	4.7			
			15	3160	nC_{28}	46.0	3160	nC_{28}	22.3
			16	3260	nC_{29}	0.3	3260	nC_{29}	Trace
			17	3320	nC_{28}	10.5			
			18	3360	nC_{30}	14.6	3360	nC_{30}	15.8
			19	3510	nC_{30}	0.6			
			20	3560	nC_{32}	0.3	3560	nC_{32}	1.7
							3760	nC_{34}	1.5
							3960	nC_{36}	Trace
		100.0				100.0			100.0

(a) 1 % OV-17 at 230°; 5′ column
(b) 1 % OV-17 at 240°; 5′ column
(c) 1 % OV-17 at 260°; 5′ column

to the leaves of Brassica spp. resulted in the incorporation of the isotope into a fraction whose composition was unknown. Schmid and Bandi[373] have shown that this fraction contained aldehydes and they drew attention to the fact that the major components of the alkane, ketone and secondary alcohol fractions of cabbage wax were C_{29} compounds, whereas n-triacontanal was the major aldehyde. Although no direct precursor/product relationship could be demonstrated, it was suggested that aldehydes might be implicated in alkane biosynthesis.

The results presented here show no obvious biosynthetic correlation between aldehydes and n-alkanes. Thus the major aldehydes in L. perenne and C. album are n-hexacosanal and n-octacosanal respectively, whilst the major alkane in both species is n-nonacosane. Furthermore, a similar situation has been reported for grape wax. It is, therefore, possible that the apparent correspondence in chain length between the major components of the aldehyde and hydrocarbon fractions in cabbage and apple may be fortuitous.

In addition, the fraction from C. album was found to contain acetates of alcohols with even-numbered chain lengths n-C_{22}-n-C_{30}. We believe that whilst acetates have not been reported in plant waxes, it is possible that they may be of much wider distribution than has been realised, since saponification has often been the first step in previous workers' analysis.

V

AIR POLLUTION

GC-MS ANALYSIS OF AIR POLLUTANTS : A FEASIBILITY STUDY

A preliminary investigation has been carried out to explore the possibilities of carrying out small scale analyses of organic constituents of particulate air pollution. The method used involved solvent extraction, TLC, GLC and GC-MS.

Experimental

A total of 10 g. of dust was collected from the filters of the air-conditioning units ("Airking Aries" Model; Stewart King Industries Ltd., Waterlooville, Hants.) in the GC-MS laboratory of this Department. These units had only recently been installed and the filters had not previously been cleaned: consequently, they were relatively "clean". The dust was removed from the filters only by gentle tapping so as not to collect particles of filter material (about 10-20% of the dust was collected for study).

1 g. of the dust was shaken with 7.5 ml ether, allowed to stand at room temperature for 0.5 hr, and centrifuged. The ether extract was concentrated to a volume of 0.5 ml under a stream of nitrogen, and applied to a TLC sheet (Chromar 1000). Six fractions were obtained by development with n-heptane (Table 37). The TLC sheet was cut up and individual fractions were extracted with chloroform/methanol. Yields are given in Table 37.

Fraction 1 was examined by GC and GC-MS and found to contain a series of n-alkanes: $C_{20}H_{42}$ to $C_{35}H_{12}$, with n-$C_{30}H_{62}$ predominating. Aliphatic hydrocarbons have been identified in other dust samples[398] during a study by Gelpi et al. on the ubiquity of hydrocarbons in nature.

Fraction 3 gave many peaks on GLC, with retention indices in the range 2000-3500. On examination by GC-MS, the majority of these

TABLE 37.

TLC separation of ether extract of filter dust

Fraction 1	absorbed U.V.	40 mg
" 2	fluoresced blue under U.V.	0.3 mg
" 3	visible yellow	0.7 mg
" 4	visible yellow	0.2 mg
" 5	fluoresced "white" under U.V.	3.6 mg
" 6	absorbed U.V.	0.8 mg

components were found to give extremely intense molecular ions, with little additional fragmentation. They are probably polycyclic aromatic hydrocarbons. There is considerable interest in polycyclic aromatic hydrocarbons, because of their potential carginogenic properties, and various procedures have been devised for their identification.[399] They have been found in the atmosphere in some large American cities,[400] Sydney,[401] Merseyside,[402] Newcastle-on-Tyne,[403] and Naples.[404]

In an attempt at characterising the suspected polycyclic aromatic hydrocarbon substituents of the dust, several authentic samples (CLXIV-CLXXV) (from the collection of Prof. Sir James Cook, by courtesy of Prof. J.D. Loudon) were examined by TLC (Table 38), GLC and GC-MS (Figs. 102-113). The multiple elution - TLC technique devised by Petrowitz[405] afforded no better resolution than the conventional single elution method when chromar 1000 sheet was used.

Component 1 gave a mass spectrum with an intense ion (m/e 215, 65%) corresponding to methyl radical loss from the molecular ion (m/e 230, 100%). This would indicate a structure such as 10-methylbenzanthrene (CLXXVI).

Component 2, with molecular ion at m/e 228 (100%), gave no significant fragment ions. It may be an isomer of chrysene (CLXX).

Component 3 provided a molecular ion at m/e 242 (100%) which indicates a molecular formula $C_{19}H_{14}$. No reasonable multihexagonal aromatic ring structure is possible for this compound.[406]

In the spectrum of component 4, there is an ion of m/e 239 (35%) which may be formed by loss of HO$^{\bullet}$ from the molecular ion (m/e 256, 100%). A possible structure for this component is 10-hydroxynaphtho-[2'.7':1.8]-anthrene (CLXXVII).

Component 5 (molecular ion: m/e 252, 100%) has mass spectrum and retention index similar to those of perylene (CLXIV), 1,2-benzpyrene

TABLE 38.

Perylene (CLXIV)	$C_{20}H_{12}$	252	2680	0.40	0.64	0.78
1,2-Benzpyrene (CLXV)	$C_{20}H_{12}$	252	2680	0.46	0.68	0.78
3,4-Benzpyrene (CLXVI)	$C_{20}H_{12}$	252	2680	0.46	0.68	0.78
Coronene (CLXVII)	$C_{24}H_{12}$	300	3310	0.44	0.68	0.76
1,2-Benzanthracene (CLXVIII)	$C_{18}H_{12}$	228	2330	0.48	0.72	0.82
1,2-Benzonaphthacene (CLXIX)	$C_{22}H_{14}$	278	3010	0.34	0.54	0.66
Chrysene (CLXX)	$C_{18}H_{12}$	228	2350	0.46	0.68	0.80
1,2-Dimethylchrysene (CLXXI)	$C_{20}H_{16}$	256	2580	0.42	0.66	0.78
1,2:5,6-Dibenzanthracene (CLXXII)	$C_{22}H_{14}$	278	3000	0.34	0.52	0.64
1,2:7,8-Dibenzofluorene (CLXIII)	$C_{21}H_{14}$	266	2710	0.36	0.56	0.68
10-Methyl-1,2-benzanthracene (CLXIV)	$C_{19}H_{14}$	242	2480	0.44	0.66	0.78
9,10-Dimethyl-1,2:5,6-dibenzanthracene (CLXXV)	$C_{24}H_{18}$	306	3200	0.34	0.52	0.66

* A = single elution
B = double elution
C = triple elution

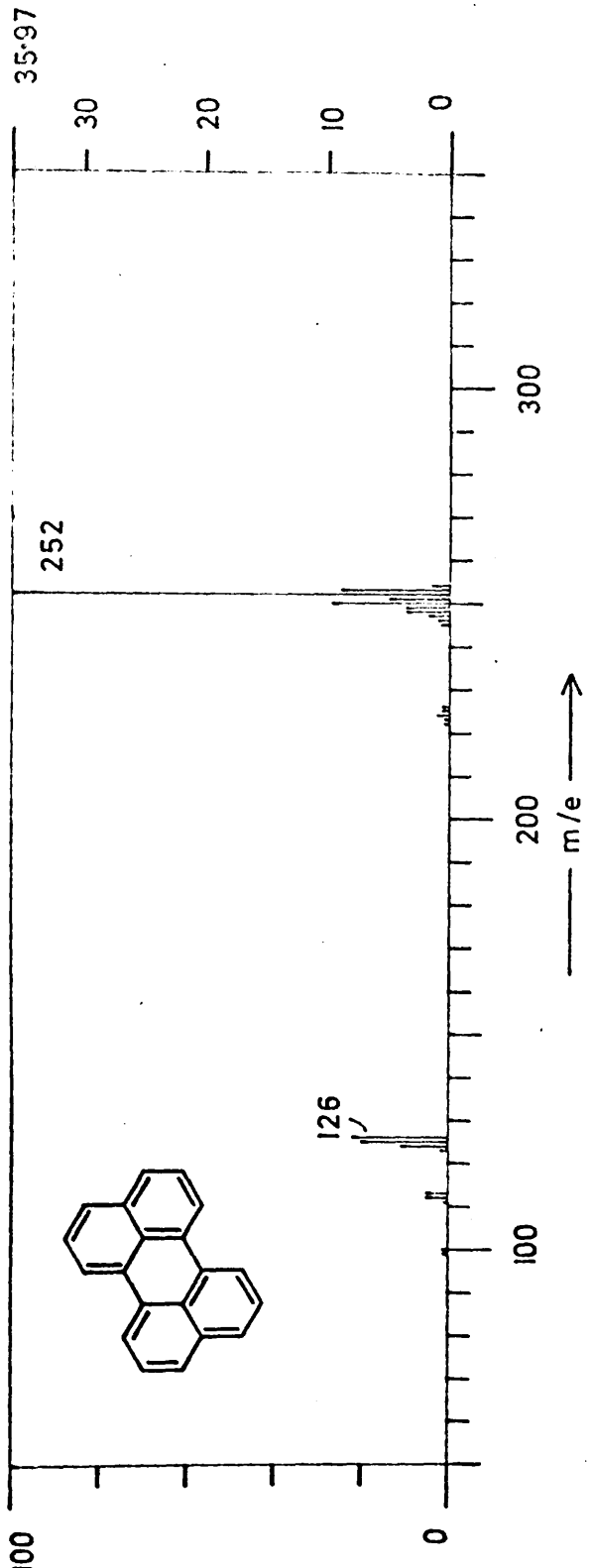
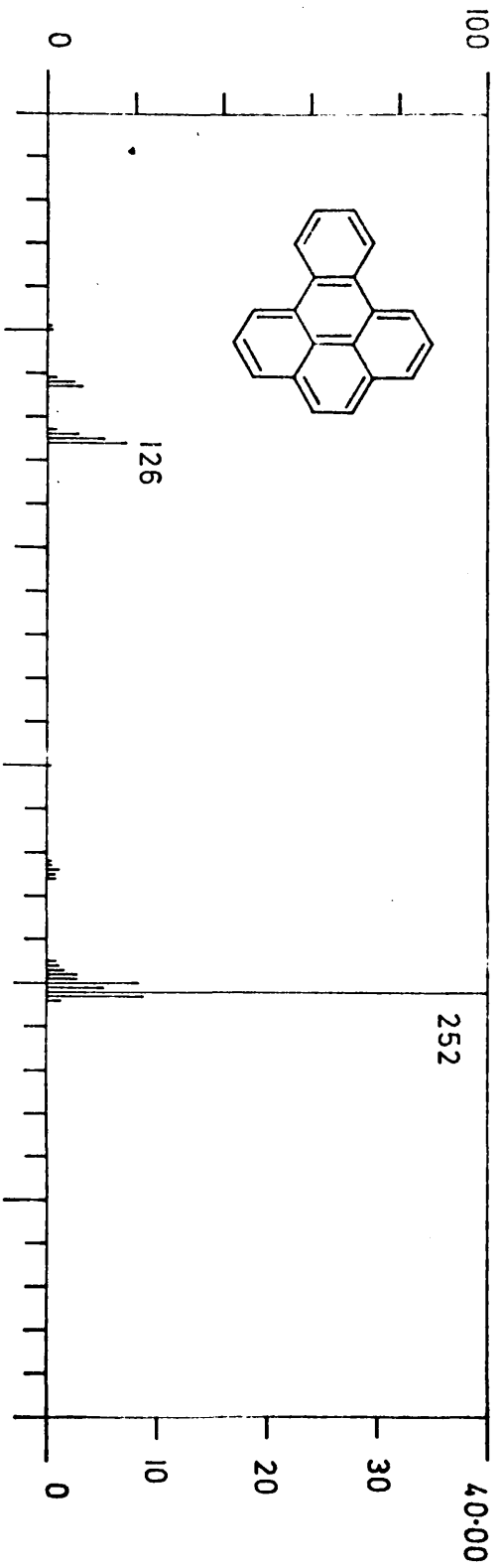


Fig 102



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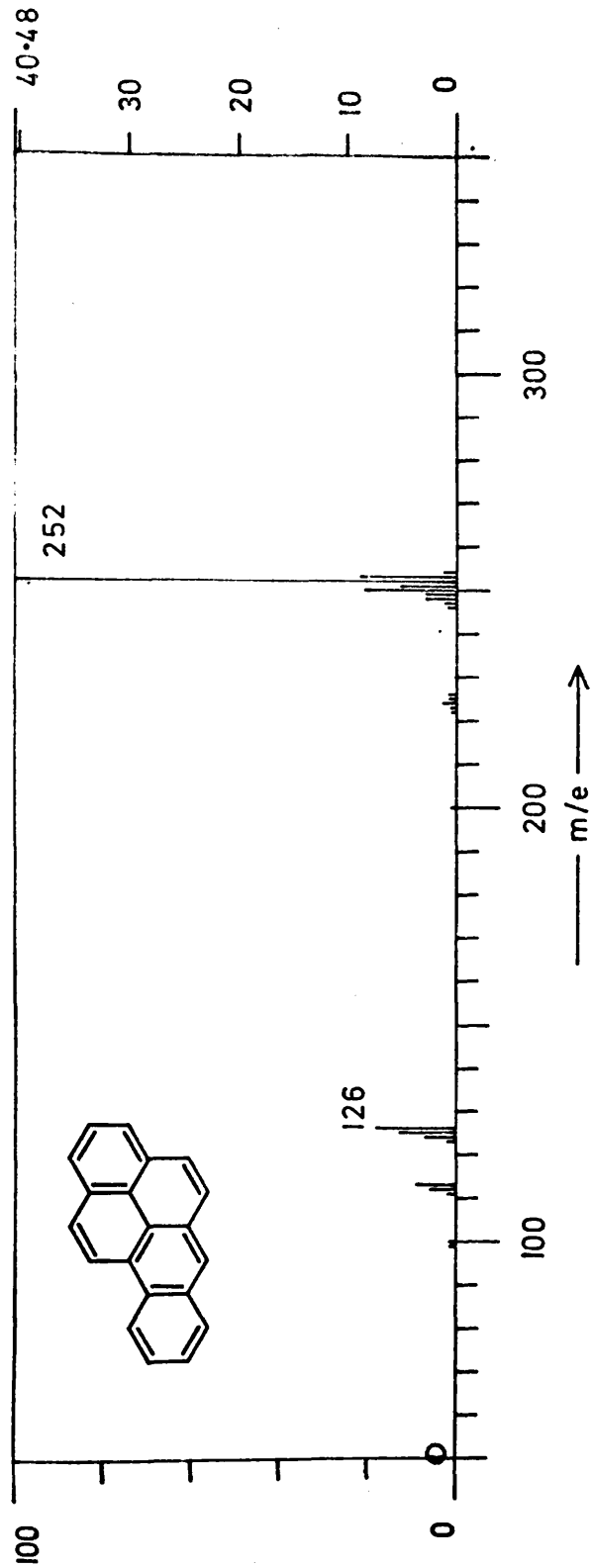

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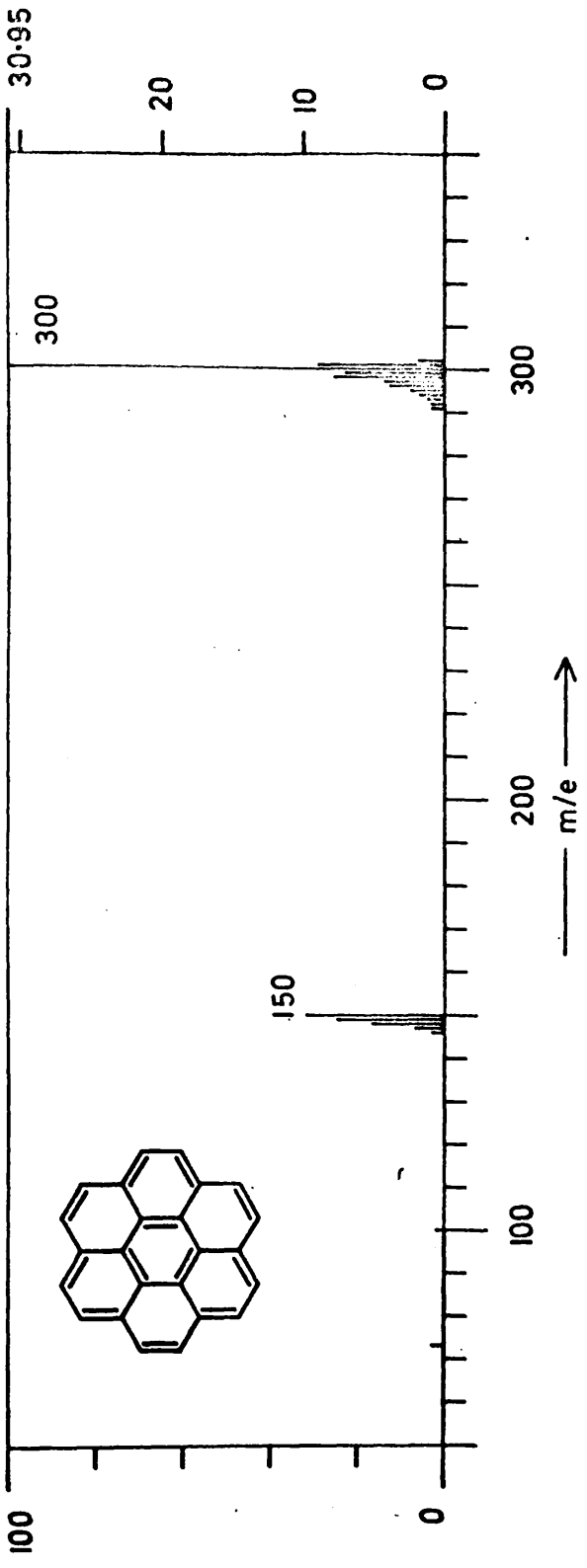

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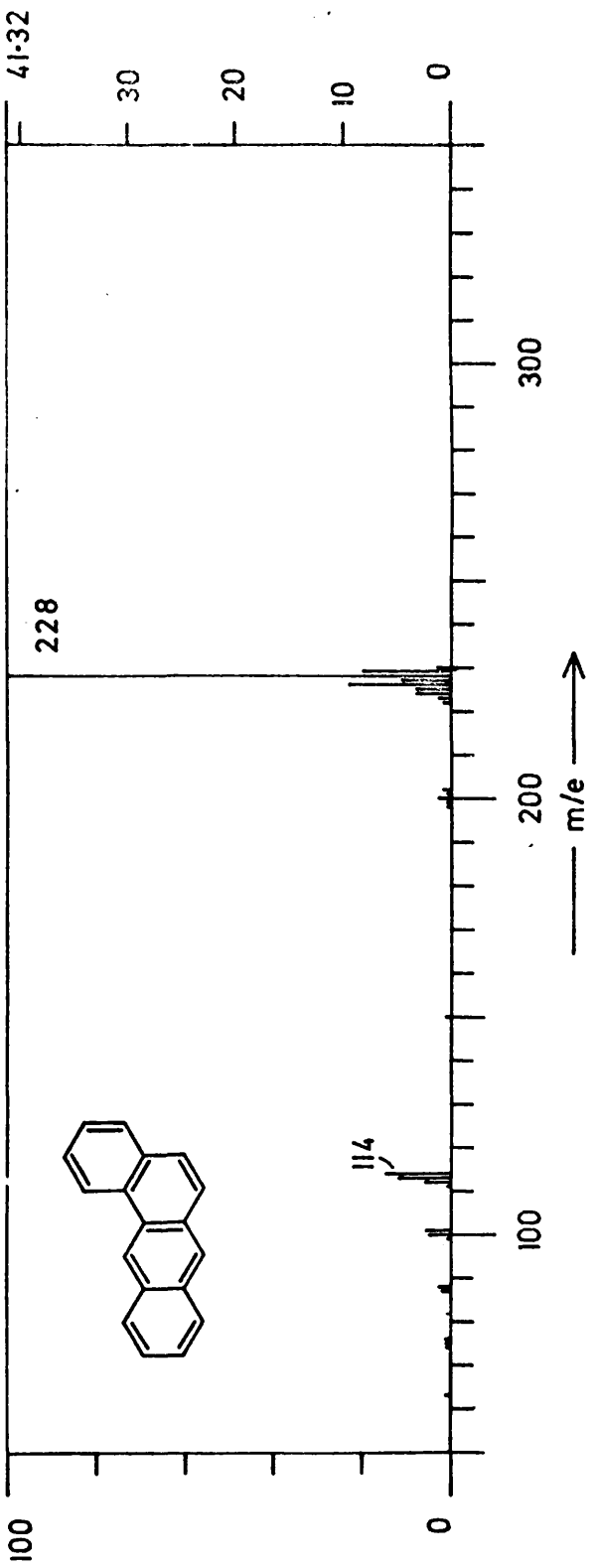


Fig. 106

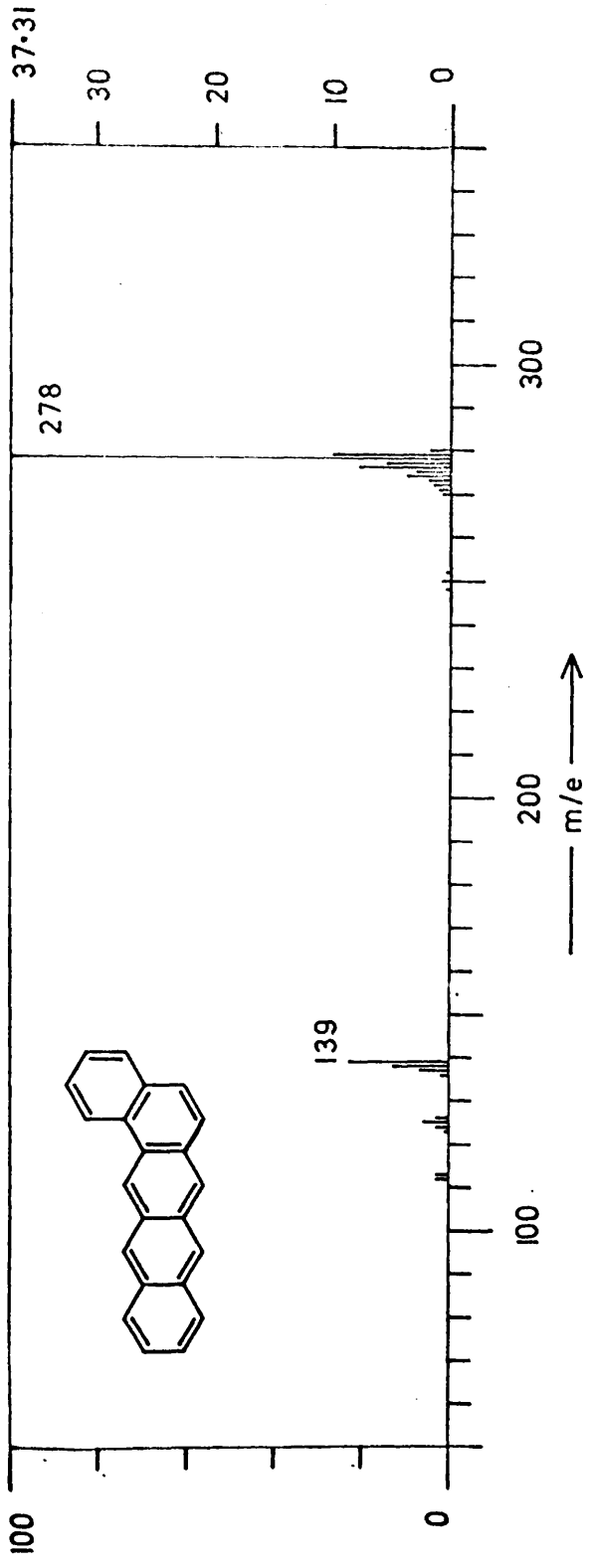


Fig. 107

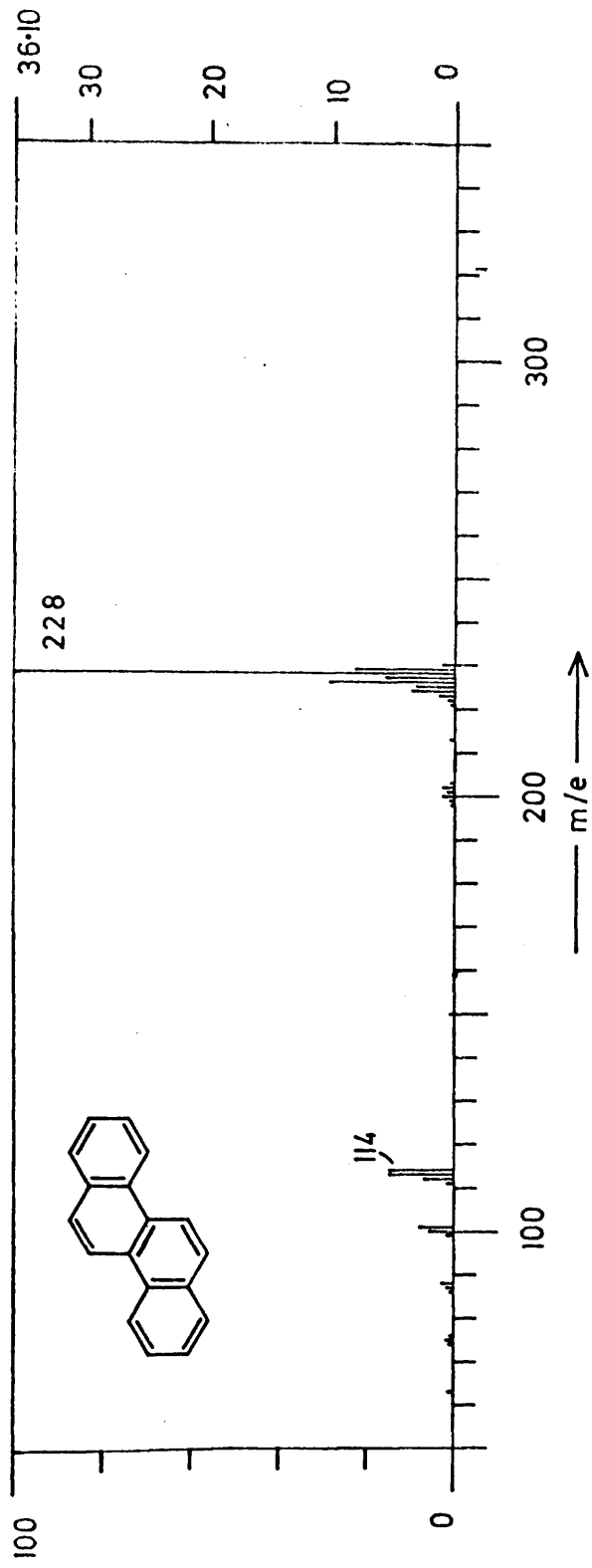


Fig 108

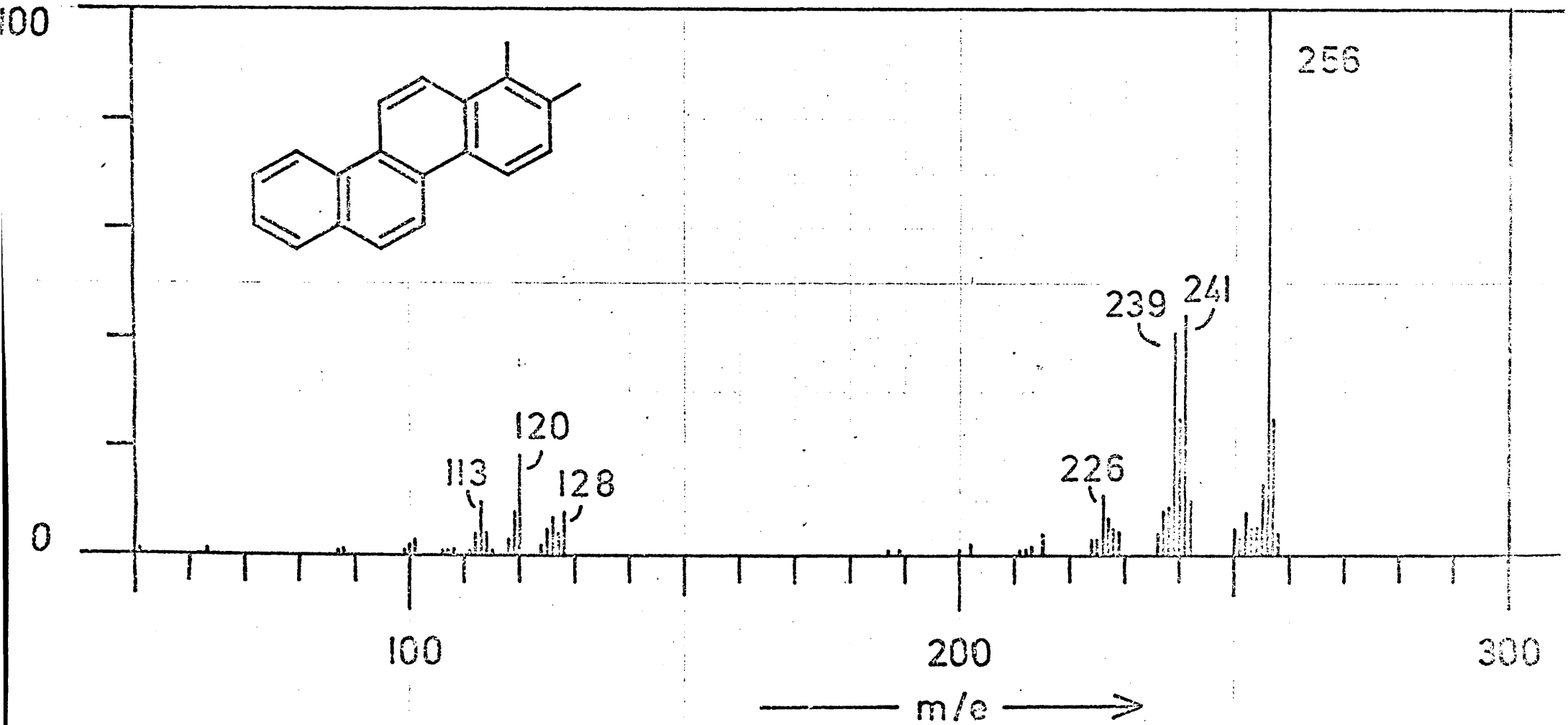
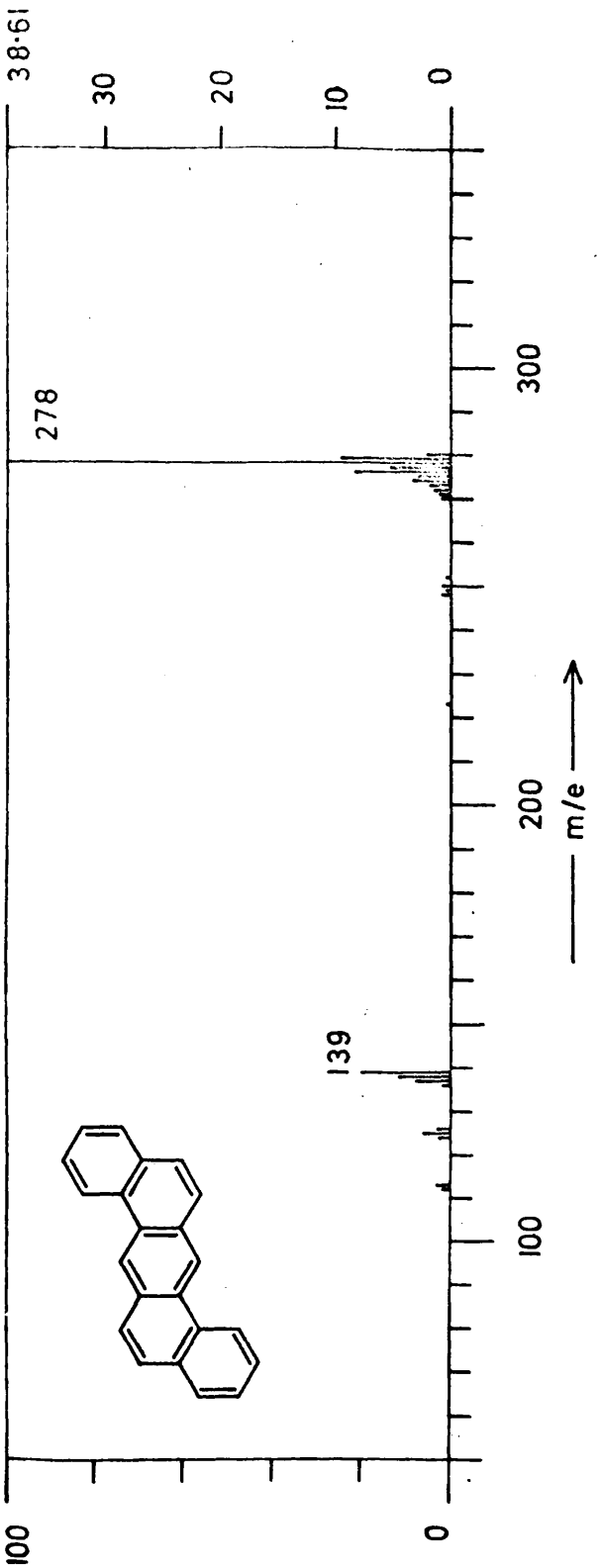


Fig. 109



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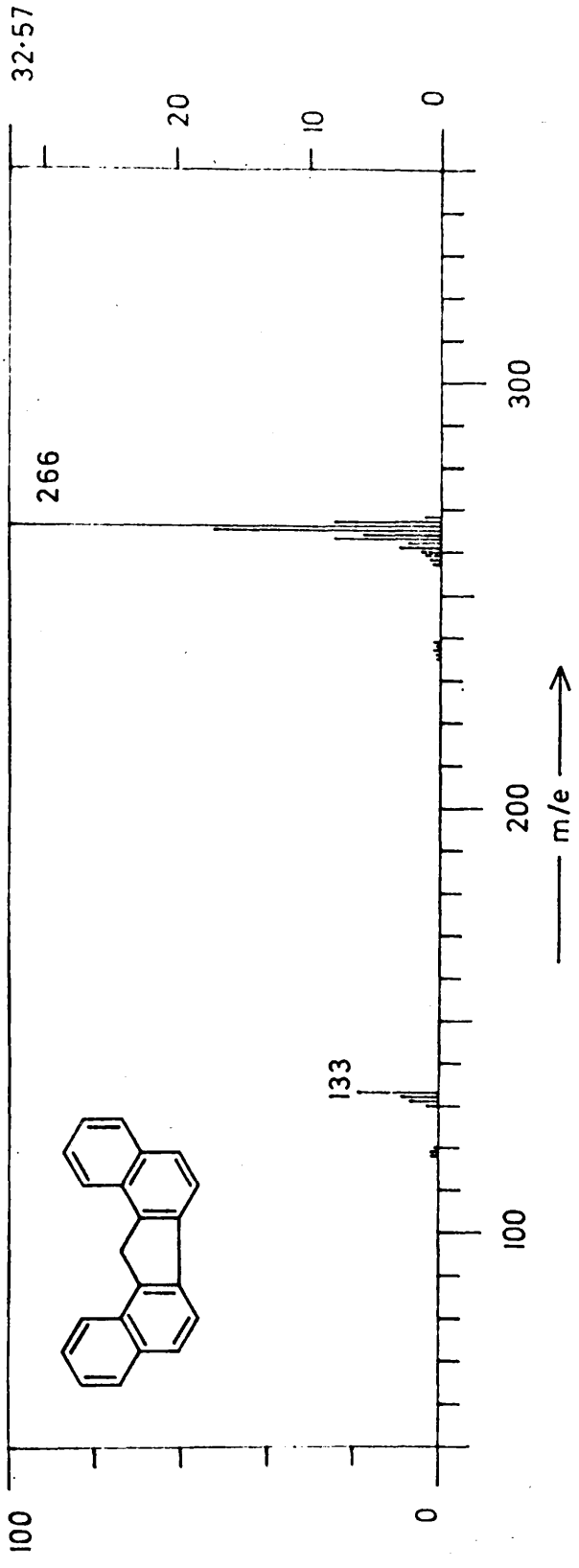


Fig 111

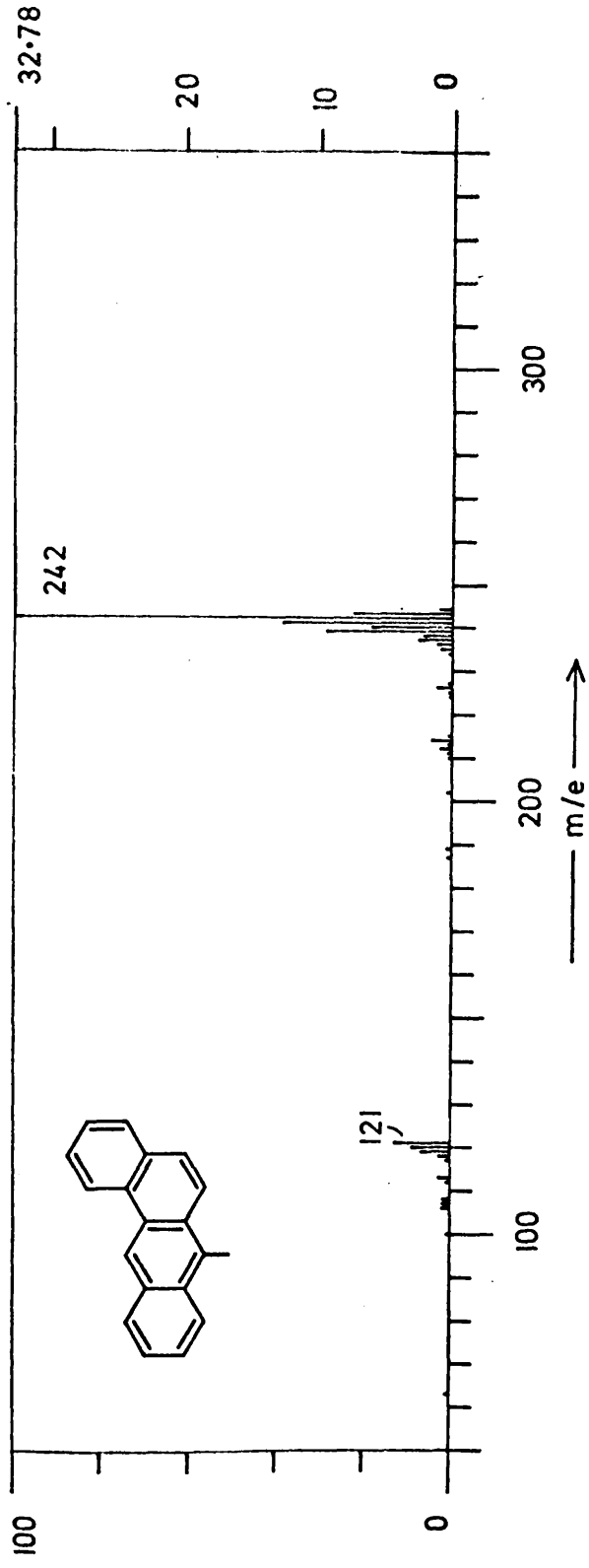


Fig 112

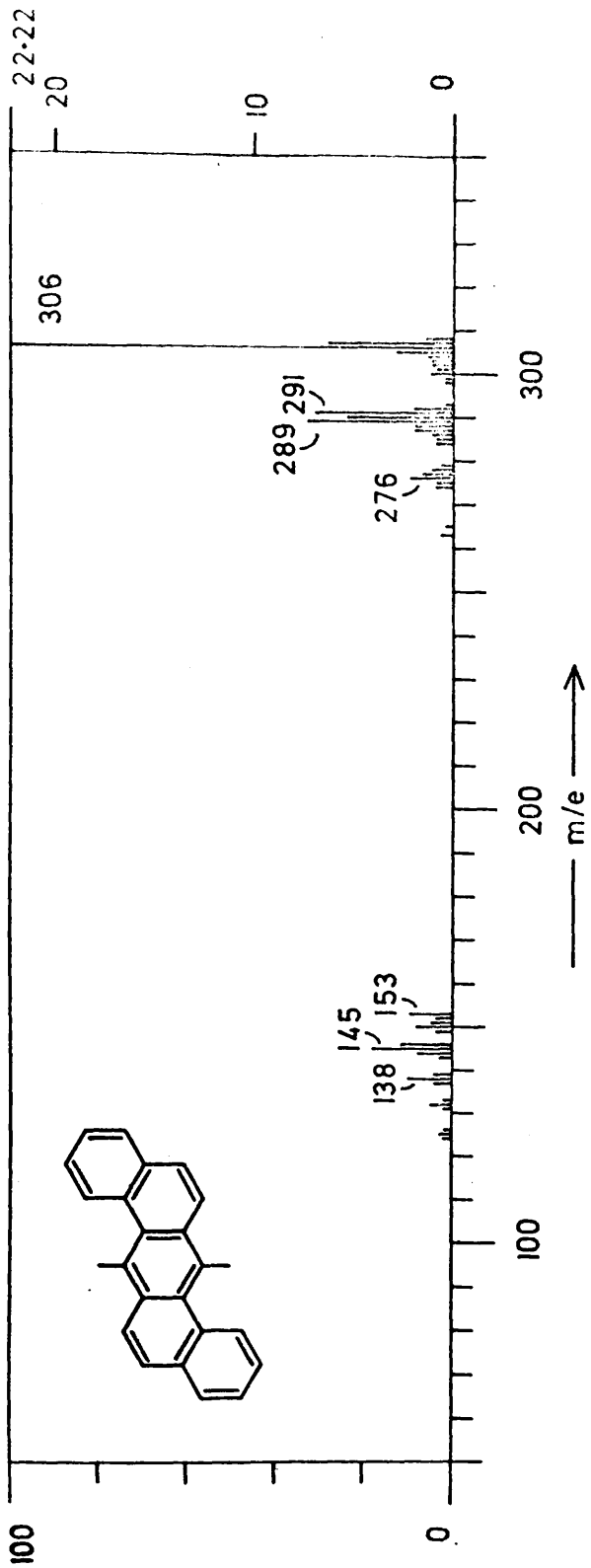
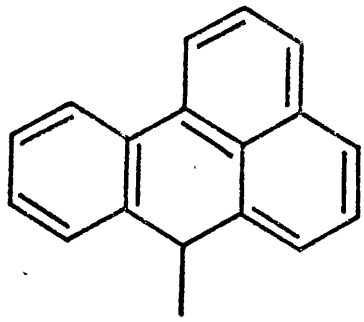
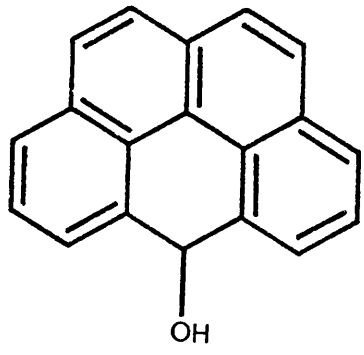


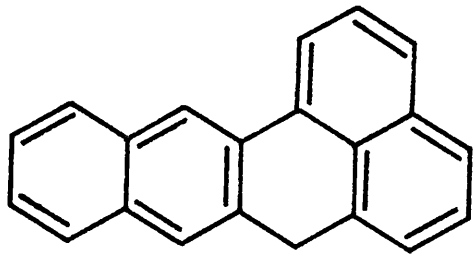
Fig 113



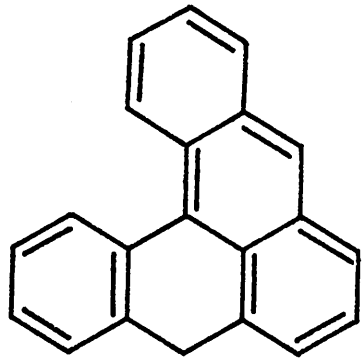
CLXXVI



CLXXVII



CLXXVIII



CLXXIX

(CLXV) and 3,4-benzpyrene (CLXVI).

Component 6 gives an intense peak at m/e 252, but this may be the "tail" of component 5. In fact, it was observed that CLXIV-CLXVI "tail" rather badly on GLC. An intense ion is observed at m/e 268 which may be ascribed to a molecular ion of composition $C_{21}H_{16}$.

The mass spectrum of Component 7 (molecular ion; m/e 266, 100%) suggests a dibenzanthrene (e.g. CLXXVIII) or coeranthrene (CLXXIX) structure.

Fraction 5 gave two major peaks on GLC, but these could not be readily identified by GC-MS: ions of m/e greater than 400 were observed and molecular ions were not distinct.

Conclusions

This brief study has shown that it is possible to analyse fairly small quantities of pollutants in a short time. It would thus be suitable especially for comparative studies.

It has been demonstrated that the air conditioner filters contain appreciable quantities of n-alkanes and significant amounts of polycyclic aromatic hydrocarbons. This filter dust was chosen because it was a ready supply of material suitable for a preliminary feasibility study. It is doubtful, however, whether dust collected in a chemistry laboratory is representative of city air pollution. Further investigations could be carried out on samples collected elsewhere, possibly with a portable vacuum cleaner fitted with a glass wool filter.

More efficient and selective extraction and "clean-up" procedures could be devised. Liberti et al.[404] carry out Soxhlet extraction with cyclohexane andremove polycyclic aromatic hydrocarbons with nitromethane. It was found that the authentic samples partially decomposed during TLC, probably with formation of peroxides. Better results may be obtained in the absence of light. GLC was carried out using OV-1 as stationary phase, effecting separation mainly by molecular

weight differences. Consequently, perylene and the benzpyrenes were not resolved. Using OV-17, marginally different retention indices were observed for these isomers, but they were not resolved in admixture. SE-52 has been suggested[407] as a more selective phase and the additional phenylsiloxane groupings present in OV-25 may be expected to improve distinctions between isomeric polycyclic aromatic hydrocarbons.

With improved extraction and separation methods, more sophisticated investigations could be carried out by GC-MS. In particular, components of selected molecular weights could be located by single ion monitoring. Fuller interpretation of the mass spectral data would be possible if a wider range of reference samples were examined.

VI

DATA HANDLING

DATA-HANDLING AND GC-MS

The major practical problem of GC-MS analysis is a consequence of a factor contributing to the efficiency of the technique: viz., the rate at which data are produced. It is not unusual for hundreds of spectra to be obtained in the course of one day, particularly if complex natural product mixtures are being investigated. The difficulties formerly encountered in assigning mass numbers to peaks in the spectra have now largely been overcome. (For a brief discussion, see the following section). There remains the onerous task of converting the data to a form in which they can be readily utilised (as tabulated ion abundances, or as line diagrams) and of correlation and interpretation of the spectra. These procedures are efficiently effected with the aid of a digital computer.

At the outset of this project, comparatively little work had been done on computerised data handling systems for mass spectrometry. Semi-automatic devices had been devised for measuring and drawing mass spectra, and these were capable of adaptation to provide punched paper tape for off-line computer processing.[408-410] An alternative approach involved recording of the data in analog form for processing by a computer with an analog-to-digital converter (ADC),[411] or recording of digitised data.[412-413] It was, however, considered desirable to carry out data acquisition and processing on-line and in real-time.

At this time, Hites and Biemann had published details of a data-acquisition system which involved regularly repetitive scanning of spectra throughout chromatographic runs.[414] Mass calibration was performed by comparison of arrival times (at the detector) of ions in spectra of known and of unknown compounds. In such a system, much information which is largely redundant must be processed if spectra of only the components of mixtures under investigation are required,

as is usually the case in GC-MS analysis. Moreover, large-scale computing facilities are required for all but the briefest of GC-MS runs (Hites and Biemann used an IBM 1800 computer with a relatively large magnetic disc store). Ryhage and co-workers at the Karolinska Institutet in Stockholm were, at this time, investigating the feasibility of incorporating the LKB 9010 mass marker, which they had earlier developed, into a data-handling system in which only one intensity value per mass number would be recorded. As a result of initial discussions with Dr. R. Ryhage, Dr. S. Wikström and Mr. S. Melkersson (January 1968), it was decided to adopt this system. (A generous grant from the Science Research Council provided for the purchase of a mass marker unit). A major factor which influenced this decision was the limited nature of on-line computing facilities available. No dedicated computer was available for mass spectral data acquisition and processing, but we were offered the part-time use of a PDP-8 computer (linked directly to a KDF 9 computer) situated in the Computing Services Department (at that time, part of the Department of Computing Science). This led to the initiation of the required computer programs and to the installation of the necessary cables and interfacing components during 1968-70. Dr. R.N. Stillwell, a visiting worker on leave from Baylor College of Medicine, Houston, U.S.A. (July-September 1969) made a major contribution to the programs, while valuable advice and help was provided until September 1970 by Mr. A.D. Wilkinson of the Department of Computing Science.

Two output signals are produced by a gas chromatograph-mass spectrometer: mass spectra and TIC. The latter can be constructed from the former so, in general, only the mass spectral output need be measured. Spectra may be scanned from m/e 10 to 1,000 in 1 second, but the scan rate is usually somewhat lower. High frequency electrical noise can be filtered from the signal via a capaciter.

Noise of frequency less than 120 cps, associated with mechanical vibration and the A.C. mains supply, cannot be removed without also damping the spectral record and is a factor limiting sensitivity. Although tabulations of spectra rarely contain significant ions of relative abundance less than a few percent of that of the base peak, it is desirable to record spectra with a dynamic range of about 10,000 to take account of components of widely varying quantity throughout the chromatogram.

Any effective system of data handling should be capable of carrying out all or the majority of the operations which are normally performed manually. A comprehensive system would collect data concerning mass numbers, ion abundances, and metastable ions from spectra, together with their standardised retention times. The output should comprise "normalised" tabulations and line diagrams of individual spectra, with background spectra subtracted and facilities for scale expansion and/or contraction. It is also desirable to automate the "bookkeeping" aspect of GC-MS when large numbers of spectra are handled. Records of scan number, retention time (or retention index) and salient mass spectral data (e.g. base peaks or molecular ions) should be compiled to assist correlation of spectra. A degree of dialogue between the user and the system is desirable at this stage.

In designing the system for the Glasgow University GC-MS Unit, priority was given to development of an effective means of data acquisition with the available hardware with a view to later expansion if and when further facilities become available. The PDP-8 computer used was fitted with a single-channel ADC with no multiplexer. Pulses from the mass marker were carried to the interrupt bus of the PDP-8. The scan start and stop were signalled to the computer by superimposing a potential of -10 V. on the analog channel: this line was at OV. between scans, and the mass spectrum was added to the -10 V. signal during scans. These values were convenient since the analog

output from the LKB 9000 was within the range 0 to 10 V., whereas the ADC on the PDP-8 accepted signals within the range -10 to 0 V.

It was not possible to obtain a grounded output directly from the LKB 9000, so an interface was constructed (based on a design kindly supplied by S. Melkersson, with modifications suggested by J.A. Hardy) incorporating an active filter network of variable bandwidth. This interface also incorporated a relay, operated in turn by the "scan start" relay in the LKB 9000 control unit, which was used to apply -10 V. to the output during scans.

The PDP-8 was programmed to carry out an initial data reduction, and to place on file a set of ion abundances correlated with nominal mass numbers. A teletype unit was provided in the GC-MS laboratory to permit a degree of remote control over the PDP-8 during the data acquisition phase. It is envisaged that the system will be further developed so that subsequent data handling and output (on line printer, incremental plotter, and magnetic tape) could be performed by the KDF 9 or other large computer.

The viability of the system was demonstrated in September 1969, but work was virtually suspended pending the appointment of a full-time programmer (Dr. J.A. Wilson) in October 1970. In the meantime, as a result of further discussions with Drs. Ryhage and Wikström, and Mr. G. Jälkemo (LKB Produkter AB), a more heavily shielded coaxial cable was installed between the LKB 9000 and PDP-8 in an attempt to reduce noise.

There is no provision in the present system for the recognition of metastable ions or ions of non-integral m/e value. This could be effected only with a more complex program and a computer with more core-storage.

Mass spectra are usually produced in hard copy and stored in tabulated form and/or as line diagrams. This method is only wholly suitable if relatively few spectra are involved: otherwise, information

retrieval tends to be slow and inefficient. It has been suggested that spectra be stored on edge-punched cards,[167,415] but rapid sorting is not possible with large numbers of cards. The use of IBM punched cards and a mechanical sorter[416] has been found to be impracticable.[417] Initial trials with optical coincidence systems,[417-418] whereby large numbers of partial spectra are stored on relatively few cards, appear to show promise.

With the increasing availability of large collections of reference spectra, it is apparent that computer-assisted retrieval systems are necessary. Various approaches have been discussed,[419-421] and the methods now employed appear to be quite satisfactory.[422] Procedures have been devised for computer-assisted characterization of spectra which do not require full searches of reference spectra,[423-424] and a recent innovation has been the application of computerized learning machines to the problem.[425] Even though GC-MS usually provides spectra of relatively "pure" components, computers can be used to identify components of mixtures from their mass spectra.[426-427]

PERFLUORODECALIN AS A MASS CALIBRATION STANDARD FOR LOW RESOLUTION MASS SPECTRA

In low-resolution mass spectrometry, the integral mass of each peak has to be determined beyond doubt. Several automatic mass markers are commercially available, but each is designed for use only with a particular type of instrument. A more widely applicable method of mass calibration involves the simultaneous introduction of a reference standard into the spectrometer. The most commonly used reference standards are the perfluoroalkanes.[428] However, there is a predominance of ions of low mass in their spectra, and a relatively high partial pressure of standard must be employed if the peaks of higher m/e are to be used for mass calibration. The detector, amplifier, and recorder will then be overloaded at the low mass end of the spectrum. Other compounds such as heptacosafluorotri-n-butylamine and perfluoroalkyl-s-triazines have been used[75,429,430] but these suffer from the same disadvantage: the perfluoroalkyl substituents readily undergo cleavage to form fragment ions of low mass.

In a search for more suitable markers, the mass spectrum of a bicyclic fluorocarbon, commercial perfluorodecalin, was examined (Fig. 114).[431] Although the ion of m/e 69, which may be ascribed to CF_3^+, is the base peak, its relative contribution to the total ionization is much less than for the standards previously employed because it cannot be produced by simple fragmentation. The mass range extends conveniently to m/e 462 (molecular ion), and peaks are well distributed throughout the spectrum. At high concentrations a peak is observed at m/e 481, due to an ion-molecule reaction in the ion source. Several metastable peaks are present, and these are an aid to the rapid location of fragment peaks. Perfluorodecalin is a liquid with sufficient volatility at room temperature to obviate

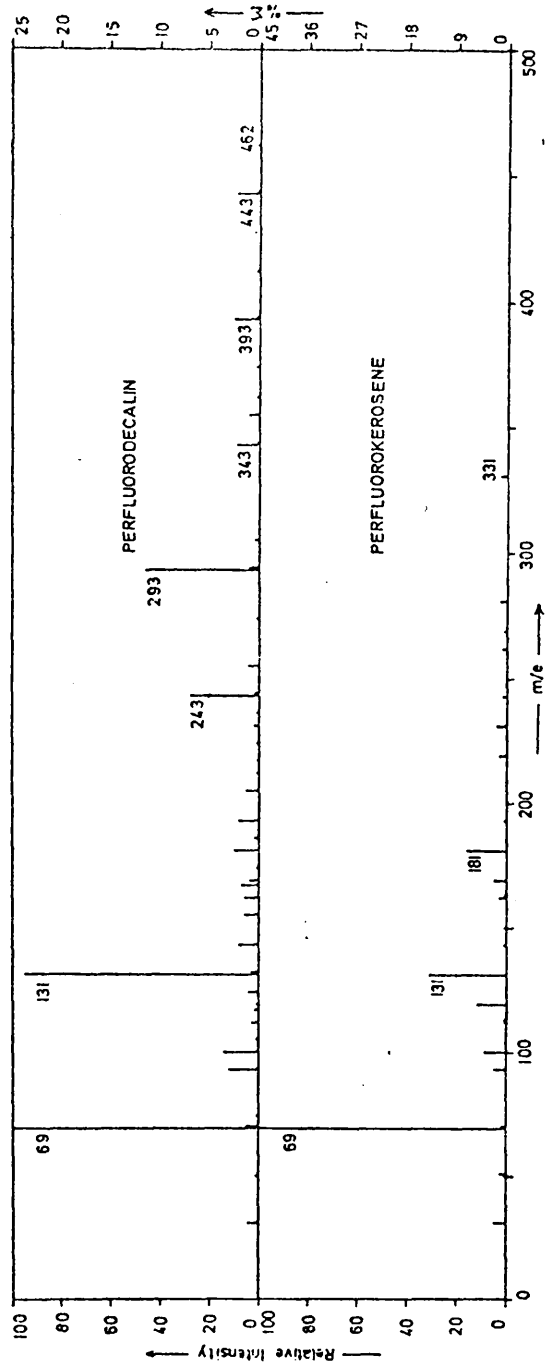


Fig.114 Line Diagrams Representing Mass Spectra of Perfluorodecalin and Perfluorokerosene.

the need for a heated inlet system; it can be introduced from a cold reservoir fitted with a suitable valve and pumping line. It is chemically inert and can be pumped away from the spectrometer within seconds.

Perfluorodecalin is accordingly a suitable calibration standard for use in mass spectrometry, particularly with low resolution instruments which are extensively used for the mass range m/e 1-500. Perfluorodecalin has been used in this department and elsewhere for several years as a reference for accurate mass measurement by the peak matching method, its advantages in this respect being essentially as outlined above.[432]

EXPERIMENTAL

The perfluorodecalin was obtained from Ralph Emanuel Ltd, Middlesex, England.

The mass spectrum was obtained using an LKB 9000 mass spectrometer under the following conditions: electron energy, 70 eV; ion source temperature, 270°C; accelerating voltage, 3.5 kV. The sample was introduced from a cold glass reservoir fitted in place of the gas chromatograph column.

VII

CONCLUSIONS

CONCLUSIONS

The work described in this thesis was based on applications of the combined technique of GC-MS to a variety of topics in organic chemistry and biochemistry. The research included studies of the scope of the technique (based on model compounds) and applications to actual analytical problems.

Following the introduction, a section of the thesis was devoted to work on steroids. Results obtained with progesterone and testosterone analogues confirmed the value of GC-MS in distinguishing isomers. The use of TMS ether derivatives in GC-MS was well established, but the advantages of (chloromethyl)dimethylsilyl ethers as derivatives for GC-MS had been little explored. The utility of these derivatives was illustrated and discussed for the example of 17α-alkyl-17β-hydroxy steroids. The mass spectral fragmentations of TMS ether derivatives of androst-5-en-3β-ol analogues and of other unsaturated 3β-hydroxy steroids have been investigated. The results of this survey were applied to the characterisation of yeast sterols, sterols from a bacterium (Methylococcus capsulatus) grown on methane, steroidal drug metabolites, and a steroidal enzyme-reduction product.

Corticosteroids cannot be examined directly by GC-MS because of the low thermal stability of the side chain. Earlier work had shown that their boronate derivatives are quite stable. The mass spectra (recorded by GC-MS) of representative corticosteroid boronates were discussed in respect of their use in structural assignments.

Similar difficulties are encountered in the GC-MS of β-hydroxy amines because of their relatively high polarity and low thermal stability. The use of boronate derivatives in the characterisation of catecholamines and related β-hydroxy amines by GC-MS was discussed, and a more detailed investigation of the mass spectral fragmentations of the derived 1,3,2-oxazaborolidines has been carried out.

O-methyloxime (MO) derivatives are of value in the analysis by GC-MS of aldehydes and ketones. Salient features of the spectra of MO derivatives of aliphatic aldehydes and ketones were enumerated. Aldehydes from the cuticular leaf waxes of Chenopodium album L. and Lolium perenne L. have been identified by GC-MS of their MO derivatives. Unsaturated aliphatic hydrocarbons from the green form of the freshwater alga Botryococcus braunii have been ozonised and cleaved to form aldehydes which were identified as their MO derivatives. The structures of the hydrocarbons were thus inferred.

An exploratory study of the use of GC-MS in the analysis of air pollutants was carried out. The gas chromatographic and mass spectrometric properties of some polycyclic aromatic hydrocarbons were surveyed and a number of these compounds were tentatively identified in dust collected from air conditioner filters.

Perfluorodecalin was found to be a convenient mass calibration standard for low-resolution mass spectra. The need for, and problems associated with, computer-assisted data handling in GC-MS were discussed. The development of an on-line real-time data acquisition system has been described.

VIII

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REFERENCES

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