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Some aspects of mass spectrometry in research on steroids

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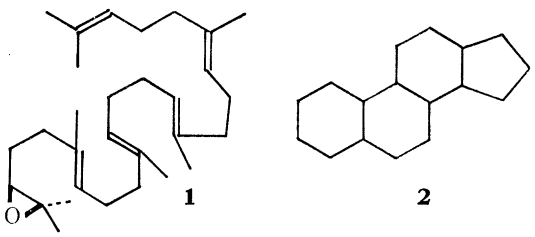
The application of mass spectrometry to steroids has been extensively developed since its inception in 1956. Detailed studies based on pure steroids and their isotope-labelled analogues have established the major pathways of fragmentation under electron impact. The resulting correlations have been of great value in the analytical characterization of steroids and their metabolites. The combination of mass spectrometry with gas chromatography is particularly powerful: the latter technique reduces the complexity of samples, and provides independent characterization by virtue of retention regularities. These features are essential for distinguishing many stereoisomeric or otherwise closely related steroids.

Modifications of functional groups can augment the utility of mass spectrometry in qualitative and quantitative analysis. Derivatives may be chosen to alter fragmentation modes, or to produce informative shifts in ion masses within an essentially unchanged fragmentation pattern. Enhancement of molecular ion abundance is usually achievable with derivatives, or by means of milder ionization techniques such as chemical ionization.

Steroids (Fieser & Fieser 1959) may be concisely defined as a class of natural products formed via cyclization of squalene (3*S*)-2,3-epoxide (**1**) and possessing the tetracyclic perhydrocyclopentanophenanthrene skeleton (**2**) or a biogenetic variant thereof. Numerous synthetic compounds of cognate constitution are also described as steroids. Representative natural steroids are depicted in figure 1: in these examples the molecular masses are between 270 and 386.



Although the tetracyclic nucleus is common to all, variations in the side chain and in substituents give rise to a diversity of properties. Sterols (typically monohydroxylic steroids) such as cholesterol are relatively non-polar and show considerable thermal and chemical stability. On the other hand cortisone, with a 'dihydroxyacetone' type of side chain, is much more susceptible to alteration.

The first applications of mass spectrometry to the characterization of organic compounds were made in the early 1950s (Beynon 1954, 1956). The feasibility of extending the technique to steroids and related natural products of comparatively high molecular mass was demonstrated by de Mayo & Reed (1956), who examined several steroid hydrocarbons and one ketonic steroid. Samples were introduced as solids directly into the ion source so that vaporization occurred close to the filament. This early technical innovation (Reed 1958, 1960), in the form

of a probe that can be inserted and withdrawn through a vacuum lock, has been of pre-eminent importance in the study of large molecules, especially in the field of natural products. The work of de Mayo & Reed showed that molecular masses in the range 370–414 could be measured, and that some indication of the side chain mass could be obtained, even with the instruments of modest specification then available. In the ensuing two decades, the development and accessibility of mass spectrometers with higher resolving power has encouraged extensive and detailed investigations of the modes of fragmentation of steroids under electron impact. Many of the results have been collected and discussed in monographs (Budzikiewicz *et al.* 1964; Zaretskii 1976) and reviews (see, for example, Horning *et al.* 1968; McCloskey 1969; Spiteller-Friedmann & Spiteller 1969; Djerassi 1970, 1978; Sjövall *et al.* 1971; Budzikiewicz 1972; Elliott 1972; Brooks & Middleditch 1973). In general, the pathways of fragmentation are complex, but reveal many regularities that can be diagnostic for structural features.

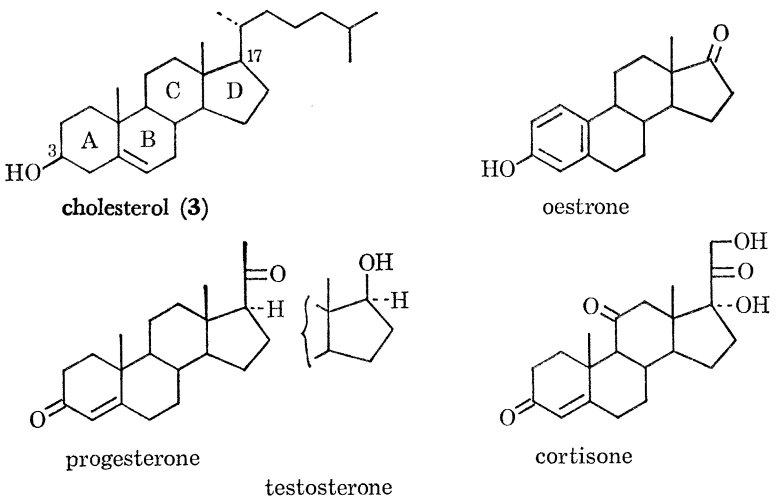


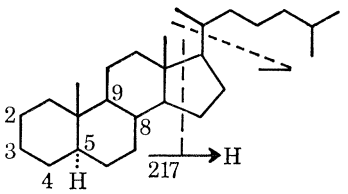
FIGURE 1. Representative natural steroids possessing the common tetracyclic nucleus (rings A, B, C and D). Cholesterol (**3**) is the principal mammalian sterol: other sterols differ chiefly in the degree or site of unsaturation and in the structure of the side chain. Cholesterol is the biosynthetic precursor of the four hormones depicted: progesterone is formed initially and is converted by hydroxylation to cortisone, or by further catabolism to testosterone and oestrone.

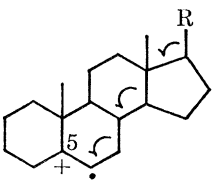
Before 1964, mass spectrometry found limited use in steroid analysis. The advent of effective methods for the dynamic combination of gas chromatography with fast-scanning mass spectrometry (Ryhage 1964; Stenhagen 1964; Watson & Biemann 1964) made possible the study of minute samples of complex organic mixtures. The unique potency of the combined technique (g.c.–m.s.) has been of especial importance in the analysis of steroids for two main reasons: first, because of the subtlety of structural details that determine physiological or pathological activity, and secondly, by virtue of the need to determine, in many instances, quantities less than 1 ng (10^{-9} g).

Thus three main strands can be discerned in the present fabric of steroid mass spectrometry: the basic study of fragmentation in relation to structure; the use of g.c.–m.s. in separation and characterization of mixtures; and the adaptation of mass spectrometry (usually g.c.–m.s.) to the highly sensitive determination of specific steroids. In this paper, emphasis is placed upon characterization, and the principal examples are taken from studies of sterols.

Some basic features of the mass spectra of steroids

5α-Cholestane, the parent hydrocarbon (sterane) of many natural sterols, is shown in figure 2. Of the 30 C–C bonds in the molecule, it is reasonable to expect that those linking more highly substituted atoms would be particularly prone to cleavage, and that effects of steric strain would also play a role. Indeed, the base peak (at 70 eV) of the mass spectrum of 5α-cholestane is at m/z 217 and results from the indicated cleavage of the five-membered ring D, initiated by fission of the C(13)–C(17) bond (Tökés *et al.* 1968). Similarly, ions $[M-15]^+$ result from cleavage of an angular methyl group. These fragmentations occur in many steroids and in modified structures, such as steranes with ring D enlarged to six or seven members (Eadon *et al.* 1972).



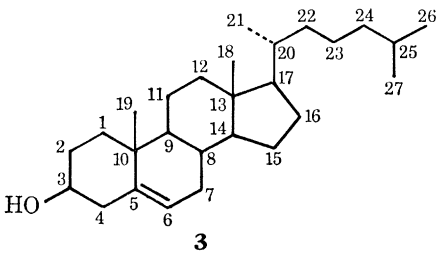


	% of base peak			% of base peak	
	$M^{+\cdot}$	$[M-s.c.]^+$		$M^{+\cdot}$	$[M-s.c.]^+$
5α	28	1	Δ^4	48	19
5β	33	1	Δ^5	100	59
5α-Δ^2	86	32	5α-$\Delta^{8(9)}$	100	45
5α-Δ^3	95	31			

Figure 2. The effect of nuclear unsaturation on cleavage of the side chain. Nuclear double bonds afford sites for charge localization as illustrated for Δ^5-cholestene. Data refer to 70 eV electron impact.

It may be noted that in 5α- and 5β-cholestane, cleavage of the side chain is not a major process. Introduction of unsaturation into the nucleus affords more abundant molecular ions and also enhances the side chain cleavage: these effects can be rationalized by the hypothesis of charge localization (Budzikiewicz *et al.* 1964; McLafferty 1966; cf. Williams & Beynon 1976) as exemplified in figure 2 for loss of the side chain in cholest-5-ene (Djerassi 1970). Analogous fragmentations of Δ^7- and $\Delta^{8(14)}$-sterenes have been examined in detail (Partridge *et al.* 1977).

The great majority of natural steroids contain oxygen, and the ubiquitous animal sterol cholesterol (**3**) serves to illustrate the complexity of mass spectrometric fragmentation modes that are encountered. The eight strongest peaks in the high mass range as shown in figure 3.



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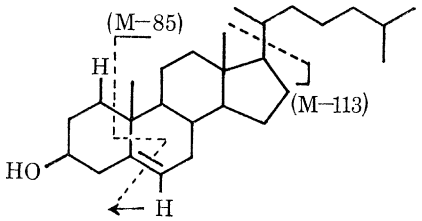
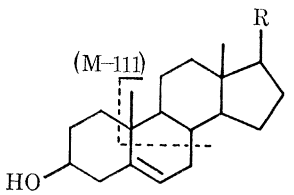
In addition to ions expected by analogy with cholest-5-ene, and to those arising via loss of H_2O, there are two ions, m/z 301 and 275, arising from complex and unpredictable processes that

have been elucidated only by elaborate studies of cholesterol specifically labelled with deuterium (Wyllie *et al.* 1977; cf. Budzikiewicz & Ockels 1976).

While the mass spectrum of cholesterol is highly distinctive as a 'fingerprint', and also affords information on the masses of the nucleus and side chain, it lacks any prominent feature indicative of the double bond position. The 3-hydroxy-Δ^5-steroid structure can, however, be detected readily by conversion to an ether. The most convenient derivative is the trimethylsilyl





ion	*m/z*	%	ion	*m/z*	%
M	386	100	M − 85	301	29
M − 15	371	21	M − 111	275	34
M − 18	368	31	M − 113	273	14
M − 33	353	17	M − 173	213	16

FIGURE 3. Major fragmentations of cholesterol (Wyllie *et al.* 1977). The eight most abundant ions in the higher mass range are cited. Ions $[M-173]^+$ result from cleavage through ring D (cf. figure 2) with concomitant loss of H_2O.

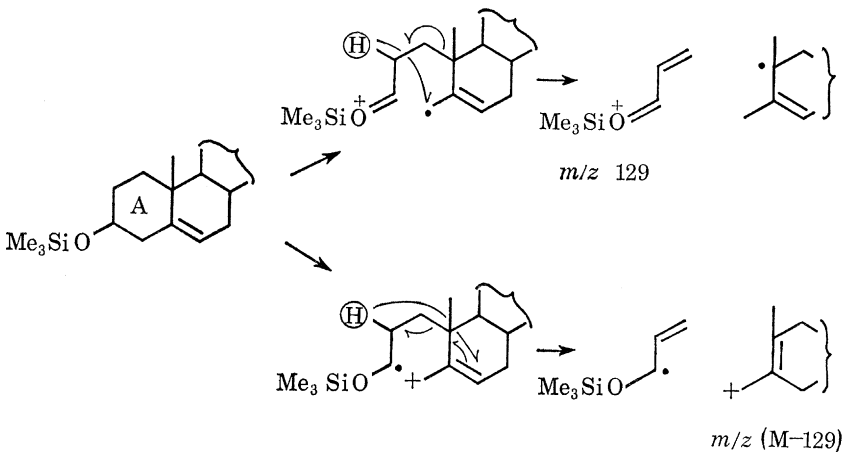
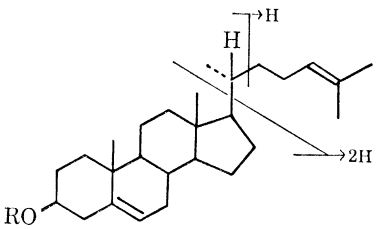


FIGURE 4. Major fragmentations of cholesteryl trimethylsilyl ether, general for 3-hydroxy-Δ^5-steroid trimethylsilyl ethers.

ether, which strongly promotes the fragmentations outlined in figure 4 to yield the characteristic complementary pair of ions at *m/z* 129 and $[M-129]^+$ (Eneroth *et al.* 1964; Brooks *et al.* 1967; Dickman & Djerassi 1967; Brooks *et al.* 1968). Although ions of *m/z* 129 can arise from other types of trimethylsilyloxysteroid, the accompanying ions of $[M-129]^+$ almost always denote the 3-hydroxy-Δ^5-derivatives. (They have been observed for a Δ^9-sterol trimethylsilyl ether (Brooks *et al.* 1973).) Distinction between epimeric 3β- and 3α-sterols is much more satisfactorily made by gas chromatography, since their retention times are widely different. Methyl ethers yield analogous ions, $[M-71]^+$, but none of prominence at *m/z* 71 (Idler *et al.* 1970*a*; Narayanan & Lala 1972).

An illustration of the effects of derivative formation in 3-hydroxy-Δ^5-steroids is given in figure 5 for desmosterol (cholesta-5,24-dien-3β-ol) (Brooks *et al.* 1968). Sterols containing unsaturated side chains readily afford ions resulting from side chain cleavages with hydrogen rearrangement. In certain instances these correspond to products of McLafferty rearrangement, but in desmosterol cleavage occurs at the C(20)–C(22) bond giving $[M-84]^{+\cdot}$. A second characteristic ion at m/z 271 results from loss of the side chain together with two nuclear hydrogen atoms (Wyllie & Djerassi 1968). In desmosteryl acetate, just as in cholesteryl acetate (Partridge & Djerassi 1977) the mass spectrum is dominated by the ion $[M-60]^{+\cdot}$ and the molecular ion is absent. Of the two typical desmosterol fragmentations, $[M-\text{side chain}-2H-CH_3COOH]^+$ is represented in the mass spectrum of the acetate at m/z 253, but $[M-84]^{+\cdot}$ is very weak. In the trimethylsilyl ether, the ions $[M-\text{side chain}-2H]^+$ and $[M-84]^+$ remain prominent together with m/z 129 and $[M-129]^+$: this derivative thus preserves a balance between the two types of diagnostic information.



R	$M^{+\cdot}$	%	base peak	$[M-84]^{+\cdot}$	%
H	384	30	271 (M−s.c.-2H)	300	26
CH_3CO (Ac)	426	—	366 (M−ROH)	342	—
Me_3Si (TMS)	456	32	129	372	15

FIGURE 5. Characteristic ions from desmosterol, its acetate, and its trimethylsilyl ether. The relative abundance data are for 20 eV electron impact.

GAS CHROMATOGRAPHY – MASS SPECTROMETRY OF STEROLS AS TRIMETHYLSILYL ETHERS

(*a*) *Application to sterols of marine organisms*

Many natural sterols occur as complex mixtures of closely related structures, and it is imperative that a high degree of separation be achieved, if mass spectrometric data are to be reliably interpreted. Furthermore, full use is desirable of the structural evidence provided by gas chromatographic retention data. In the most powerful analytical technique so far devised, sterol trimethylsilyl ethers are separated on gas chromatographic columns of high efficiency, and mass spectra are recorded at precisely defined retention times. The following examples, drawn from studies of marine sterols, briefly illustrate the scope and limitations of the method.

The gas chromatographic analysis of a (trimethylsilylated) sterol fraction from *Dentalium entale*, isolated in D. R. Idler's laboratory at St John's (Newfoundland) is shown in figure 6. The packed column fails to resolve all the components, but has the merit of tolerating the heavy (*ca.* 25 μg) load of sample needed because of the preponderance of cholesterol. Retention index values, measurable under these conditions to the nearest 5 units, are indicated, and figure 7 depicts salient features of the corresponding mass spectra, leading to partial structures as noted (Idler *et al.* 1978). Diagnostic ions include those resulting from allylically promoted

side chain cleavage in the Δ^{22}-sterol ethers, and that attributable to McLafferty rearrangement in the 24-methylene sterol of retention index 3225. Gas chromatography is of obvious value in disclosing the presence of carbon skeletons which are deficient in, or augmented by, carbon atoms as compared with 'normal' sterols. Mass spectrometry readily shows whether the differences are in the side chain (as in the first compound cited in figure 7) or associated with the nucleus, e.g. in the isomeric 19-norcholesterol (Popov *et al.* 1976).

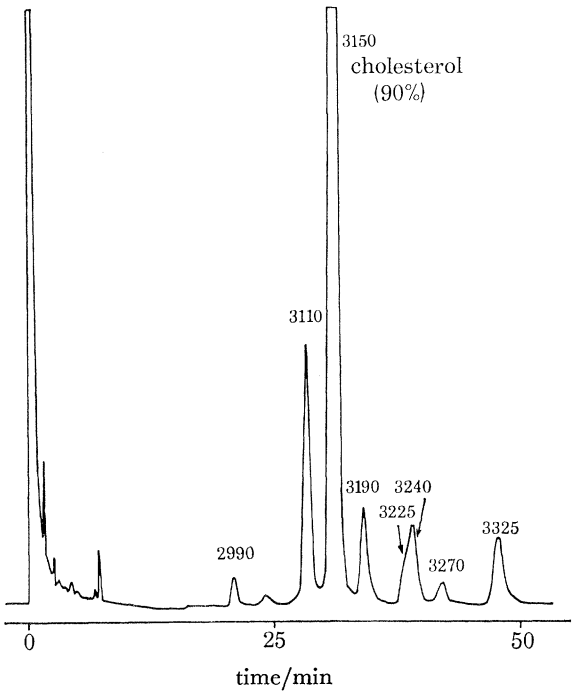


FIGURE 6. Gas chromatographic separation of trimethylsilyl ethers of a 4-demethylsterol fraction isolated from the scaphopod *Dentalium entale*. A 6 m glass column packed with 1 % SE-30 on Gas Chrom Q (125–150 μm) was used at 260 °C with nitrogen as carrier gas. Retention index values, determined in separate analyses with *n*-alkanes as internal standards, are cited above the peaks.

I(SE-30)	M+·	base peak	other		side chain in Δ^5-sterol
2990	442	97	129	313	
3110	456	111	129	327	
3150	458	129	329		
3190	470	125	129		
3225	470	129	386		
3240	472	129	343		
3270	484	83	129		
3325	486	129	357		

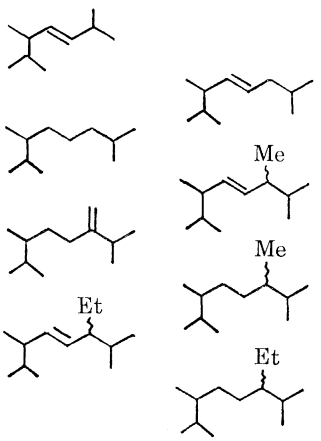


FIGURE 7. Partial structures of *Dentalium* sterols inferred from gas chromatographic and mass spectrometric data, salient features of which are indicated.

24-Norsterols were among the first discovered examples (Idler *et al.* 1970*b*) of natural sterols having 'abnormal' side chains, and marine organisms have since yielded an increasing variety of types: some of these are represented in figure 8. While gas chromatography and mass spectrometry have played a major role in their analysis and characterization, discrimination between certain closely similar steroids requires additional evidence, e.g. from n.m.r. spectrometry. Thus the sterols 5,*Z*-22-cholestadien-3β-ol and occelasterol (Kobayashi & Mitsuhashi 1974) have similar retention times on OV-1 phase (I = 3092 for both sterol trimethylsilyl ethers at 265 °C), and similar mass spectra (figure 9) (C. G. Edmonds, T. M. T.

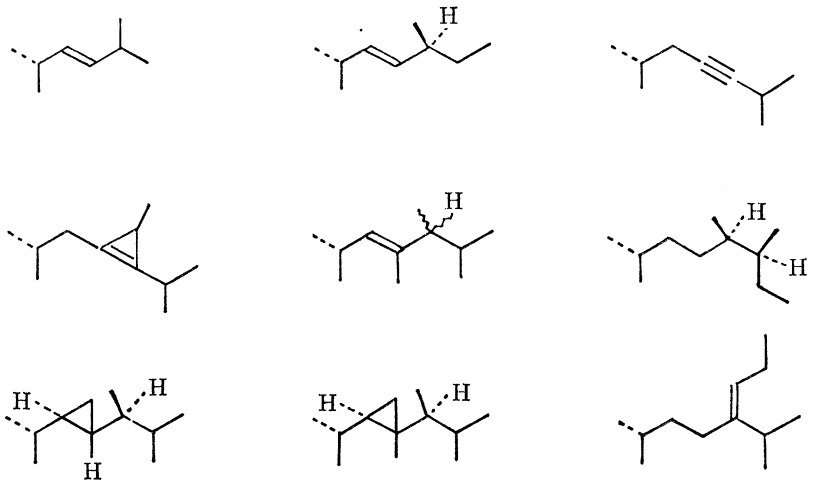


FIGURE 8. Side chain variants in marine sterols. Wavy lines indicate uncertain configurations.

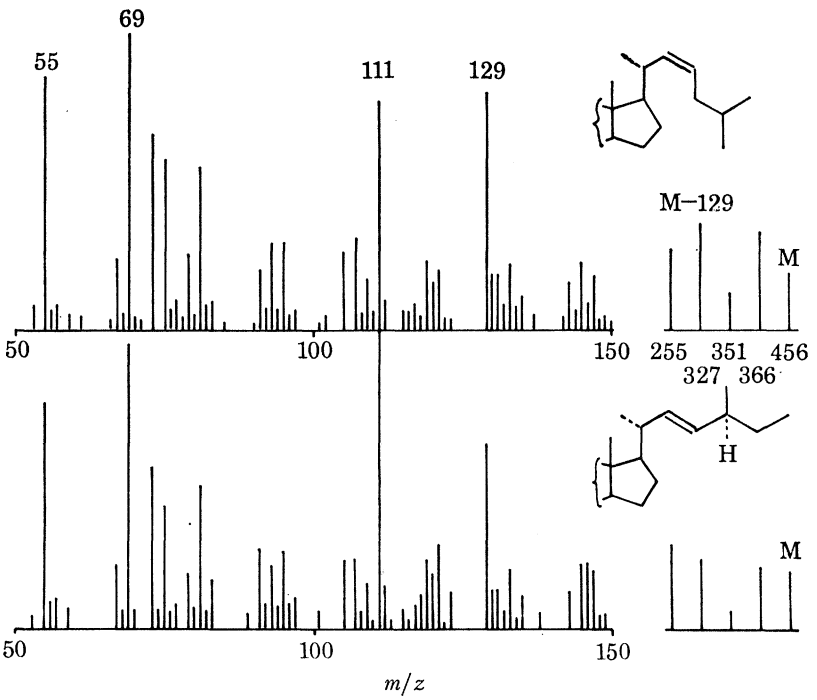


FIGURE 9. Partial mass spectra (70 eV) of 5,*Z*-22-cholestadienol and occelasterol trimethylsilyl ethers: above m/z 150 only the five major ions are compared.

Sheehan & C. J. W. Brooks 1977, unpublished). The discovery of occelasterol and its congeners (Kobayashi & Mitsuhashi 1974, 1975) thus casts doubt on assignments of the 5,Z-22-cholestadienol structure based on g.c.–m.s. (see, for example, Idler & Wiseman 1971; Idler *et al.* 1976).

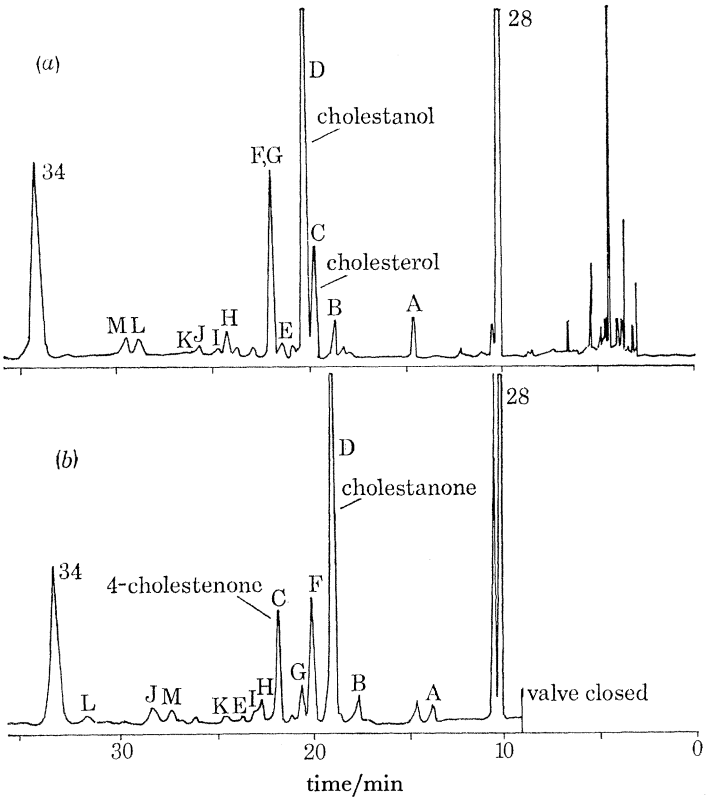


FIGURE 10. Total ion current chromatograms indicating the separation (*a*) of trimethylsilyl ethers of sterols from the sponge *Grantia compressa*; (*b*) of ketones formed by enzymic oxidation. A 50 m glass open-tubular column coated with OV-1 was used at 275 °C in an LKB 9000 instrument with helium (6 ml/min) as carrier gas. (From Edmonds *et al.* (1977); reproduced with permission.)

It is possible to improve the analytical distinction between similar sterols by means of the higher chromatographic efficiency attainable with glass open-tubular columns in g.c.–m.s. Figure 10*a* shows the 'total ion current' chromatograms recorded for trimethylsilylated sterols of a marine sponge, *Grantia compressa* (Edmonds *et al.* 1977). Retention index values are determinable to within one or two units, and peaks differing by only ten index units are almost completely separable. It is important to note that the resolution depicted in figure 10*a* is that at the 'total ion current' detector within the mass spectrometer. Even after the passage of a relatively massive peak such as D, the mass spectra measured correspond to components appropriate to the particular retention time, and show few signs of 'memory' effects. Since fortuitous coincidence of some peaks is generally to be expected, it is prudent to conduct separate analyses under different conditions. One particularly convenient and effective procedure applicable to sterols is selective enzymic oxidation with 3β-hydroxysteroid oxidase (Smith & Brooks 1974, 1976; Brooks & Smith 1975) which converts 3β-hydroxy-Δ^5-steroids into the conjugated 4-en-3-ones, and 3β-hydroxy-5α-steroids into 5α-3-ones. The attendant changes in chromatographic separation afford valuable retention–structure correlations, and

changes in the elution pattern facilitate correlation of the mass spectral data. The measurements on the two families of derivatives, as illustrated in figure 10*b* and table 1 for peaks E to I of the *Grantia* sterol mixture, afford stringent evidence of structure and composition. Thus only peak E is a 3β-hydroxy-Δ^5-steroid, as indicated by the increased retention time typical of the derived conjugated enone (figure 10*b*). Peaks F and G, overlapping in figure 10*a*, are both 5α-sterols, but after oxidation are adequately separated as the respective 3-ones.

TABLE 1. 4-DEMETHYLSTEROLS OF *GRANTIA COMPRESSA*: GAS CHROMATOGRAPHIC DATA FOR TMS ETHERS OF STEROLS IN PEAKS E–I, AND FOR KETONES FORMED BY ENZYMIC OXIDATION

	I(OV-1) (TMS ether)	M$^{+\cdot}$	*I*(OV-1) (ketone)	Δ*I*	M$^{+\cdot}$	sterol†
E	3196	470	3245	+49	396	24-Me $\Delta^{5,22}$
F	3207	458	3163	−44	384	5α Δ^7
G		472	3178	−29	398	24-Me 5α Δ^{22}
H	3256	472	3223	−33	398	24-CH_2=5α
I	3264	474	3230	−34	400	24-Me 5α

† Substituents in cholestan-3β-ol.

It is clear that the study of sterol mixtures by g.c.–m.s. is capable of yielding substantial information. Nevertheless it is often necessary, if rigorous identification is to be established, to secure more reliable evidence concerning details of the skeletal structure, particularly of the side chains. At present, n.m.r. spectrometry is the method of choice: it would, however, be of interest to try to improve the diagnostic power of g.c.–m.s. because of the unique applicability of this technique to extremely small samples.

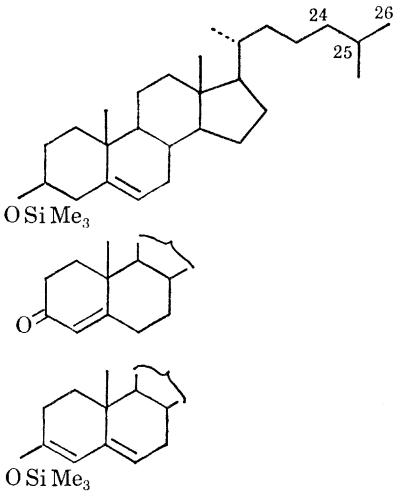
(*b*) *Application to hydroxylated sterols*

Natural sterols containing additional hydroxyl groups have been known for many years, but their significance is only gradually becoming appreciated. Side chain hydroxylated cholesterols include cerebrosterol (24*S*-hydroxycholesterol) from brain tissue (Ercoli & de Ruggieri 1953; Lin & Smith 1974); 26-hydroxycholesterol from human atherosclerotic plaques (Brooks *et al.* 1966); 22-hydroxycholesterols, which are among the intermediates in the side chain cleavage of cholesterol to pregnenolone by adrenocortical enzymes (Morisaki *et al.* 1976); and 25-hydroxycholesterol, an autoxidation product of cholesterol, which is a potent inhibitor of sterol biosynthesis in a variety of systems (see, for example, Kandutsch & Chen 1974).

Gas chromatography–mass spectrometry of trimethylsilyl ethers is particularly effective for sterols with hydroxylated side chains by virtue of the ready separability of positional isomers (Gaskell *et al.* 1975) and the prominence of ions formed by α-cleavage. The incorporation of oxygen from $^{16}O_2$ and $^{18}O_2$ in the enzymic formation of (20*R*,22*R*)-dihydroxycholesterol from cholesterol was readily determined by measurement of the ions arising from the trimethylsilyl ether by cleavage at C(17)–C(20) (Burstein *et al.* 1974). A specific method for determining the *seco*-sterol, 25-hydroxy-vitamin D_3, by g.c.–m.s. has been based on the abundant ion at m/z 131 resulting from cleavage of the C(24)–C(25) bond in the trimethylsilyl ether (Björkhem & Holmberg 1976).

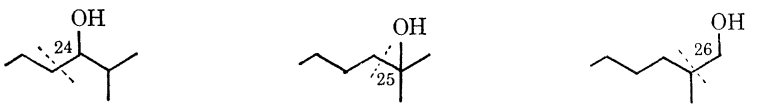
The strengthening of characterization that ensues from specific analytical transformations is exemplified for 24-, 25- and 26-hydroxycholesterol in figures 11 and 12 (Gaskell *et al.* 1975). Data are cited for the diol di-trimethylsilyl ethers, for the monoethers of the 4-en-3-ones

obtained by enzymic oxidation, and for the corresponding 3-enol ether derivatives. The three sets of retention indices in figure 11 are significantly more distinctive than any single set. In the mass spectra (figure 12), the α-cleavages are prominent throughout but the enol ethers are of special value in yielding abundant molecular ions from the 24- and 25-isomers.



	retention indices (I)		
	24-*O*TMS	25-*O*TMS	26-*O*TMS
	3465	3510	3555
	3700	3715	3780
	3560	3550	3645

FIGURE 11. Partial structural formulae and retention index values (1% OV-17, 265 °C) for derivatives of side chain hydroxycholesterols and the 4-en-3-ones derived therefrom by selective enzymic oxidation.



	24-OSiMe$_3$		25-OSiMe$_3$		26-OSiMe$_3$	
derivative	M$^{+\cdot}$	145	M$^{+\cdot}$	131	M$^{+\cdot}$	103
diol di TMS	2	94	2	100	13	28
enone TMS	0.1	82	0.1	100	33	33
enone enol TMS	75	17	25	55	59	11

FIGURE 12. Intensities (as percentages of respective base peaks) of molecular ions and of ions formed by α-cleavage of the side chain trimethylsilyloxy group, in the derivatives cited in figure 11. Data refer to 70 eV electron impact.

(*c*) *Rearrangement ions in mass spectra of steroidal trimethylsilyl ethers*

In the course of verifying the structure of 26-hydroxycholesterol isolated from human brain (Smith *et al.* 1974; cf. Smith *et al.* 1973), the sample was oxidized by cholesterol oxidase to 26-hydroxycholest-4-en-3-one. The mass spectrum of the trimethylsilyl ether unexpectedly gave as the base peak an ion at m/z 196, which was found to result from a remarkable intramolecular migration of the side chain trimethylsilyl group to a fragment formed by cleavage through ring B (Gaskell *et al.* 1975). In effect, the trimethylsilyl group replaced a hydrogen atom in the typical ion at m/z 124 from 4-en-3-ones (Shapiro & Djerassi 1964). Ions at m/z 196 were also observed from other side chain hydroxy-enone trimethylsilyl ethers such as the 24-hydroxychol-4-en-3-one derivative (figure 13).

The trimethylsilyl group rearrangements under electron impact exemplified above would appear to involve *macrocyclic* transition states, unless they proceed in a stepwise manner. Very

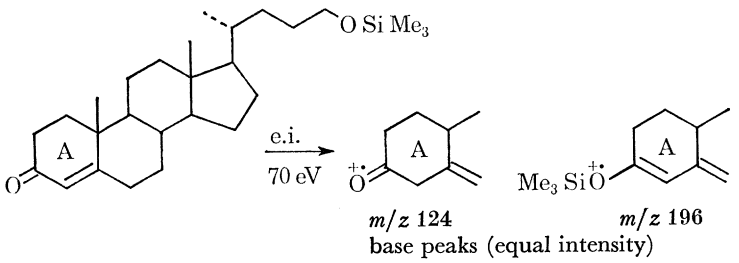


FIGURE 13. Long-range trimethylsilyl group rearrangement in the electron impact (70 eV) spectrum of 24-trimethylsilyloxychol-4-en-3-one.

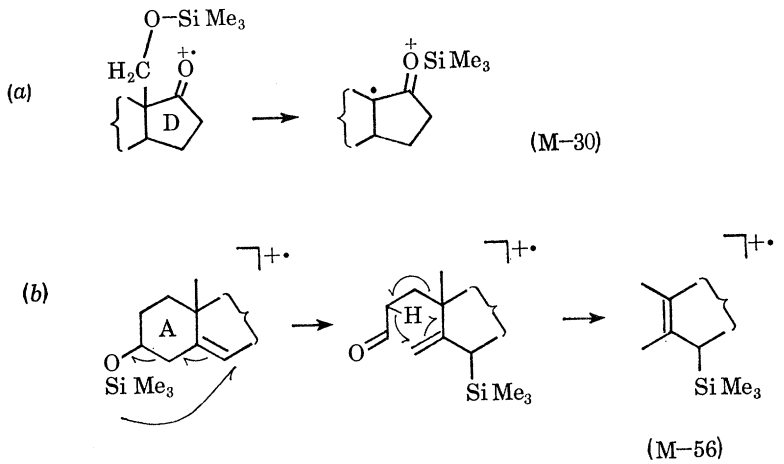


FIGURE 14. Trimethylsilyl group rearrangements occurring under electron impact (*a*) in 18-hydroxy-17-oxo-steroid trimethylsilyl ethers; (*b*) in 3β-hydroxy-Δ^5 steroids (chiefly observed in C_{19}-steroid 17-ketones).

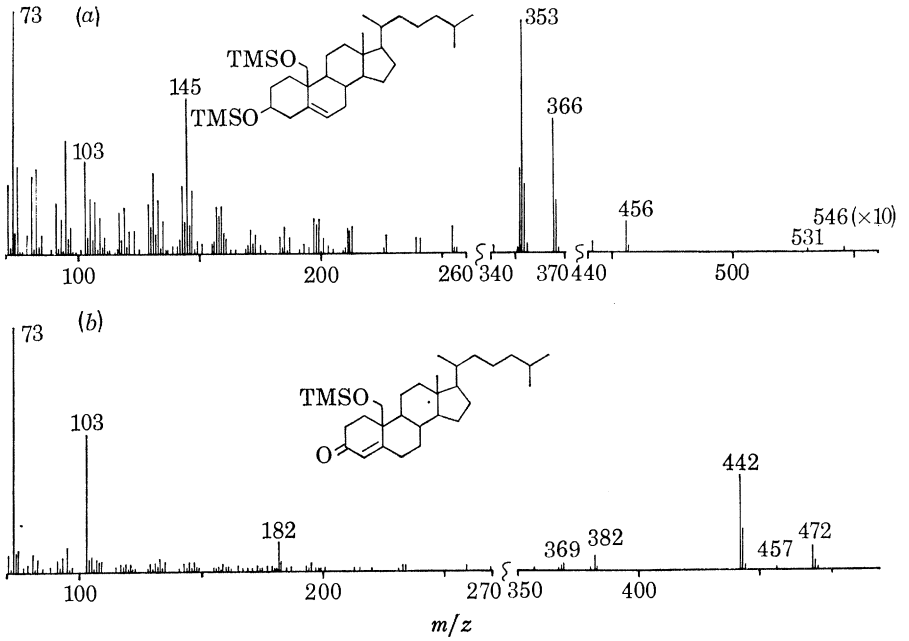


FIGURE 15. Mass spectra (70 eV) of (*a*) 19-hydroxycholesterol di-trimethylsilyl ether and (*b*) 19-hydroxycholest-4-en-3-one trimethylsilyl ether.

much more common are trimethylsilyl group rearrangements associable with *mesocyclic* transition states involving between five and eight members. Several of these processes are of diagnostic value. Thus 18-trimethylsilyloxyandrostan-17-ones are characterized by ions of type $[M-30]^{+\cdot}$ (figure 14*a*) (Laatikainen & Vihko 1969), while 3β-trimethylsilyloxyandrost-5-en-17-ones undergo ring A cleavage with trimethylsilyl group rearrangement, yielding $[M-56]^{+\cdot}$ ions (figure 14*b*) (Brooks *et al.* 1972; Björkhem *et al.* 1973). A particularly interesting case is presented by the trimethylsilyl ether of 19-hydroxycholest-4-en-3-one, a compound readily obtained by enzymic oxidation of 19-hydroxycholesterol. The mass spectrum (figure 15*b*) yields a much more prominent molecular ion than is observed from the trimethylsilyl ether of the parent sterol (figure 15*a*). It also shows the expected ion $[M-30]^{+\cdot}$ and, in addition, a prominent ion at m/z 182, shifted to m/z 191 in the [$^2H_9$]-trimethylsilyl analogue (A. G. Smith & C. J. W. Brooks 1977, unpublished). A reasonable mode of formation is depicted in figure 16.

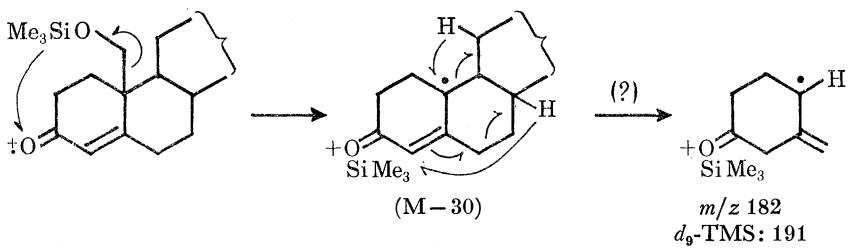


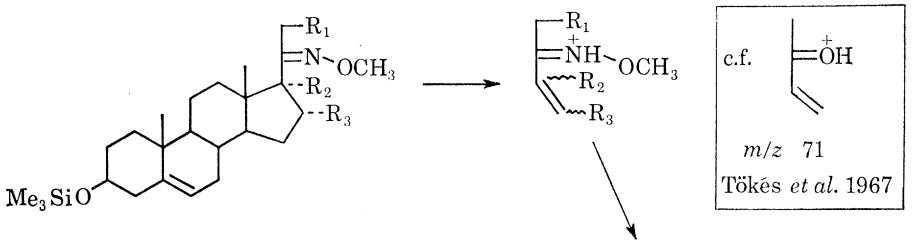
FIGURE 16. Postulated mode of fragmentation affording the ion of m/z 182 in the electron impact mass spectrum of 19-trimethylsilyloxycholest-4-en-3-one.

GAS CHROMATOGRAPHY – MASS SPECTROMETRY OF KETONIC STEROIDS OF THE 20-OXOPREGNANE CLASS

The important groups of hormones exemplified by the progestogenic steroid, progesterone, and the adrenocortical steroid, cortisone (cf. figure 1), give rise to a wide variety of metabolites, many of which retain the 20-oxo group. Steroids containing hydroxyl substituents adjacent to the ketonic group, particularly those substituted at both C(17) and C(21), are unstable towards gas chromatography. The most generally successful method of stabilization was devised by Gardiner & Horning (1966) and consists in conversion of the ketone to a methyloxime, a derivative introduced by Fales & Luukkainen (1965), followed by trimethylsilylation of hydroxyl groups, preferably including the tertiary 17α-hydroxyl. It is, of course, possible to select any suitable *O*-substituted oximes: thus *O*-benzyloximation confers long retention times which give rise to a complete gas-phase separation of reactive ketones from other steroids (Devaux *et al.* 1971), while *O*-isopentyloximation bestows intermediate properties (Baillie *et al.* 1972).

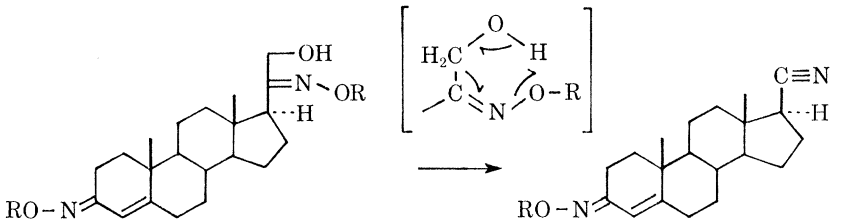
In addition to their value in gas chromatography, oximes and related nitrogenous derivatives of 20-oxopregnanes have the virtue of promoting certain regular fragmentation modes. One of these, illustrated in figure 17, parallels the formation of the ion of m/z 71 from the parent ketone (Tökés *et al.* 1967) but is much more prominent (Horning *et al.* 1968; Brooks & Harvey 1970; Dray & Weliky 1970). Moreover, it persists in the presence of a wide variety of additional substituents as exemplified in figure 17. Since the regularity of fragmentation holds good for a variety of oximes, verification of the ions ascribed thereto is readily achieved

by comparing methyloximes with ethyloximes, benzyloximes, trimethylsilyloximes, etc. Good evidence for the substituents present at C(16), C(17) and C(21) is thus obtained, and ion masses can be shifted to almost any desired value to improve qualitative or quantitative measurements.



R_1	R_2	R_3	m/z	percentage
H	H	H	100	100
H	OH	H	116	68
H	OH	CH_3	130	26
OAc	H	H	158	40
$OSiMe_3$	H	H	188	48
$OSiMe_3$	$OSiMe_3$	$OSiMe_3$	364	10

FIGURE 17. Characteristic fragmentation of 20-ketosteroid methyloximes, analogous to the formation of ions of m/z 71 from the parent ketones. Regularity of fragmentation is preserved for many substituted analogues, five of which are exemplified.



R = CH_3; C_2H_5†; $CH_3CH(CH_3)CH_2CH_2$; $C_6H_5CH_2$

† For ethyloxime, identity confirmed by collection of g.l.c. peak (I = 2850 on OV-1, 260 °C). Infrared: $\nu_{C\equiv N}$ = 2235 cm^{-1}

FIGURE 18. Schematic indication of thermal Beckmann fission observed during gas chromatography of 3,20-dimethyloximes and other dioximes of 11-deoxycorticosterone (DOC). The identity of the nitrile formed from the ethyloxime was confirmed by collection of the eluted peak and its examination by infrared spectrometry and gas chromatography in comparison with an authentic sample.

It may be appropriate to conclude by emphasizing the need for vigilance in the use of derivatives of intrinsically labile steroids for g.c.–m.s. Decomposition of oximes, even those of simple oxosteroids, occurring via Beckmann fission, has been observed on gas chromatographic columns that have developed catalytic sites at the site of injection (Thenot & Horning 1972*a*, *b*). Alkyloximes of 20-, 21-ketols with the hydroxyl group unprotected are unstable towards gas chromatography, undergoing upon injection an almost instantaneous thermal Beckmann fission to afford the 20-nitrile, as exemplified in figure 18 (R. C. Glass & C. J. W. Brooks 1973, unpublished). As already mentioned, trimethylsilylation prevents this decomposition: however, C. H. L. Shackleton (personal communication) has reported his observation of anomalous mass spectra, on occasions, from corticosteroid methoxime trimethylsilyl ethers during glass capillary column g.c.–m.s. These spectra were dominated by ions clearly attributable (as instanced in figure 19) to products of Beckmann fission, but since the retention times were those of the original derivatives, the process was evidently occurring in the g.c.–m.s.

connection, and appeared to be associated with unduly elevated temperatures therein. In my view there is always a distinct risk of encountering untoward effects such as these, especially in g.c.–m.s. analyses necessitating high operating temperatures. The parallel analysis of two or more derivatives or specific transformation products of each sample, as illustrated in this article, is one of the simplest ways of monitoring the validity of the observations.

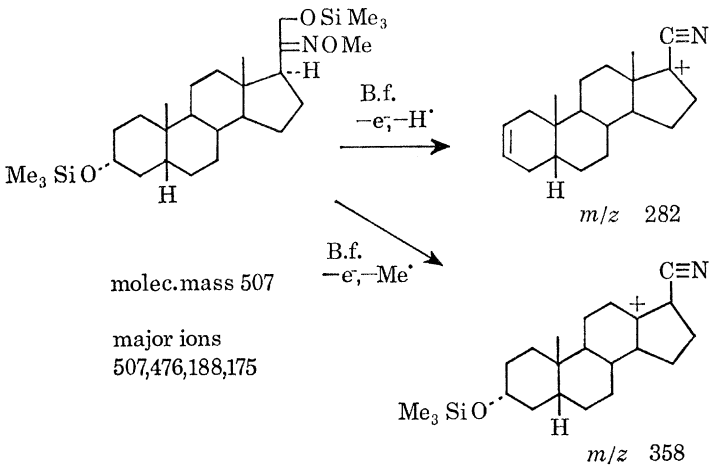


FIGURE 19. Ion structures postulated by the author for anomalous ions observed under certain conditions by C. H. L. Shackleton, during the analysis of tetrahydro-DOC methyloxime di-trimethylsilyl ether. Normal retention time prevailed and the ions at m/z 282 and 358 are ascribed to Beckmann fissions occurring in the heated line leading to the mass spectrometer.

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