AN INVESTIGATION INTO THE ANALYSIS OF ECDYSONES AND OTHER STEROID HORMONES

by

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ABSTRACT

As an aid to the analysis of ecdysones (arthropod moulting hormones), an investigation has been carried out to find an efficient and sensitive method for determining them by chromatography.

A series of fluorocarbonsilanes were synthesized and tested as electron capturing derivatives for gas chromatography. Flophemesyl * derivatives of mammalian steroids were volatile and sensitive to detection. The derivatives produced characteristic mass spectra with a greater proportion of the ion current carried by hydrocarbon fragments than with other siloxy derivatives making flophemesyl derivatives more suitable for structural determinations. A series of flophemesyl reagents were developed for selectively protecting hydroxyl groups in different steric environments. New methods of synthesis of some of these reagents and analytical techniques for their identification were established. In their mass spectra, unusual rearrangements were found to occur between the fluorine atoms of the pentafluorophenyl ring and the methyl groups bonded to silicon, giving hydrofluorocarbon tropylium-type ions.

Flophemesyl derivatives, although useful for mammalian steroids did not give volatile derivatives of ecdysones.

The introduction of the pentafluorophenyl ring into steroids as pentafluorophenylhydrazones or pentafluorophenylboronic esters was attempted. The hydrazones, as ketone derivatives, had poor GC characteristics and limited stability when exposed to light or the atmosphere. The pentafluorophenylboronic esters were susceptible to disproportionation in the presence of traces of water or other strong nucleophiles.

Boron can be selectively detected with a nitrogen thermionic detector. Boronic esters of model steroids were detected at lower levels than with

* pentafluorophenyldimethylsilyl

the FID, but the estimated sensitivity for ecdysones was insufficient for trace level analysis.

As ecdysone TMS ethers fragment to produce mass spectra containing a few ions of relatively high intensity, the possibility of using the mass spectrometer as a gas chromatographic detector was investigated. The ecdysone derivatives could only be chromatographed at high temperatures as low carrier gas flow rates had to be used to meet the vacuum requirements of the mass spectrometer. An excessive column background made quantification difficult at trace levels. Optimum GC-MS conditions for steroid analysis were established.

The model steroids 2β,3β-dihydroxy-5α-cholestane and 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one were prepared by published procedures; 5α-cholest-7-en-6-one and 14α-hydroxy-5α-cholest-7-en-6-one were synthesised for the first time in good overall yield.

In an investigation of the formation of ecdysone TMS ethers and the formation of heptafluorobutyrate esters by an exchange reaction with the TMS ethers, it was discovered that the ecdysone nucleus contains the features necessary for electron capture without the need for the formation of halogenated derivatives. The electrophore was identified as the unsaturated ketone, the C-14 oxy substituent with a smaller contribution from the more remote 2β,3β-oxy substituents. The rate of formation of the TMS ethers of ecdysterone hydroxyl groups with trimethylsilylimidazole was found to be 2,3,22,25>20>>14. The degree of TMS ether formation in ecdysone and ecdysterone was confirmed by mass spectrometry of the derivatives and also by selective silylation of model steroids. Ecdysones in biological samples were determined as their TMS ethers after a preliminary extraction, solvent partition and TLC separation from impurities. The method has been applied to determine the daily changes in ecdysone content in the desert

locust, Schistocerca gregaria.

A brief survey of high pressure liquid chromatography illustrated the potential of this technique for the detection and separation of ecdysones.

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INTRODUCTION

The arthropods (insects, spiders and crustaceans), have a hard exoskeleton, which serves to support and contain their internal organs but is incapable of growth or modification. The larva develops from the egg, predisposed to a career of feeding, and to grow it must shed its restrictive exoskeleton. This shedding of the old exoskeleton and the formation of the new larger covering is the process known as "moulting" or "ecdysis".

Kopéc was the first to recognize that the initiation of moulting in insects was a process under hormonal control.[1] Extensive work by Wigglesworth on the tropical bug Rhodnius prolixus provided convincing support for this proposition.[2] The first isolation and chemical identification of a moulting hormone was achieved by Butenandt and Karlson from the pupae of the silkworm Bombyx mori.[3] From 0.5 tons of pupae they isolated 25 mg. of a pure crystalline hormone which they named ecdysone. At a later date they isolated a further 2.5 mg of a more polar hormone, ecdysterone.[4] A chemical and X-ray crystallographic investigation established the structure of ecdysone as 2β,3β,14α,22α(R),25-pentahydroxy-5β-cholest-7--en-6-one, which has been confirmed by synthesis.[5] Since the initial extraction of ecdysone and ecdysterone, four other moulting hormones in arthropods have been identified; namely 20,26-dihydroxyecdysone[6], 2-deoxy-ecdysterone,[7] inokosterone[8] and makisterone A[8]. Ecdysones have also been found in the plant kingdom and over forty different types have been identified.[5,9,10] Their role in the plant kingdom is not understood, but certain species contain greater than 1% of the hormone by dry weight and these represent the best supply of hormones for biological testing.[10] The level in arthropods is usually of the order $10^{-3} \longrightarrow 10^{-5}$%.[9]

Moulting in arthropods according to current theory, is initiated by neurosecretory cells of the brain which secrete brain hormone into the blood. This hormone stimulates the prothoracic gland in the thorax to produce ecdysone. Ecdysterone is recognized as the active form of the hormone and this is produced from ecdysone by hydroxylation in various insect tissues. The prothoracic gland does not secrete ecdysterone *in vitro* nor can it convert ecdysone to ecdysterone and this has led to the suggestion that ecdysone is a prohormone.[11] Another pair of glands, the *corpora allata*, are located near the brain and are connected to it by several nerves. These glands secrete a further hormone, juvenile hormone, which directs the qualitative expression of each moult. When a relatively high titre of juvenile hormone is present in the insects blood, the ensuing moult will be larval in nature. When juvenile hormone is absent, the succeeding moult will be to the adult. In the adult the prothoracic glands degenerate and no further moulting occurs.[12]

The availability of ecdysones and their analogues through synthesis and extraction from plants has led to an imperfect understanding of those features of the hormone which are essential for biological activity.[5,9] These can be summarized as:

1) Cis fusion of the A and B ring of the steroid skeleton. Ecdysones with a 5α-H are biologically inactive.
2) A β-hydroxylic function attached to C-3. This is essential for high biological activity but hydroxyl groups at C-2 and possibly that at C-14 exert little influence on activity (cf[5,9])
3) A keto group at C-6 in conjunction with a Δ^7-double bond
4) A steroid side chain with an appropriately (R) orientated hydroxylic function at position C-22. A hydroxyl group at C-20 is not important and the biological activity is only marginally changed by a hydroxyl group at C-25. In the absence of a steroid side chain, then ecdysone

analogues are inactive.

Insects cannot synthesise steroids and need an exogenous supply for the biosynthesis of moulting hormones. For this purpose a supply of cholesterol or phytosterol which can be dealkylated to cholesterol is required.[13] It has been shown that labelled cholesterol fed to insects can be converted to ecdysones.[14] The metabolic pathway is far from fully elucidated, but an early stage is believed to be the dehydrogenation of cholesterol to cholesta-5,7-dien-3β-ol.[13] Isomerization of the C-5 position is thought to be facilitated by the interaction of the 2β-hydroxyl function with the C-19 methyl group of the steroid skeleton.[9] Introduction of the 14α-hydroxyl group can be accomplished at any stage of the biosynthesis. It is believed, though by no means proven beyond doubt, that the 14α-hydroxyl group is introduced very early in the sequence, after the formation of the unsaturated ketone. The sequence of hydroxylation of the side chain is very much species dependent. For example, in Calliphora stygia, the enzymatic hydroxylation appears to begin at C-22, to continue at position C-20 and end at positions C-25 or C-26 [9,13], while in the tobacco hornworm, Manduca sexta, hydroxylation at C-25 precedes that at C-22.[15]

The levels of moulting hormones in the insect organism vary in relation to life cycles. These levels are lowered by the action of catabolic processes which are controlled by certain enzymes. Ecdysterone in arthropods is catabolised by oxidation of the side-chain diol grouping with the formation of a C_{21} steroid, which is known as poststerone.[16,17] In the case of plants, poststerone is further degraded to the C_{19} steroid, rubrosterone.[18] An alternative mechanism of deactivation involves the formation of sulphates or glucoside derivatives.[19]

To gain a better understanding of the sequence of events in the

moulting process, the site of production and amount of the hormone, its manner of transport and target organs, an accurate chemical assay, capable of detecting sub-nanogram quantities in biological fluids was needed. Analytical techniques in current use include bioassay, fluorescence, radioimmunoassay and gas chromatography procedures. Bioassay techniques are of moderate sensitivity and lack specificity. Fluorescence and radioimmunoassay are sensitive but unspecific and technically difficult to perform. The fluorescence procedure requires relatively pure ecdysone samples which limits its areas of application for the analysis of crude biological material.[20] Radioimmunoassay requires expensive counting equipment and the up-keep of animal colonies for the supply of antigens.[21] At the onset of this work, GC was shown to be specific in that it could resolve ecdysones of similar structure but lacked sensitivity.[22]

The object of the present work was to examine the ways in which the unique separation powers of gas chromatography and its sensitivity, could be exploited to give sensitive detection of ecdysones by a simple and routine method. Such a method has been developed and shown to be applicable to physiological studies by analysis of hormone present in the desert locust, Schistocerca gregaria.

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CHAPTER 1

FLUOROCARBONSILYL ETHERS AS GC-ECD DERIVATIVES OF STEROIDS

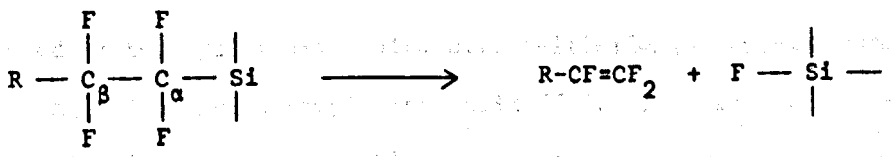
The principle problems associated with the analysis of hydroxylated steroids by gas chromatography are their limited thermal stability and poor peak shape which makes the resolution of mixtures difficult. The formation of non-polar derivatives is used to solve both these problems. At trace levels in biological tissue, the detection limit is set by the non-selective flame ionization detector. The use of the electron capture detector (ECD) allows as little as 10^{-12}g of sterol to be detected in favourable circumstances. This detector is selective for only those compounds which are able to capture thermal electrons. As steroids collect thermal electrons very poorly,[1-3] the introduction of an "electrophore" is required. Reagents for this purpose fall into two classes, namely, the haloacyl esters and the halomethyldimethylsilyl ethers.

The haloacyl esters include the chloroacetates, the fluorodichloroacetates, trifluoroacetates, pentafluoropropionates and heptafluorobutyrates.[4-14] No single reagent is ideal and they all share to some extent limited volatility, poor peak shape, hydrolytic instability and a range of sensitivity to the detector. The heptafluorobutyrates are the most popular as they combine the properties of ease of formation, produce volatile derivatives and are normally very sensitive to detection. Unfortunately they are also the least stable hydrolytically or thermally.[14-16] A further disadvantage of the haloacyl derivatives is that they are normally formed by the use of the acid anhydride which can affect unprotected ketone groups and they do not react quantitatively with hindered hydroxyl groups.[17-22] The introduction of heptafluorobutyryl-

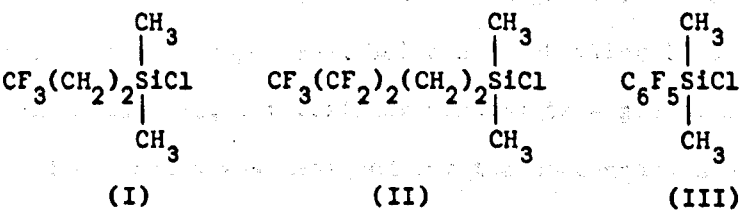
imidazole alleviated some of these problems as the by-product of the acylation is the amphoteric imidazole. However, this reagent does not react quantitatively with hindered hydroxyl groups.

The second class of reagent makes use of the trimethylsilylation reaction, in which a halogen atom is substituted for hydrogen in one of the methyl groups. This leads to the chloromethyldimethylsilyl-, bromomethyldimethylsilyl-, iodomethyldimethylsilyl-, ethers of sterols.[23-26] The chloromethyldimethylsilyl ethers are not very sensitive to ECD and are less volatile than the trimethylsilyl ethers. The bromomethyl- and iodomethyldimethylsilyl ethers are very sensitive to the ECD but have only moderate volatility on GLC, which precludes their use for polyhydroxy sterols. Only the halomethyldisilazane and the halomethylchlorosilane are available commercially which limits their use to unhindered hydroxyl groups in steroids that do not contain a readily enolizable ketone. Attempts to prepare the halomethyldimethylsilylimidazole from the halomethyldisilazane and imidazole by heating to reflux results in preferential cleavage of the halomethyl group. Both loss of the halomethyl group or substitution of the halogen can occur when silyl ether formation is attempted.[8,27]

A combination of the high sensitivity of the heptafluorobutyryl group towards ECD and the enhanced range of reactivity towards hindered hydroxyl groups of trimethylsilylimidazole suggested that a combination of these features in the same reagent would be useful. Direct attachment of the heptafluorobutyryl group to silicon is impractical because the silicon-to-acyl bond is chemically very labile and displacement of this group would occur.[28] Fluoroalkylsilanes with fluorine atoms α or β to silicon are thermally unstable at GLC temperatures necessary for sterols, giving alkenes by fluorine migration and elimination.[29-30]



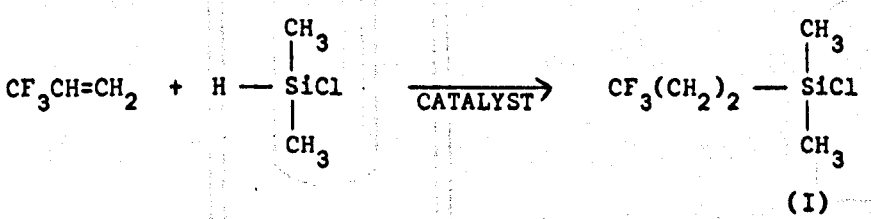
With these points in mind 3,3,3-trifluoropropyldimethylchlorosilane (I), 3,3,4,4,5,5,5,-heptafluoropentyldimethylchlorosilane (II) and pentafluorophenyldimethylchlorosilane (III) were synthesized for evaluation.



(I) (II) (III)

PREPARATION OF FLUOROALKYLSILANES

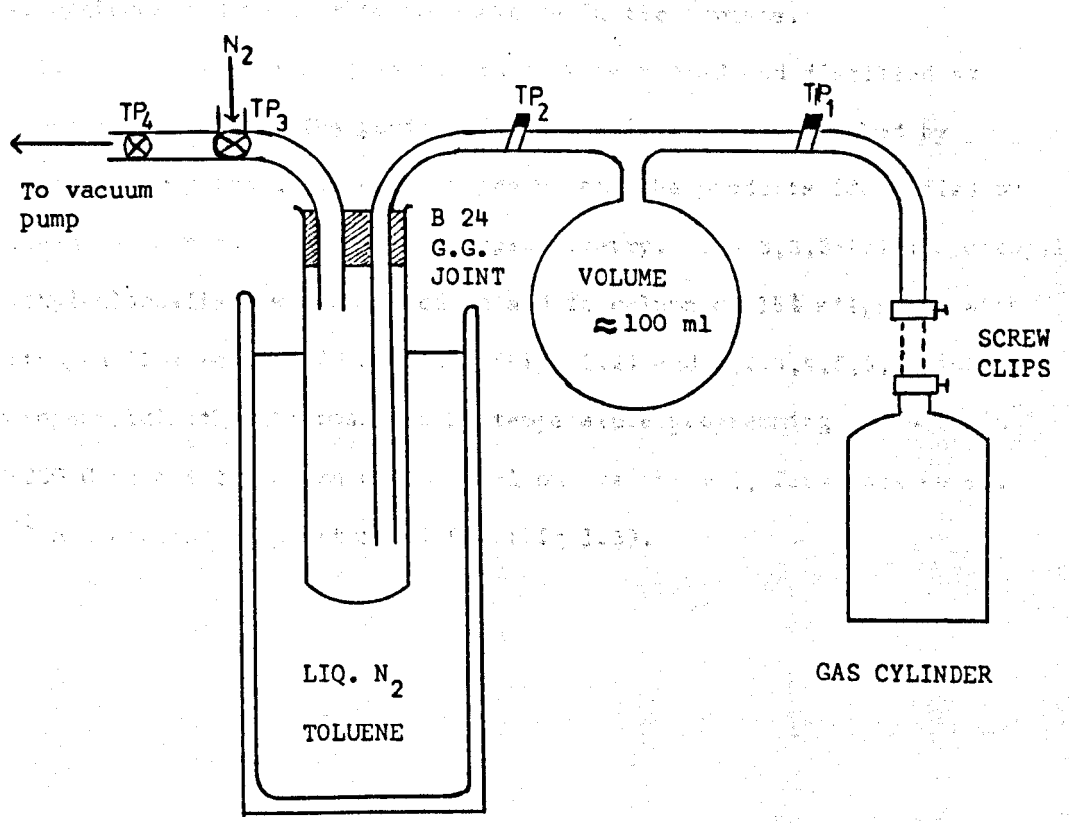
The trifluoropropyl group can be attached to silicon by the addition of the olefin 3,3,3-trifluoropropene to the appropriate silane[31] or by reaction of the Grignard reagent prepared from trifluoropropylbromide and a halosilane[32].



(I)

The olefin addition reaction was chosen because it produces a higher yield

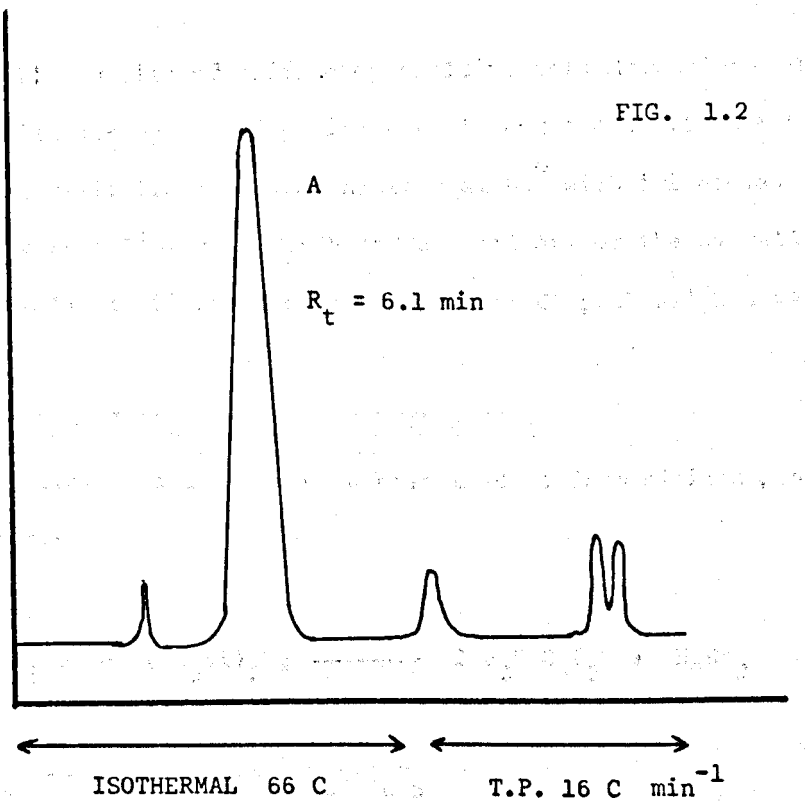
of chlorosilane with fewer by-products. The addition reaction is catalysed by t-butyl peroxide with u.v. initiation,[33] by platinum deposited on carbon[34,35] or by chloroplatinic acid[36,37]. The best conditions known for the addition reaction are high temperature and pressure in the presence of chloroplatinic acid. The use of chloroplatinic acid in the synthesis of (I) and (II) as well as many other organosilicon monomers has been reviewed[38]. The majority of these reactions are carried out in glass--lined high pressure autoclaves. As only small quantities of material were required for evaluation and a suitable autoclave was not available, the reactions were carried out in sealed glass tubes using a Carius furnace. Heptafluoropentene, being a low-boiling liquid could be handled by usual procedures, but trifluoropropene is a gas at room temperature and a special apparatus was designed for the condensation of known quantities of the gas into the Carius tube.



The Carius tube had a nominal volume of 75 cm^3 and a maximum working pressure of 16 atmospheres. It was fitted with a B24 ground glass socket to allow its convenient attachment to the apparatus. To condense the olefin in the Carius tube, the apparatus was evacuated (oil pump) and the cylinder valve and TP_1 opened to emit gas to the buffer volume V. The Carius tube was surrounded by coolant, the cylinder valve and TP_1 closed and TP_2 opened to fill the tube, TP_1 briefly opened to remove olefin from the connecting line, TP_2 was then closed and the Carius tube brought to atmospheric pressure by emitting nitrogen by TP_3. The amount of gas condensed was assumed to be the same as the difference in weight of the cylinder before and after the addition. The volume V was chosen so as to allow between 2.0 and 3.0 g of gas to be condensed per tube. The remaining reagents were added by syringe under nitrogen and the tube sealed in a gas flame without removal from the coolant. The tube was then placed in a metal cylinder and brought to temperature in the furnace.

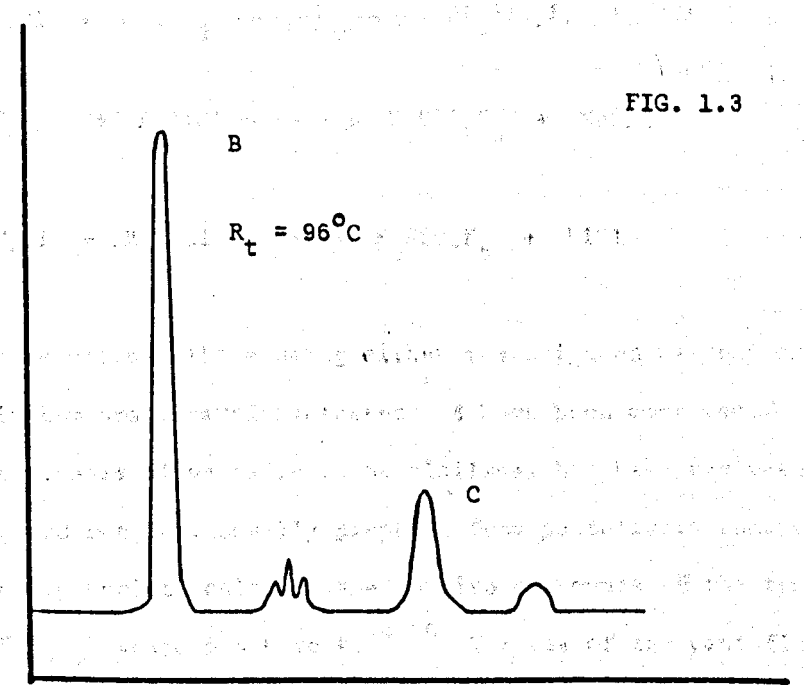
The products of more than one tube were pooled and distilled at atmospheric pressure. The purity of the distillates was checked by temperature-programmed gas chromatography and the products identified by combined gas chromatography - mass spectrometry. The 3,3,3-trifluoropropyldimethylchlorosilane was analysed on a 9 ft column of 15% silgrease with a nitrogen flow rate of 30 ml min^{-1} (Fig. 1.2) and 3,3,4,4,5,5,5-heptafluoropentyldimethylchlorosilane by temperature programming at 24°C min^{-1} (50-200 C)on a 5 ft column of 3% QF-1 on as hrom Q, flow rate 60 ml. min^{-1} and elution temperature of 96 C (Fig 1.3).

FIG. 1.2



GC of 3,3,3,-trifluoropropyldimethylchlorosilane (A)

FIG. 1.3



GC of 3,3,4,4,5,5,5-heptafluoropentyldimethylchlorosilane (B) and the disiloxane(C).

A small quantity of trifluoropropyldimethyl chlorosilane was converted to the disilazane by reaction with ammonia and the disilazane to trifluoropropyldimethylsilylimidazole by heating at 80° with imidazole. The reactions were followed by GC-MS and an estimate of the overall yield of silylimidazole of 60% based on comparison of GC peak heights was obtained.

SYNTHESIS OF SILICON PENTAFLUOROPHENYL BONDS.

The following reactions have been used to form silicon pentafluorophenyl bonds:

$$2\ C_6F_5Br + (Me_3Si)_2Hg \longrightarrow 2Me_3SiC_6F_5 + HgBr_2 \qquad \text{ref.39.}$$

$$C_6F_6 + SiF_2 \longrightarrow F_3SiC_6F_5 \qquad \text{ref.40.}$$

$$C_6F_6 + HSiCl_3 \xrightarrow{h\nu} Cl_2FSiC_6F_5 + HCl \qquad \text{ref.41.}$$

$$C_6F_5Cl + HSiCl_3 \xrightarrow[\text{irrad.}]{\gamma} Cl_3SiC_6F_5 + HCl \qquad \text{ref.42.}$$

$$C_6F_5MgX + R_3SiCl \longrightarrow R_3SiC_6F_5 + MgClX$$

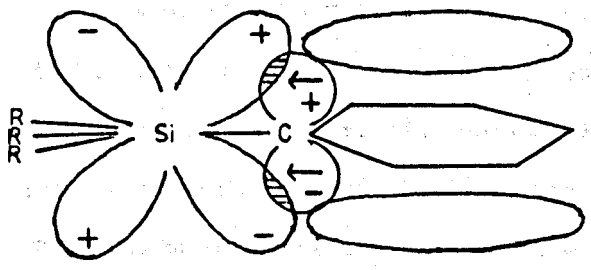
$$C_6F_5Li + R_3SiCl \longrightarrow R_3SiC_6F_5 + LiCl$$

Of these reactions, those using either the Grignard reagent or pentafluorophenyllithium are general syntheses and have been most used. The literature on the synthesis of pentafluorophenylsilanes has been reviewed up to 1969[43]. The Grignard reagent, usually prepared from pentafluorobromobenzene, reacts with the appropriate chlorosilane to give compounds of the type $R_xSi(C_6F_5)_{4-x}$, where x = 0 to 4.[44-46] The use of the pentafluorophenyllithium reagent is to be preferred as generally it gives a higher yield of

product and the reaction can be carried out at low temperatures (-65°) thus avoiding the by-products that are usually formed by the use of the Grignard reagent at higher temperatures.[47-51] As general observations, increasing pentafluorophenyl substitution on silicon appears to activate any remaining silicon halogen bonds to attack by pentafluorophenyllithium or pentafluorophenyl magnesium halide and the reactivity of the pentafluorophenyl silicon derivatives towards nucleophilic reagents rises with increase in the number of pentafluorophenyl substituents. Thus silanes with one to four pentafluorophenyl groups are all known. Those with one or two pentafluorophenyl groups are liquids while those with three and four are generally solids.

THE NATURE OF THE SILICON PENTAFLUOROPHENYL BOND.

To explain the chemical properties of pentafluorophenylsilanes, it has been necessary to propose a contribution of (p ⟶ d) π bonding between the pentafluorophenyl ring and silicon.[52]



Schematic atomic orbital representation of the dative π bond between silicon and the aromatic system.

Eaborn *et al.*[53], from a study of the kinetics of the acid cleavage of pentafluorophenyltrimethylsilane, concluded that the inductive effect of

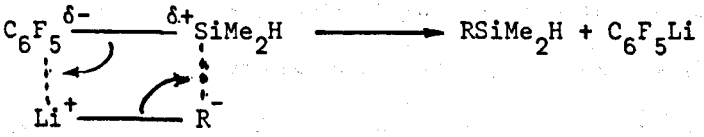
the five fluorine atoms was insufficient to explain the rate data and an interpretation in terms of (p ⟶ d) π bonding was proposed.

The ^{19}F chemical shift of pentafluorophenylsilanes have been measured[53 54]. Hogden et al.[54] developed an approach to the study of π - bonding, based on the linear relationship of $J_{2,4}$, the coupling constant between o- and p-fluorine atoms of the pentafluorophenyl group, to Øp, the chemical shift of the p-fluorine. A series of π-donor and acceptor substituents were measured and the results correlated with the Hammett-Taft reactivity parameters and to molecular orbital calculations of electron density. In the case of the pentafluorophenylsilanes, it was concluded that the inductive contribution from the silicon-pentafluorobenzene bond to the $J_{2,4}$ - Øp value was small in comparison with that expected from molecular orbital calculations from π-interactions of silicon with the benzene ring. Similar conclusions were reached by Egorochkin et al.[55 56] based on infra-red measurement of the ν(Si-H) stretching frequency. They developed a relationship between the experimentally observed ν(Si-H) stretching frequency and the calculated frequency for the same silane by an equation which sums all the Taft constants which affect that bond. The magnitude of the difference (Δν), between the two values was directly related to the conjugation effect. In those cases in which (p ⟶ d) π interaction was the main conjugative effect then the values of (Δν) were positive. This was shown to be the case for the pentafluorophenylsilanes.

In terms of electron-withdrawing power, in neutral molecules, a pentafluorophenyl ring is somewhat less electron-withdrawing than bromine. In transition states involving the generation of a partial negative charge at silicon, the electron-withdrawing power can be considerably greater than bromine.[50]

CLEAVAGE OF SILICON PENTAFLUOROPHENYL BONDS.

Smith and Gilman[57] have shown that cleavage of the C-Si bond in pentafluorophenyldimethylsilanes proceeded smoothly and cleanly under very moderate conditions (-70°) and in short times with methyl- or n-butyllithium. The product is the alkyldimethylsilane and the mechanism of the cleavage reaction can be visualized as a nucleophilic attack on the silicon atom viz:



The mechanism is favoured by electron-withdrawal by the aryl centre which increases the positive nature of the silicon atom. For bis(pentafluorophenyl)methylsilane cleavage of one pentafluorophenyl group from silicon proceeded readily with a variety of Grignard reagents at 0° in tetrahydrofuran or ether.[58] Cleavage of the remaining pentafluorophenyl group was much more difficult. With methyllithium or n-butyllithium cleavage of both pentafluorophenyl groups was effected rapidly under mild conditions. However, nucleophilic displacement of para-fluorine atoms occurred predominantly with t-butyllithium. The steric bulk of the t-butyllithium lessens its ability to cleave the Si-C bond and shifts attack mainly to the relatively more accessible *p*-fluorine atoms. Attack of the *p*-fluorine atom has been observed in the reaction between n-butyllithium and pentafluorophenyldiphenylsilane, although with methyllithium, displacement of the pentafluorophenyl group occurred.

The formation of $(C_6F_5)_2SiH_2$ and C_6F_5H when $(C_6F_5)_3SiCl$ was reacted

with $LiAlH_4$ at room temperature is presumably due to cleavage of the pentafluorophenyl groups from silicon by $LiAlH_4$. At -30°, cleavage in the above reaction is minimal.[50]

$$2(C_6F_5)_3SiCl + LiAlH_4 \longrightarrow 2(C_6F_5)_2SiH_2 + 2C_6F_5H$$

In a heterogeneous medium tetrakis (pentafluorophenyl) silane has been shown to be stable to strong acid and base at reflux temperatures.[47] In an homogeneous medium cleavage of the silicon pentafluorophenyl bond occurs slowly in acid or base conditions and also in water-acetone mixtures. The reactivity of pentafluorophenyl-silicon compounds towards nucleophiles increases with the number of pentafluorophenyl substituents. Thus pentafluorophenyltrimethylsilane is more stable under the above conditions, but cleavage still occurs.

THE SYNTHESIS OF PENTAFLUOROPHENYLDIMETHYLSILANES.

The method of Oliver and Graham[51] was used to introduce the pentafluorophenyl silicon bond into the dimethylsilanes reported here. Typically, pentafluorophenyllithium[51,59] at -70° was reacted with the appropriate dimethylchlorosilane in a nitrogen atmosphere, the reaction mixture allowed to reach room temperature, lithium chloride filtered off and the products fractionated by distillation. When dimethylchlorosilane, chloromethyldimethylchlorosilane and dichloromethyldimethylchlorosilane were used the products were pentafluorophenyldimethylsilane, pentafluorophenyldimethylchloromethylsilane, and pentafluorophenyldimethyldichloromethylsilane respectively. The synthesis of pentafluorophenyldimethylsilane has been reported previously[51,60].

It has been reported that attempts to prepare pentafluorophenyldimethylchlorosilane from pentafluorophenyllithium and dimethylchlorosilane resulted

in the formation of bis(pentafluorophenyl)dimethylsilane.[51] Pentafluorophenyldimethylchlorosilane was conveniently prepared by the room temperature chlorination of pentafluorophenyldimethylsilane in the dark and in almost quantitative yield.[61] In a similar manner, pentafluorophenyldimethylbromosilane was prepared by the room temperature addition of bromine to pentafluorophenyldimethylsilane.[51] An attempt was made to prepare pentafluorophenyldimethyliodosilane, initially by reaction of the silane with iodine or iodine with aluminium powder or potassium iodide catalysis without success. Refluxing 1,3-bis(pentafluorophenyl)-1,1,3,3-tetramethyldisilazane with aluminium triodide showed consumption of the disilazane but GC-MS of the reaction products indicated only material of higher molecular mass than required by the iodosilane. It was found that GC of the bromosilane produced one peak for authentic material but the mass spectrum produced by the GC-MS method was of higher molecular weight than that calculated for the bromosilane. Analysis of the mass spectrum did not reveal any bromine isotope peaks indicating that the bromosilane was not stable to GC. The GC-MS technique, therefore, was probably not suitable for monitoring the formation of the iodosilane. The decomposition of alkyliodosilanes at moderate temperatures and catalytically on glass surfaces has been reported.[63] Infra-red and N.M.R. spectra of the reaction mixture indicated the absence of disilazane, but as more than one product was formed, positive evidence for iodosilane formation could not be obtained.

The general procedure for the formation of disilazanes is the reaction of ammonia with the appropriate chlorosilane in a suitable inert solvent.[64] The reaction is believed to proceed through the silylamine which condenses with itself to form the disilazane. The nature of the product is determined by steric factors and triethylchlorosilane is taken as the steric transition,

yielding the silylamine instead of the disilazane.[65] Phenyldimethylbromosilane reacts with ammonia to produce the disilazane.[65] Pentafluorophenyldimethylchlorosilane reacted with ammonia to produce pentafluorophenyldimethylsilylamine as the sole product. The GC-MS technique could not be used with this compound, as spectrally pure material produced several peaks, none of which corresponded to the silylamine. Introduction of the same sample through the liquid inlet of the mass spectrometer gave a spectrum interpretable in terms of the silylamine with no major unassigned peaks or ions of m/e greater than the parent ion of the silylamine. Silbiger and Fuchs[66] have described a procedure for the synthesis of 1,3-bis(phenyl)-1,1,3,3-tetramethyldisilazane by refluxing hexamethyldisilazane and phenyldimethylchlorosilane in the presence of a crystal of aluminium chloride[66]. In an analogous procedure using pentafluorophenyldimethylchlorosilane, 1,3-bis(pentafluorophenyl)-1,1,3,3-tetramethyldisilazane was obtained in 55% yield.

Pentafluorophenyldimethyliodomethylsilane was prepared by the addition of sodium iodide to pentafluorophenyldimethylchloromethylsilane in acetone. The iodomethylsilane was the only product detected by GC and was identified by its mass spectrum.

Pentafluorophenyldimethylsilyldiethylamine was prepared by the addition of diethylamine to pentafluorophenyldimethylchlorosilane at -70°C, the mixture allowed to reach room temperature and fractionated to give the product in 67% yield.

A general procedure for the preparation of alkylsilylimidazoles has been described by Kuhn *et al.*[67] in which imidazole is refluxed for several hours with the alkyldisilazane or alkylsilylamine, to give the product in good yield. Neither 1,3-bis(pentafluorophenyl)-1,1,3,3-tetramethyldisilazane nor pentafluorophenyldimethylsilylamine under similar conditions

yielded any silylimidazole. Addition of pentafluorophenyldimethylchlorosilane as a catalyst had no effect on the reaction and in all cases starting material could be recovered in good yield. Pentafluorophenyldimethylsilyldiethylamine with or without chlorosilane catalysis did not react with imidazole. The addition of imidazolyllithium or imidazolyl magnesium halide[68] to pentafluorophenyldimethylchlorosilane in ether at -70°C resulted in the consumption of the chlorosilane with the production of an undistillable polymeric product. Chalk[69] has shown that secondary amines can be trimethylsilylated by dicobalt octacarbonyl catalysis of the reaction between the amine and trimethylsilane. The reaction can be represented by the following equations:

$$Co_2(CO)_8 + 2R_3SiH \longrightarrow 2R_3SiCo(CO)_4 + H_2$$

$$R_3SiCo(CO)_4 + Me_2NH \longrightarrow Me_2NSiR_3 + HCo(CO)_4$$

$$HCo(CO)_4 + R_3SiH \longrightarrow R_3SiCo(CO)_4 + H_2$$

The addition of an ether solution of dicobalt octacarbonyl to imidazole and pentafluorophenyldimethylsilane in ether caused formation of a deep blue colour with the evolution of gas. Analysis of the reaction mixture by GC indicated that the silane had not been consumed. The addition of dicobalt octacarbonyl to imidazole produced a similar colouration and the evolution of gas. It is likely that a complex of the type

$$\left[Co(Im)_6\right]^{2+} \quad 2\left[Co(CO)_4\right]^{-} \qquad \text{Im = Imidazole.}$$

is formed. A similar complex is known containing pyridine.[70]

In an attempt to prepare chloromethyldimethylsilylimidazole by refluxing 1,3-bis(chloromethyl)-1,1,3,3-tetramethyldisilazane and imidazole the only volatile product observed was 1,3-bis(imidazoyl)-1,1,3,3-tetramethyldisilazane in moderate yield formed by nucleophilic attack by imidazole on silicon with expulsion of chloromethane. Advantage of this displacement reaction could be taken for the synthesis of pentafluorophenyldimethylsilylimidazole. Pentafluorophenyldimethylchloromethylsilane was heated at 80°C with imidazole for up to twenty hours. Analysis by GC indicated the presence of two peaks as well as starting material.

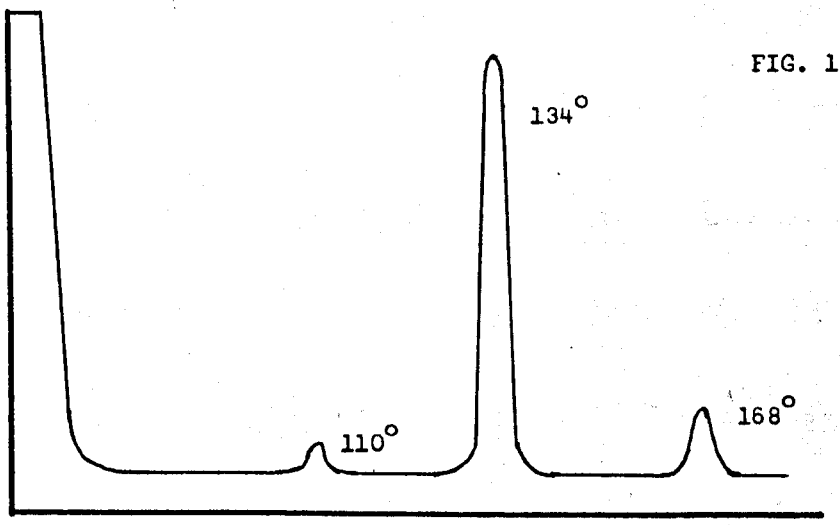


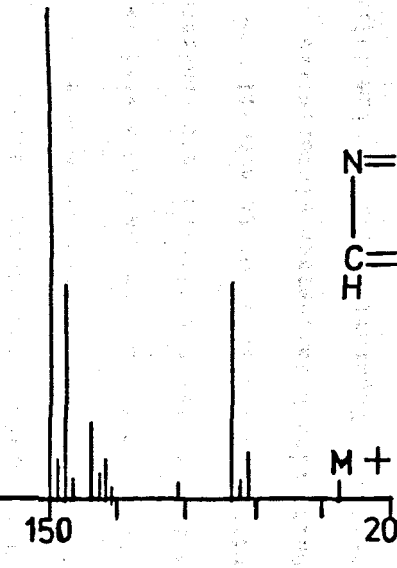
FIG. 1.4

GC of reaction product from pentafluorophenyldimethylchloro--methylsilane and imidazole on a 5 ft. column of 3% QF-1, 60 ml. min^{-1}, 60-200°C at 16°C min^{-1}. R_t = 110°C dimethyldiimidazolylsilane, R_T = 134°C pentafluorophenyl--dimethylchloromethylsilane and R_T = 168°C pentafluorophenyl--dimethylsilylimidazole.

The sample was analysed by GC-MS and the peaks identified as follows; the compound with elution temp. 110°C was identified as dimethyldiimidazoylsilane (Fig 1.5), that at 134°C as pentafluorophenyldimethylchloromethylsilane and at 168°C as pentafluorophenyldimethylsilylimidazole (Fig 1.6).

40
50
100

60
100
150



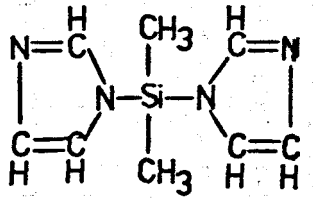


FIG. 1.5

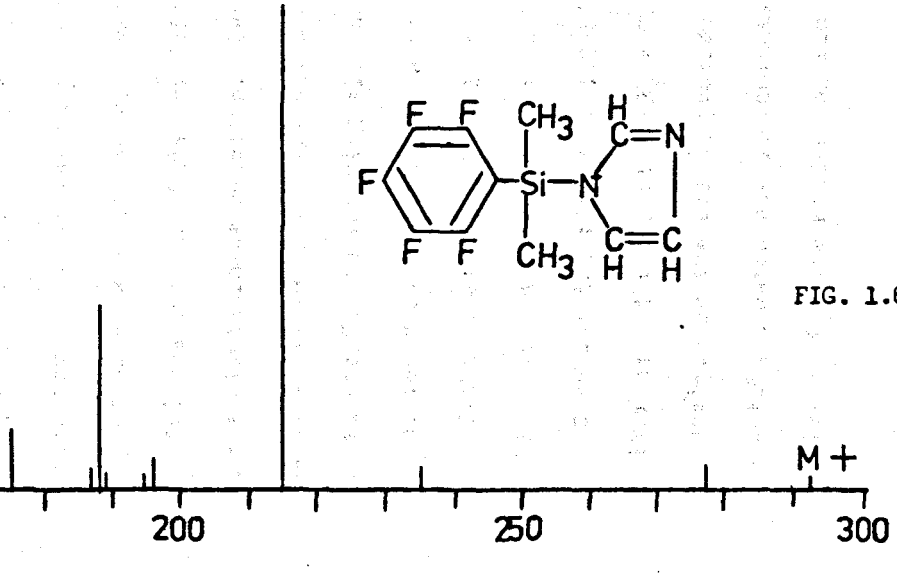


FIG. 1.6

The total yield of products was low and estimated to be 5-10%. In order to improve the yield of product, pentafluorophenyldimethyldichloromethylsilane was treated with imidazole, but as in the previous case, much starting material remained. The addition of imidazoyllithium to the dichloromethylsilane and GC of the reaction mixture indicated that all the starting material had been consumed to produce the silylimidazole in approximately 30% yield (overall based on pentafluorobenzene). Addition of imidazoyllithium at -70°C and allowing the mixture to warm slowly to room temperature did not improve the yield. Dimethylformamide, dimethyl sulphoxide or diglyme were all useful as solvents but the reaction in acetonitrile was very slow. Dimethyl sulphoxide was difficult to remove by vacuum distillation and led to decomposition of the product at high temperatures. Dimethylformamide is more volatile but distillation at 35°C/15mm also showed decomposition of the pentafluorophenyldimethylsilylimidazole. Monitoring the distillation procedure by GC indicated that decomposition only occurred when the solvent was reduced to a small volume and a visible precipitate formed. This precipitate is presumably a lithium salt generated in the displacement reaction. Evaporation of the solution to a small volume followed by precipitation of salts with hexane-ether mixtures was not successful in removing all the material. Separation from lithium salts was achieved by passage down a short column of neutral alumina, eluting with dichloromethane. Thin layer chromatography on neutral alumina in dichloromethane gave two spots absorbing in the U.V.. The spot at R_f = 0.84 was identified as 1,3-bis(pentafluorophenyl)-1,1,3,3-tetramethyldisiloxane and the spot at R_f = 0.54 as pentafluorophenyldimethylsilylimidazole. By the column procedure a small quantity of the silylimidazole was obtained which was contaminated with the disiloxane. This was sufficient for the studies on reaction rate with steroids undertaken.

The separation procedure as it stands is unsuitable for obtaining pure samples of pentafluorophenyldimethylsilylimidazole in high yield. A pure sample could be obtained by preparative gas chromatography. The use of alumina leads to hydrolysis which might be avoided by sephadex chromatography (LH-20) or by reversed-phase chromatography on amberlite XAD-2. The overall yield could possibly be improved by increasing the electronegativity of the leaving group, i.e. if pentafluorophenyldimethyltrichloromethylsilane were used as an intermediate. Imidazole forms many types of metallic salts[111] and advantage might be taken of a study of these for the purpose of providing the imidazoyl anion. The use of a larger cation than lithium might favour its precipitation from dimethylformamide by the addition of less polar solvent.

PHYSICAL AND SPECTRAL PROPERTIES OF PENTAFLUOROPHENYLDIMETHYLSILANES.

Infra red spectra.

The infra red spectra of seven pentafluorophenyldimethylsilanes are summarized in Table 1.1. Characteristic absorption for ν(Si-H), 2170 cm^{-1}, ν(SiN-H_2) 3485, 3410 cm^{-1}, ν(Si_2N-H) 3370 cm^{-1} and ν(Si-N-Si) 1180, 930 cm^{-1} are easily distinguished in the appropriate spectra. The absorptions at 1038 and 940 cm^{-1} in pentafluorophenyldimethylsilyldiethylamine are probably due to ν(SiN-alkyl) stretching. A doublet in the region 2970-2900 cm^{-1} is characteristic of the C-H stretch of the methyl groups and bands in the region 1250 and 840 cm^{-1} are due to ν(Si-CH_3) absorption.[51] The strong band in the region 1650-1635 cm^{-1} has been assigned by Oliver and Graham[51] to a vibration of the pentafluorophenyl ring perturbed by the central silicon atom. Although this band is common to all silanes investigated, it is also present in pentafluorobenzene and pentafluorophenylalkyldimethylsilanes[71] and is therefore probably caused by a pentafluorophenylaromatic deformation stretching. The region 1400-1000 cm^{-1} is dominated by

TABLE (1.1)

TABLE OF I.R. ABSORPTIONS OF PENTAFLUOROPHENYLDIMETHYLSILANES.

COMPOUND	ABSORPTION CM^{-1}	
$C_6F_5Si(CH_3)_2H$	2960 m, 2920 m, 1641 s, 1465 s, 1375 s, 1284 s, 1252 m, 1082 s, 961 m, 855 m, 695 m, 630 m.	ν(Si-H) 2170 s
$C_6F_5Si(CH_3)_2Cl$	2960 m, 2910 m, 1644 s, 1518 s, 1468 s, 1387 m, 1290 m, 1265 m, 1081 s, 972 s, 860 m, 843 m, 680 m.	
$C_6F_5Si(CH_3)_2Br$	2955 m, 2900 m, 1640 s, 1465 s, 1380 m, 1288 m, 1262 m, 1088 s, 971 s, 863 m, 681 m.	
$C_6F_5Si(CH_3)_2CHCl_2$	2960 m, 2900 m, 1638 s, 1510 m, 1460 s, 1378 s, 1284 s, 1255 s, 1082 s, 962 s, 855 m, 695 s, 670 s.	
$C_6F_5Si(CH_3)_2NH_2$	2960 m, 2905 m, 1640 s, 1515 m, 1465 s, 1371 m, 1282 s, 1260 s, 1083 s, 972 s, 855 m, 652 m.	ν(SiN-H_2) 3485 m 3410 m
$C_6F_5Si(CH_3)_2N(CH_2CH_3)_2$	2970 m, 2930 m, 2870 m, 1642 s, 1515 s, 1465 s, 1380 m, 1282 m, 1082 s, 975 s, 845 m, 800 s, 700 m.	ν(SiN-X) 1038 s 940 m
$[C_6F_5Si(CH_3)_2]_2$-NH	2955 m, 2900 m, 1640 s, 1512 m, 1460 s, 1371 m, 1285 s, 1255 s, 1080 s, 972 s, 850 m, 680 m.	ν(N-H) 3370 m ν(Si-N-Si) 1180 s 930 s

m = medium
s = strong

absorption due to C-F bonds, these absorptions showing their usual high intensity.

Nuclear magnetic resonance spectra.

The ^{1}H proton N.M.R. spectra of nine pentafluorophenyldimethylsilanes are summarized in Table 1.2. The methyl absorptions occur in the region 0-1.0 ppm and are influenced by the electronegativity of the fourth group attached to silicon. The NH and NH_2 absorptions were very broad and could not be unequivocally assigned. Spectra were run at 60 MHz and measured as the neat liquid with 5% benzene as internal standard, quoted in ppm from TMS.

Gas chromatography

With the exception of the pentafluorophenyldimethylsilyl-amine, -bromide and possibly the -iodide, all the silanes studied were stable to gas chromatography. An evaluation of suitable GC phases, indicated that QF-1 gave better resolution than Apiezon L, silgrease, OV-101 or OV-17. A 5 ft. 3-% QF-1 on gas chrom. Q column was used throughout this study. By temperature programme gas chromatography, 60-210°C at 16°C min^{-1} with a nitrogen flow rate of 60 ml. min^{-1} adequate separation of reaction mixtures was obtained. With the standard conditions, described above, the silanes could be identified by their elution temperature. These temperatures for nine pentafluorophenyldimethylsilanes are summarized in Table 1.3. The resolution of the system is illustrated by a separation of a synthetic mixture (Fig.1.7)

TABLE (1.2)

PROTON N.M.R. SPECTRA OF PENTAFLUOROPHENYLDIMETHYLSILANES

COMPOUND	$Si(CH_3)_2$	OTHER
$C_6F_5Si(CH_3)_2H$	0.46[a]	H 4.64[b]
$C_6F_5Si(CH_3)_2Cl$	0.92	
$C_6F_5Si(CH_3)_2Br$	0.99	
$C_6F_5Si(CH_3)_2CH_2Cl$	0.55	CH_2Cl 3.05
$C_6F_5Si(CH_3)_2CHCl_2$	0.67	$CHCl_2$ 5.60
$C_6F_5Si(CH_3)_2NH_2$	0.57	
$C_6F_5Si(CH_3)_2N(C_2H_5)_2$	0.66	C_2H_5 1.19[c] 3.13[d]
$[C_6F_5Si(CH_3)_2]_2NH$	0.60	
$[C_6F_5Si(CH_3)_2]_2O$	0.65	

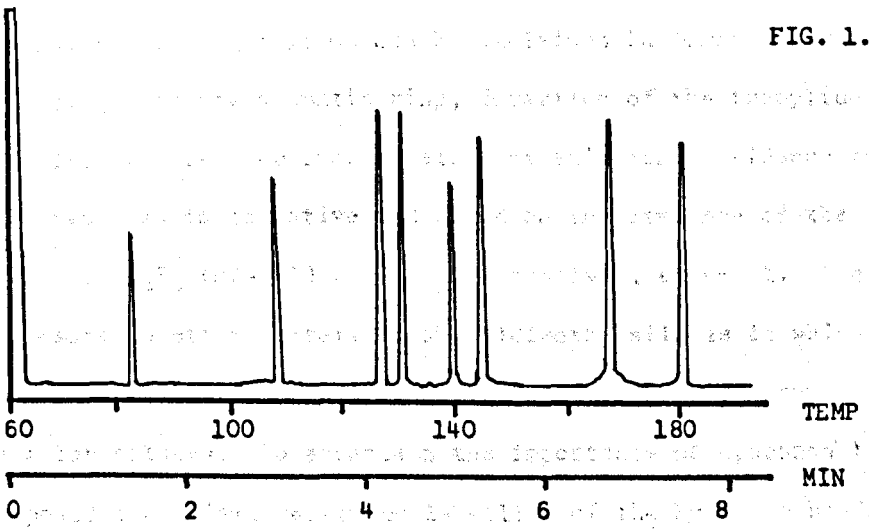
a = doublet, b = septet, c = triplet, d = quartet.

TABLE (1,3)

GC ELUTION TEMPERATURES OF PENTAFLUOROPHENYLDIMETHYLSILANES (see text)

COMPOUND	ELUTION TEMP. ^{o}C
$C_6F_5Si(CH_3)_2H$	83
$C_6F_5Si(CH_3)_2Cl$	109
$C_6F_5Si(CH_3)_2N(C_2H_5)_2$	127
$[C_6F_5Si(CH_3)_2]_2NH$	131
$C_6F_5Si(CH_3)_2CH_2Cl$	134
$C_6F_5Si(CH_3)_2CH_2I$	140
$C_6F_5Si(CH_3)_2CHCl_2$	145
$C_6F_5Si(CH_3)_2$Imidazole	168
$[C_6F_5Si(CH_3)_2]_2O$	181

FIG. 1.7



Separation of a mixture of pentafluorophenylsilanes. The components in order of elution are RH, RCl, $RN(C_2H_5)_2$, R_2NH, RCH_2I, R $CHCl_2$, R-Imidazole and ROR where R = pentafluorophenyldimethylsilyl.

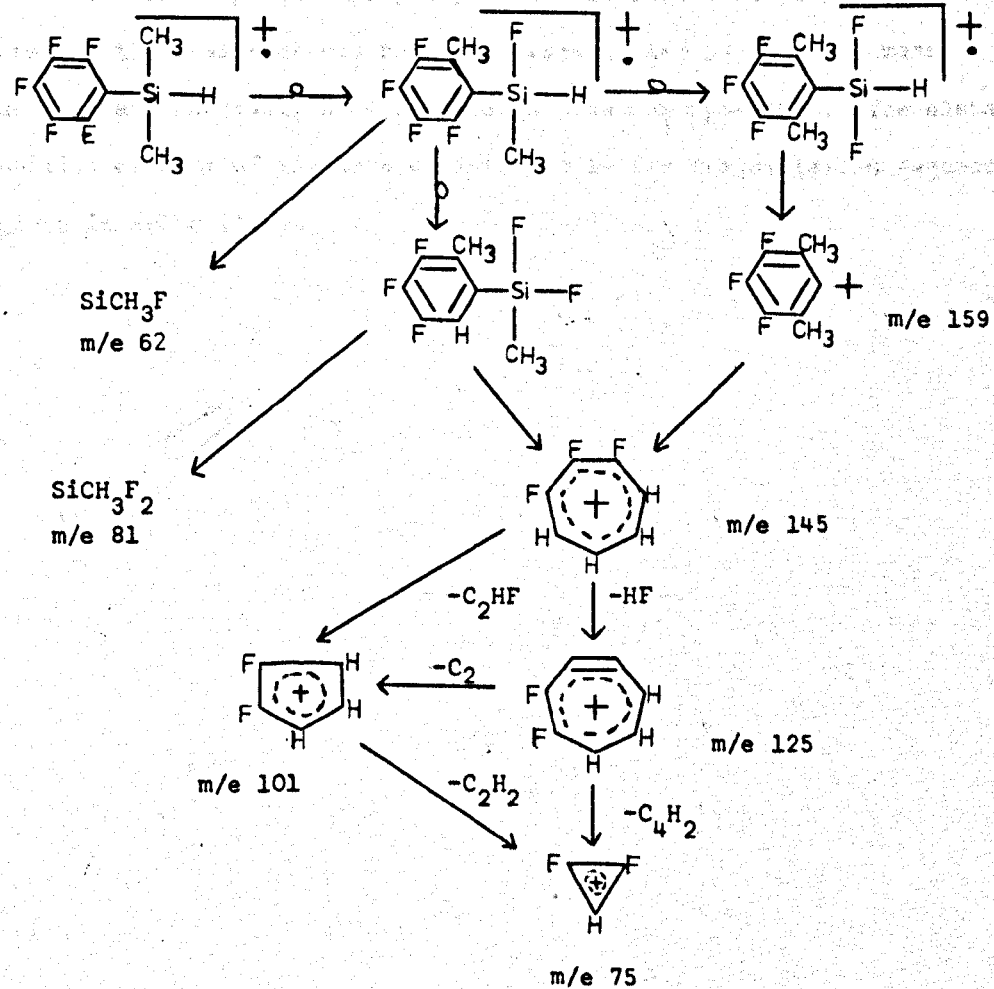
Mass spectra of pentafluorophenyldimethylsilanes.

Analysis of the mass spectra of pentafluorophenyldimethylsilanes, which were synthesised during this study, are remarkable for their lack of silicon containing ions as the charge carrying species. The preferred sequence of fragmentation involves the elimination of π-stabilized fluorocarbon rings. Their formation involves rearrangement in which fluorine and methyl groups are exchanged between the phenyl ring and silicon. The general features of the mass spectra of pentafluorophenylsilanes have not been investigated previously although reports of the spectra of pentafluorophenyltrimethylsilane,[72] tetrakis(pentafluorophenyl)silane and the related pentafluorophenol, pentafluorothiophenol[74] together with some fluorinated spirobis(silafluorene)[75] compounds have appeared.

Pentafluorophenyldimethylsilane, fragments as illustrated in scheme I. That abstraction of fluorine by silicon occurs is established by the prominent ions $Si(CH_3)_2F$ (m/e 77), $SiCH_3F_2$ (m/e 81) and SiF (m/e 47).

Those ions not containing silicon can be explained in terms of transfer of methyl or hydrogen to the aromatic ring, formation of the tropylium ion $C_7H_4F_3$ (m/e 145) and further fragmentation of this ion. Evidence for the exchange of hydrogen is tentative and based on the presence of the abundant ions $SiCH_3F_2$ (m/e 81) and $C_7H_4F_3$ (m/e 145), scheme 1. These ions are also present in other pentafluorophenyldimethylsilanes in which the only hydrogen available for transfer is the silicon-bonded methyl groups (e.g. the chlorosilane). To ascertain the importance of hydrogen transfer in the proposed mechanism, deuterium labelling of the hydrogen bonded to silicon would be required.

SCHEME 1



There is evidence from fluoroaromatic compounds to suggest that fluorohydrocarbon tropylium ions are stable species. The tropylium ion structures are formulated on the basis of the random production of fluorocarbon, hydrocarbon and fluorohydrocarbon fragments from the precursor ion. No study of the energetics of formation of such ions has appeared to support this formulation. The mass spectrum of pentafluorotoluene ($C_6F_5CH_3$) shows a ready loss of one hydrogen atom to give the base peak m/e 181 ($C_7H_2F_5$) which can be formulated as a tropylium ion[76,77]. Bruce and Thomas[78] have described the mass spectra of compounds represented by $C_6F_5CH_2X$, where X=H, Br, $CH{=}CH_2$, COCl and $CH_2C_6F_5$, in all of which the base peak was the ion at m/e 181.

In the case of pentafluorophenyldimethyldichloromethylsilane the features of the mass spectrum have been established by accurate mass measurement and observation of metastable ions where possible. The elemental composition of some of the ions of interest in the fragmentation sequence are given in table (1.4).

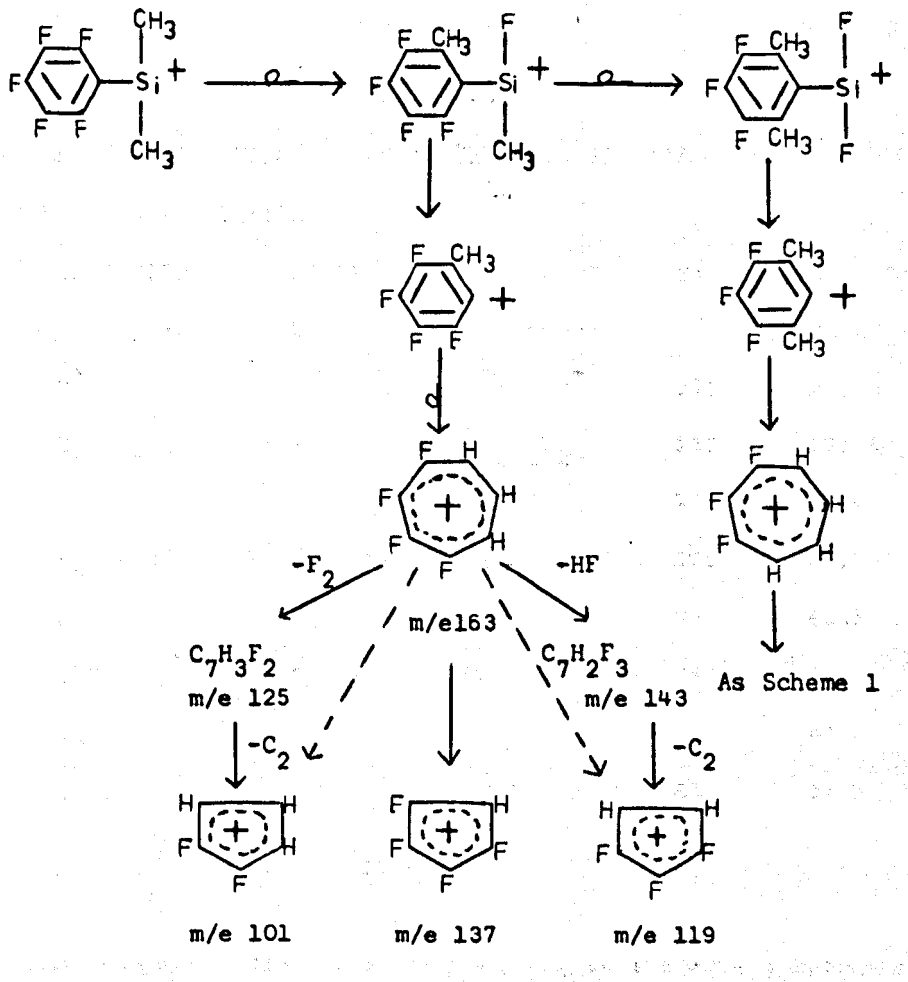
TABLE (1.4)

ACCURATE MASSES FOR FRAGMENT IONS IN PENTAFLUOROPHENYLDIMETHYLDICHLORO-METHYLSILANE.

NOMINAL MASS	MASS MEASURED	COMPOSITION	MASS CALCULATED	PERCENTAGE OF NOMINAL MASS
161	161.0010	$C_7H_1F_4$	161.0014	80
	160.9355	C_6FCl_2	160.9362	20
159	159.0421	$C_8H_6F_3$	159.0422	100
145	145.0261	$C_7H_4F_3$	145.0265	100
129	128.9950	C_6F_3	128.9952	100
125	125.0201	$C_7H_3F_2$	125.0203	100
81	81.0134	C_5H_2F	81.0141	10
	80.9967	CH_3SiF_2	80.9972	90
79	79.0188	Not assigned		35
	78.9980	C_5F	78.9984	56
	78.9767	CH_4SiCl	78.9771	9
75	75.0238	C_6H_3	75.0235	66
	75.0046	C_3HF_2	75.0046	34
63	63.0230	C_5H_3	63.0235	100

Initial fragmentation appears to take place as in Scheme 2, consisting of ions derived from a tropylium ion containing three or four fluorine atoms. The observed metastable transitions are listed in table (1.5) and are consistent with some of the transitions proposed in schemes 1 and 2.

SCHEME 2



Of particular interest is the metastable ion at 69.4 for m/e 225 ⟶ 125 which has been observed in the spectra of several derivatives of pentafluorophenyldimethylsilanes.

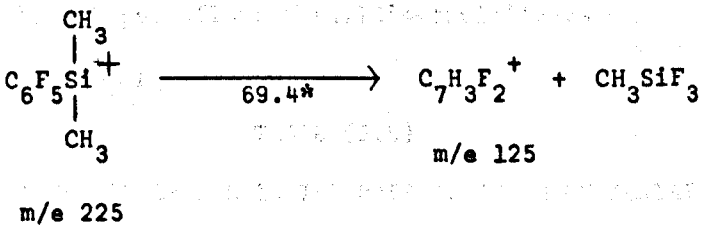


TABLE (1.5)

METASTABLE SUPPORTED TRANSITIONS IN THE MASS SPECTRA OF PENTAFLUOROPHENYLDIMETHYLDICHLOROMETHYLSILANE.

MASS	COMPOSITION	FRAGMENT LOST	FRAGMENT FORMED	MASS	METASTABLE MASS
308	M^+	Cl	$(M-Cl)^+$	273	242.3
159	$C_8H_6F_3$	HF	$C_8H_5F_2$	139	121.5
125	$C_7H_3F_2$	C_2H_2	C_5HF_2	99	78.4
225	$C_8H_6F_5Si$	CH_3SiF_3	$C_7H_3F_2$	125	69.4
137	C_5HF_4	C_2HF	C_3F_3	93	63.1
101	$C_5H_3F_2$	C_2H_2	C_3HF_2	75	55.7
125	$C_7H_3F_2$	C_4H_2	C_3HF_2	75	45.0
123	C_7HF_2	C_5H	C_2F_2	62	31.2

Miller has reported a similar metastable ion in the mass spectrum of pentafluorophenyltrimethylsilane.[72] The ion m/e 125 might also be expected to derive from fluorinated tropylium ion, but no metastable ion for that route was observed.

The silicon-containing ions in the mass spectrum of pentafluorophenyldimethyldichloromethylsilane are few in number and vary widely in intensity. The principal ions are listed in Table (1.6). The ion m/e 81 is a doublet, the principal component of which is CH_3SiF_2; normally an abundant ion in all pentafluorophenyldimethylsilanes.

TABLE (1.6)

SILICON CONTAINING FRAGMENTS IN THE MASS SPECTRUM OF PENTAFLUOROPHENYL-DIMETHYLDICHLOROMETHYLSILANE.

MASS	FRAGMENT LOST FROM PARENT ION	ION COMPOSITION	RELATIVE ABUNDANCE
308/310/312	-	$C_9H_7Cl_2F_5Si$	4(308)
273/275	Cl	$C_9H_7ClF_5Si$	6(273)
260/262	CHCl	$C_8H_6ClF_5Si$	7(260)
225	$CHCl_2$	$C_8H_6F_5Si$	100
215/217/219	C_3F_3	$C_6H_7Cl_2F_2Si$	8(215)
191	$C_2H_4FCl_2$	$C_7H_3F_4Si$	12
189	C_4HCl_2	$C_5H_6F_5Si$	38
187	CHF_2Cl_2	$C_8H_6F_3Si$	60
179	$C_3H_4FCl_2$	$C_6H_3F_4Si$	11
175	$C_2HF_2Cl_2$	$C_7H_6F_3Si$	6
77		C_2H_6FSi	77
63/65		ClSi	37(63)

In halomethyl or dihalomethylsilanes, loss of a halogen atom is frequently seen, or transfer of halogen to silicon with expulsion of the halomethyl group.

The principal silicon-containing ions of a representative series of pentafluorophenyldimethylsilanes are given in Table (1.7). Considerable variation in intensity is found between different compounds. The ion m/e 81 is not included because it is a doublet, with a variable contribution from a fluorocarbon ion. The ion m/e 77 is usually greater than 50% of the base peak in most mass spectra of the series.

TABLE (1.7)

RELATIVE ABUNDANCE OF THE PRINCIPAL SILICON-CONTAINING IONS IN PENTAFLUOROPHENYLDIMETHYLSILANES.

COMPOUND	SiF	$(CH_3)_2Si$	$(CH_3)_2SiF$	$C_6F_5Si(CH_3)_2$
	m/e 47	m/e 58	m/e 77	m/e 225
$C_6F_5Si(CH_3)_2H$	69	100	48	3
$C_6F_5Si(CH_3)_2Cl$	59	9	50	9
$C_6F_5Si(CH_3)_2NH_2$	32	2	100	32
$C_6F_5Si(CH_3)_2N(CH_2CH_3)_2$	62	15	87	-
$C_6F_5Si(CH_3)_2CH_2Cl$	32	3	100	61
$C_6F_5Si(CH_3)_2CH_2I$	25	35	57	62
$C_6F_5Si(CH_3)_2CHCl_2$	10	-	77	100

The principal fluorohydrocarbon ions in the same series of compounds are given in Table (1.8). The ions m/e 125 ($C_7H_3F_2$) and 129 (C_6F_3) are usually of moderate intensity with m/e 125 invariably the stronger.

TABLE (1.8)

THE RELATIVE ABUNDANCE OF THE PRINCIPAL FLUOROHYDROCARBON IONS IN PENTAFLUOROPHENYLDIMETHYLSILANES

MASS	H	Cl	NH_2	$N(C_2H_5)_2$	CH_2Cl	CH_2I	$CHCl_2$	COMPOSITION
163	-	12	11	30	-	-	15	$C_7H_3F_4$
159	6	28	53	3	15	71	60	$C_8H_6F_3$
145	68	50	8	8	3	18	20	$C_7H_4F_3$
143	4	28	9	36	8	16	12	$C_7H_2F_3$
137	-	20	18	2	2	18	28	C_5HF_4
129	20	55	23	13	9	14	32	C_6F_3
125	38	100	52	25	43	51	74	$C_7H_3F_2$
119	4	20	23	25	9	35	24	$C_5H_2F_3$
117	8	27	13	5	3	12	21	C_5F_3
111	42	18	5	2	12	22	13	C_6HF_2
110	10	37	6	-	9	27	18	C_6F_2
105	3	20	9	3	7	18	10	C_4F_3
101	22	30	11	40	7	3	14	$C_5H_3F_2$
99	18	60	40	16	14	39	41	C_5HF_2
97	2	80	2	25	9	5	45	$C_3H_4F_3$
95	8	30	15	20	7	8	18	$C_3H_2F_3$
93	16	52	18	10	12	7	42	C_3F_3

In the high mass region of the spectra of 1,3-bis(pentafluorophenyl)-1,1,3,3-tetramethyldisiloxane and 1,3-bis(pentafluorophenyl)-1,1,3,3,-tetramethyldisilazane are found ions for which accurate mass measurements (Table (1.9)) indicated the absence of silicon and which have a composition represented by fluorohydrocarbon ions.

The structures of these ions are proposed on the basis of their elemental composition.

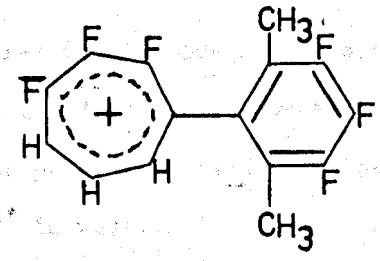
TABLE 1.9

ACCURATE MASS DATA FOR DIAGNOSTIC IONS IN THE SPECTRUM OF 1,3-BIS(PENTAFLUOROPHENYL)-1,1,3,3-TETRAMETHYLDISILOXANE.

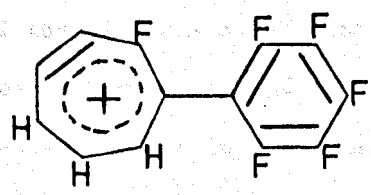
NOMINAL MASS	ELEMENTAL COMPOSITION	MASS MEASURED	CALCULATED MASS
355	$C_{12}H_6OF_7Si_2$	354.9866	354.9845
303	$C_{15}H_9F_6$	303.0572	303.0608
273	$C_{13}H_3F_6$	273.0133	273.0139
227	$C_{11}F_5$	226.9928	226.9920
203	$C_7H_9OF_2Si_2$	203.0166	203.0159
151	$C_5H_9OF_2Si$	151.0406	151.0390
141	C_6H_6OFSi	141.0168	141.0172[1]
	$C_3H_4OF_3Si$	140.9999	140.9983
109	$C_4H_5Si_2$	108.9937	108.9929[2]
	C_2F_3Si	108.9723	108.9721

1 Ratio of high to low mass 2:1

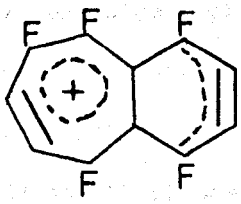
2 Ratio 1:1



m/e 303

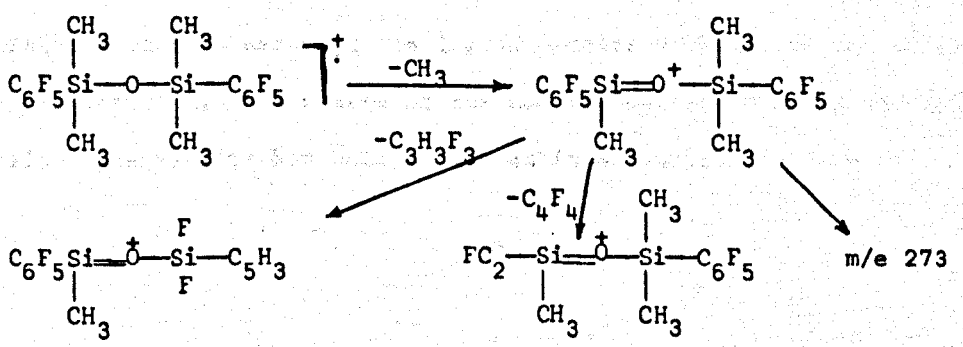


m/e 273



m/e 227

Their formation presumably involves migration of pentafluorophenyl rings to the same silicon centre prior to elimination. The formation of the ions at m/e 355,327 and 273 from m/e 451 $(M-CH_3)^+$ was established by the metastable ions at 279.4, 237.1 and 165.3 respectively.



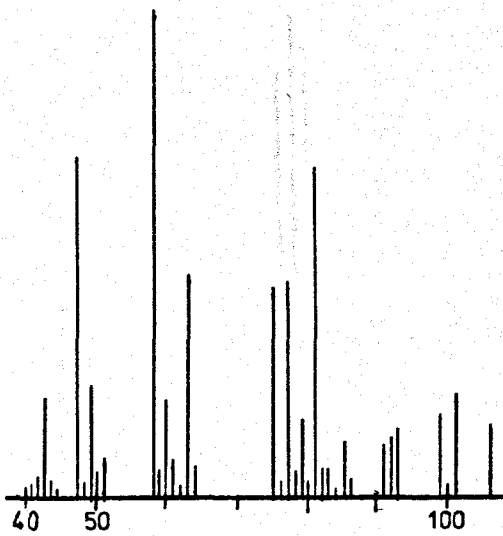
m/e 355

m/e 327

m/e 273

An interesting feature of the spectra of the disiloxane and disilazane, is that in the disiloxane, the molecular ion is very weak, and $(M-CH_3)^+$ is the base peak; for the disilazane, the molecular ion is the base peak and $(M-CH_3)^+$ is weak.

All of the silanes studied here give recognizable molecular ions, which fragment by loss of methyl to give ions of moderate intensity. In all cases there is evidence of transfer of fluorine from benzene to silicon, recognized by ions of the type $Si(CH_3)_2F$. Exchange of fluorine and methyl groups leads to tropylium-type fluorohydrocarbon ions which with their breakdown products dominate the spectrum. Ions due to exchange of one methyl group to give the tropylium ion m/e 163 ($C_7H_3F_4$) or both methyl groups to give m/e 145 ($C_7H_4F_3$) are observed. Although exchange of both methyl groups is common to all spectra studied, the single exchange is not observed in several examples. The mechanism of this exchange probably involves the vacant d-orbitals of silicon and their interaction with the phenyl ring. Hawthorne *et al*[79] have suggested that when a metal is σ-bonded to unsaturated organic ligands, mass spectral rearrangement ions can be rationalized by assuming the ligand becomes π-bonded to the metal during fragmentation. The nature of the bonding between silicon and the pentafluorophenyl ring has been discussed in an earlier section.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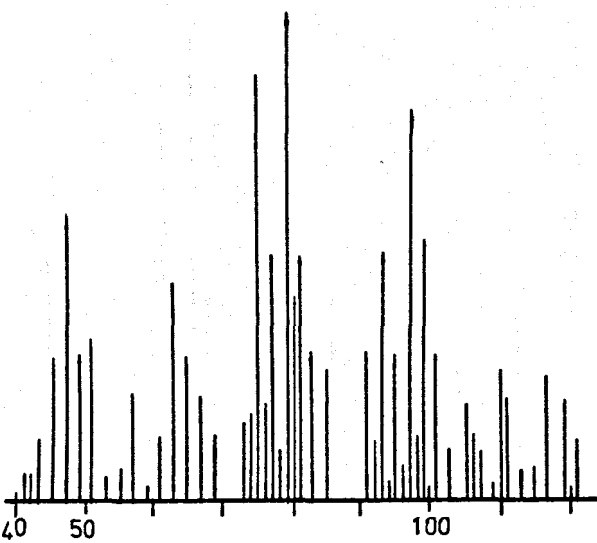
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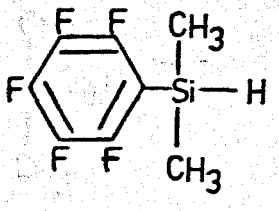
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H
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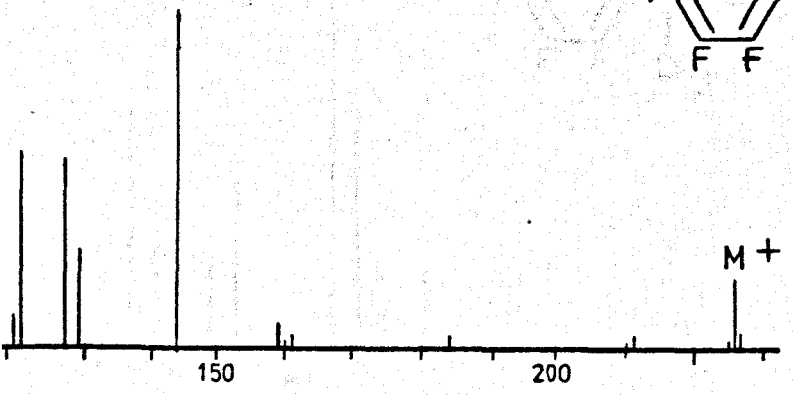
F
CH3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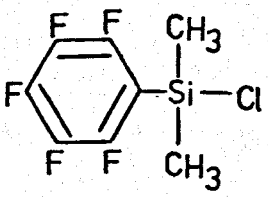
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M+
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200
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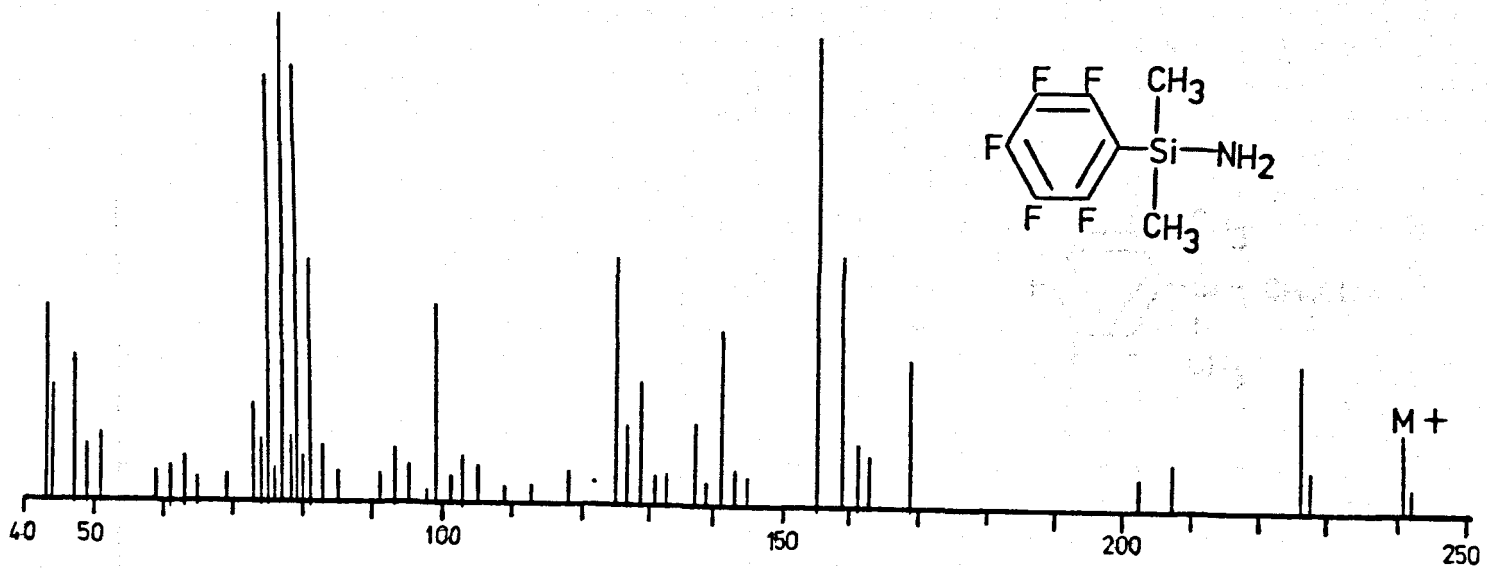
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F Si NH2
F F CH3
M+
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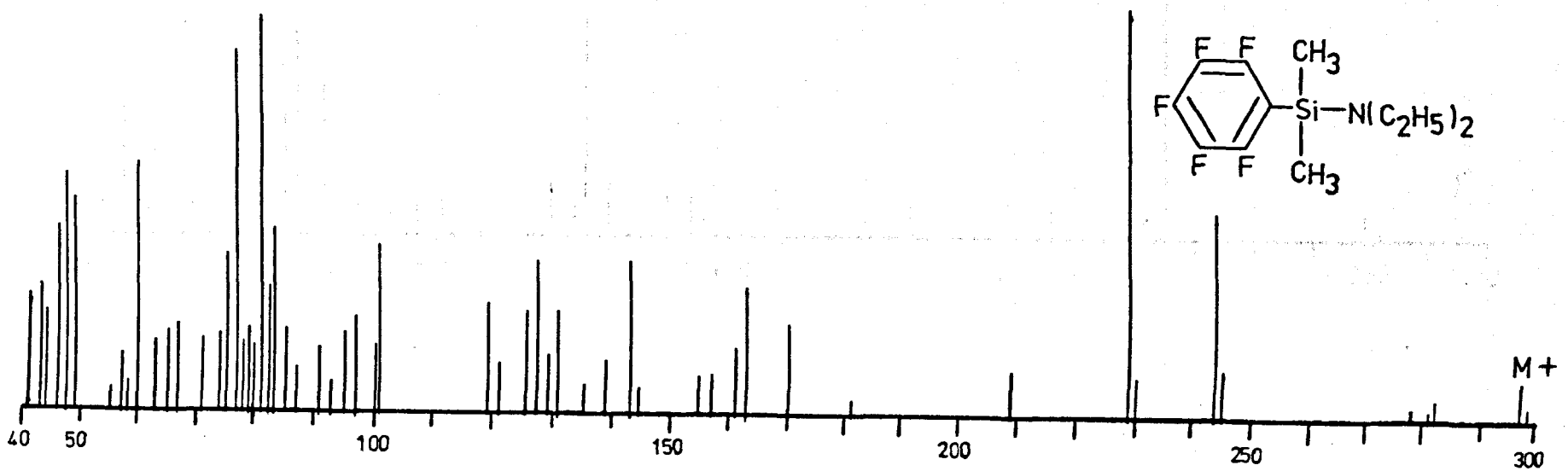
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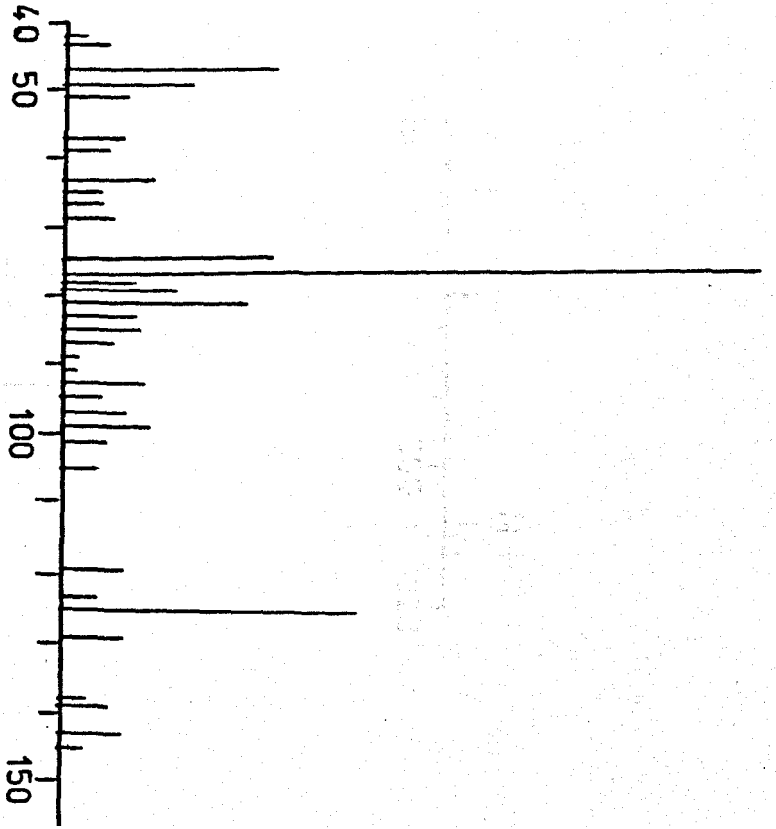
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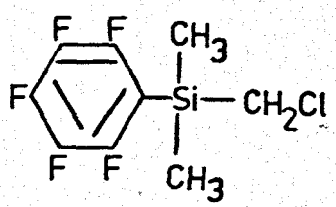
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CH3
F
Si
CH2Cl
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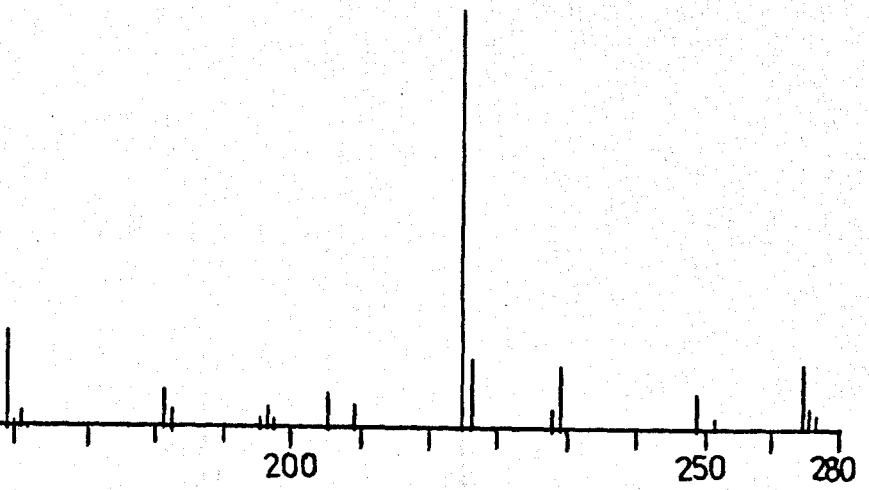
F
CH3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M+
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40
50
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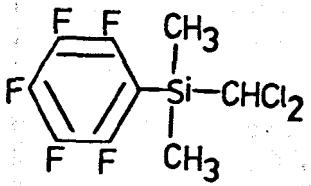
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CH3
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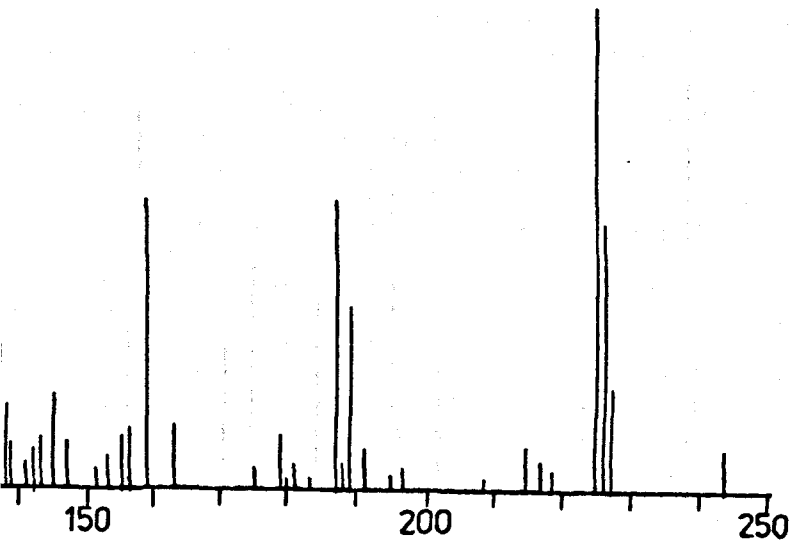
Si
CHCl2
F
F
CH3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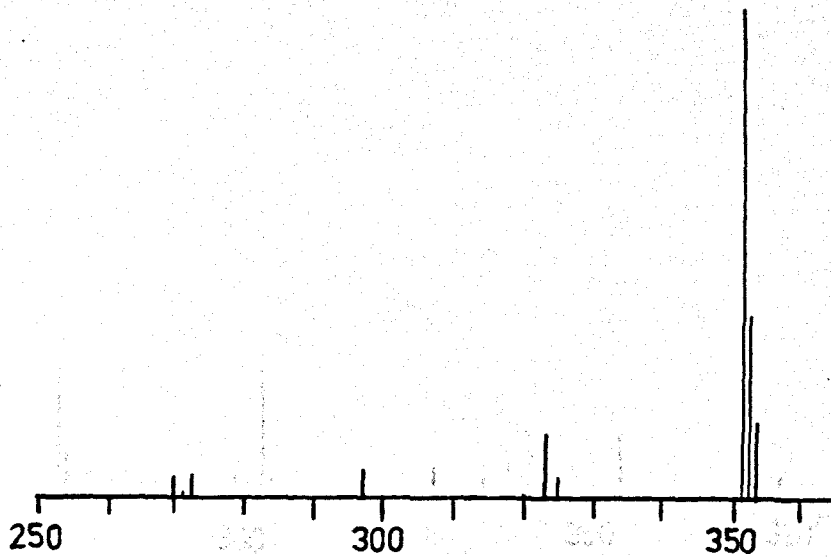
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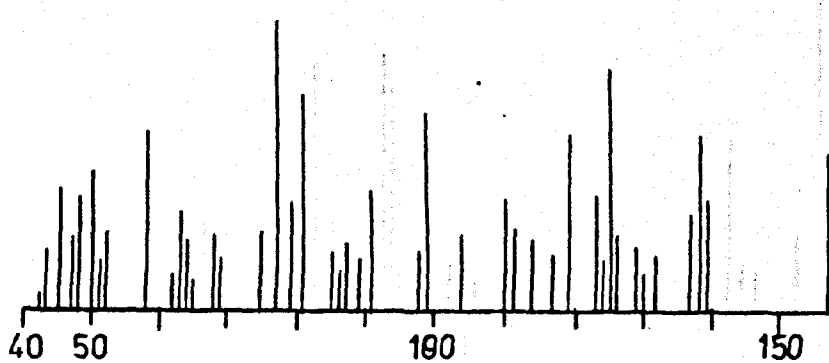
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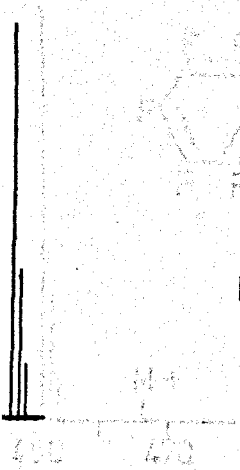

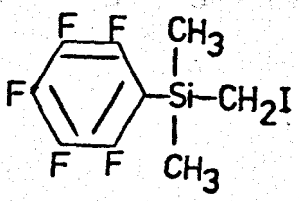
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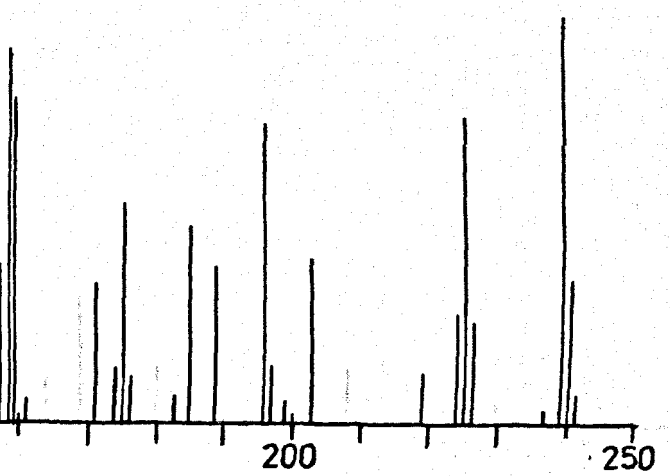
F
CH3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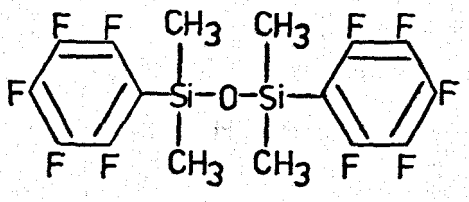
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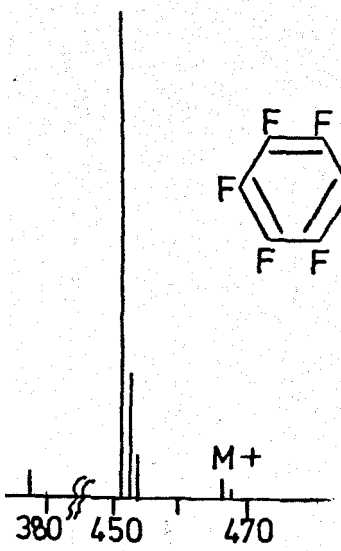
Si–O–Si
F
F F CH3 CH3 F F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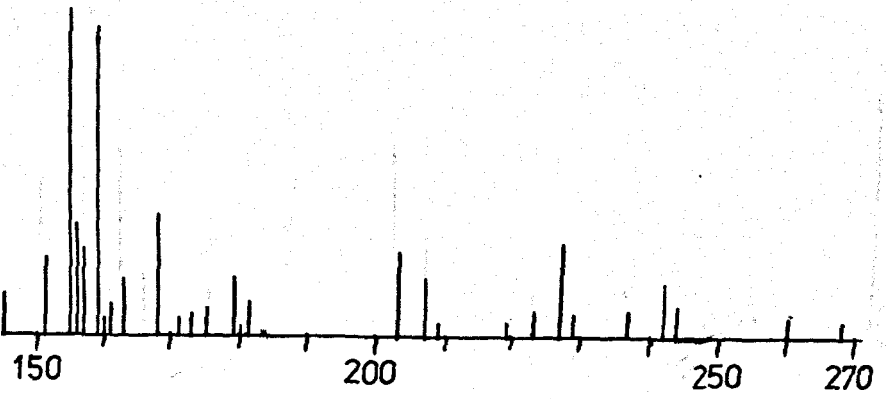
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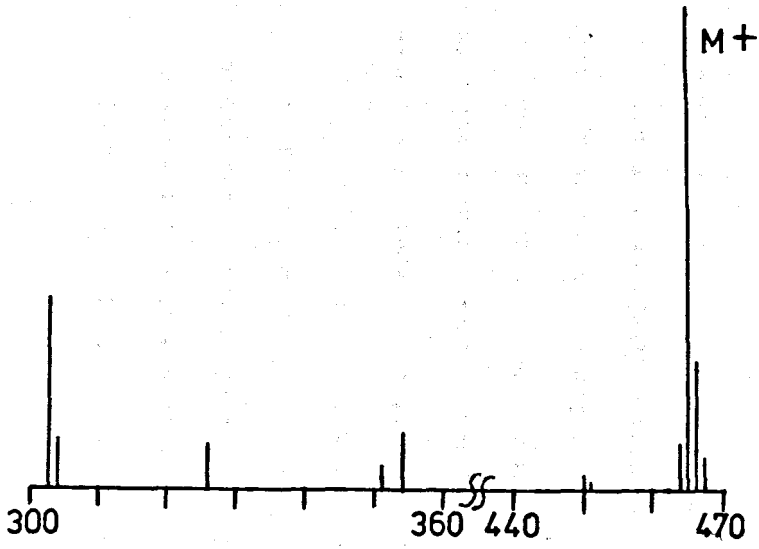
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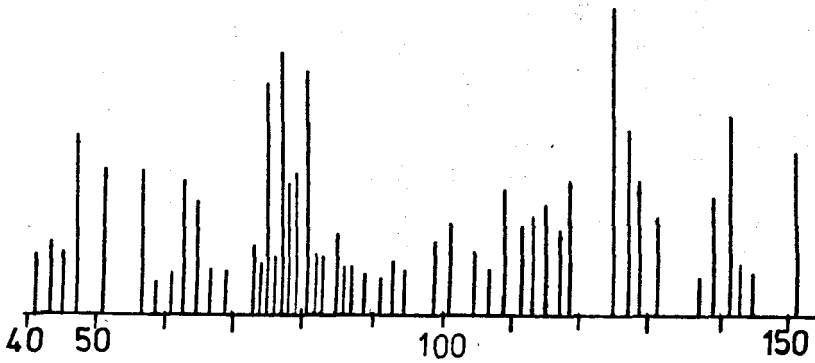
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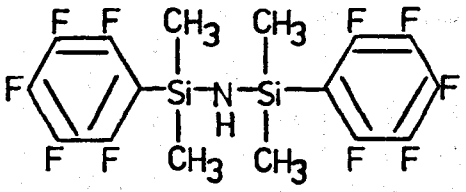
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F
Si N Si
H
F
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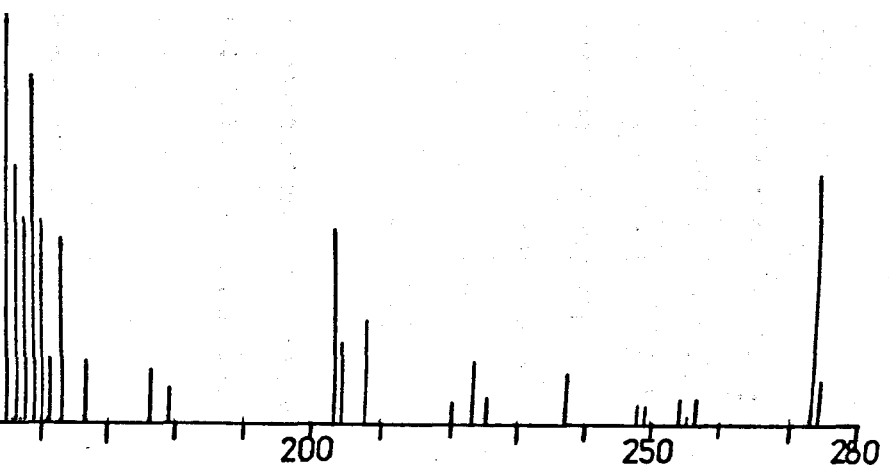
200
250
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THE FORMATION OF FLUOROCARBONSILYL ETHERS OF STEROIDS.

To assess the behaviour of the fluorocarbonsilyl ethers as GC derivatives of steroids and to establish their sensitivity towards the electron capture detector, the appropriate silyl ethers of cholesterol were prepared. Typically, 100 µl of the chlorosilane was added to 100 µl of diethylamine in 0.5 ml. of hexane under nitrogen and after thirty minutes the mixture centrifuged and 100 µl. of the supernatant added to 1-5 mg. of cholesterol in a screw-capped Reacti-vial. After heating at 60°C for 3 hours, solvent and reagent were removed in vacuo and the cholesteryl ether dissolved in ethyl acetate for gas chromatography using FID or hexane for ECD analysis. In all cases a single product was obtained which was identified by mass spectrometry as the appropriate silyl ether.

The fluorocarbonsilyl ethers prepared here are compared in terms of volatility on GC with some other silyl ethers of cholesterol in Table (1.10).

TABLE (1.10).

RELATIVE RETENTION TIMES FOR GC OF CHOLESTEROL SILYL ETHERS AND R_f VALUES ON SILICA GEL TLC, ELUTING WITH TOLUENE.

COMPOUND	COLUMNS			
	A	B	C	R_f
cholesterol	1.00	1.00	1.00	0.05
TMS-cholesterol	1.17	0.92	0.69	0.68
$CF_3(CH_2)_2Si(Me)_2$-cholesterol	1.47	1.24	1.06	0.70
$CF_3(CF_2)_2(CH_2)_2Si(Me)_2$-cholesterol	1.60	0.87	0.68	0.64
$ClCH_2Si(Me)_2$-cholesterol	2.54	1.87	1.69	0.60
$C_6F_5Si(Me)_2$-cholesterol	3.68	2.84	2.78	0.67

COLUMN A: 3ft 1% OV-101 on Gas-Chrom Q, 75 ml. min^{-1} N_2, 250°C, R_t Cholesterol = 1.58 min.

COLUMN B: 3ft 1% Dexsil 300 GC on Gas-Chrom Q, 75 ml. min^{-1} N_2, 290°C, R_t Cholesterol 1.03 min.

COLUMN C: 1.5ft., 1.5% OV-101 plus 1.0% tetramethylcyclobutanediol adipate, 90 ml. min^{-1} N_2, R_t cholesterol 2.45 min., 250°C.

The fluorocarbonsilyl ethers had excellent thermal stability and good volatility on chromatography, the non-polar nature of the fluoroalkyl groups producing relatively shorter retention times on the polar column C, in spite of their increased molecular weights. On the non-polar column A, the excellent volatility of closely bound fluorine atoms is illustrated by the similar retention times of the trifluoropropyl and heptafluoropentyldimethylsilyl ethers of cholesterol. The fluorocarbonsilyl ethers were shown to be stable to silica gel TLC with a variety of solvents.

The poor sensitivity of the fluoroalkylsilyl ethers towards electron capture (see page 52) limits their use for this purpose. Their excellent volatility may find uses in the separation of steroids by GC-FID. Further, it was noted that the heptafluoropentyldimethylsilyl ether of cholesterol fluoresced strongly in u.v. light. This strong blue emission could form the basis of a fluorescence method for the determination of steroids. A combination of the reasonable volatility and electron capture properties of pentafluorophenyldimethylsilanes was encouraging and a more detailed evaluation of its properties made. For the sake of convenience, the abreviated name FLOPHEMESYL will be used in place of the more cumbersome expression pentafluorophenyldimethylsilyl when discussing reagents and steroid derivatives in the following sections.

Uncatalysed Reactions of Flophemesyl Reagents

In pyridine solution, the order of reactivity of flophemesyl reagents

towards steroid hydroxyl groups is:
flophemesylamine > flophemesyl chloride > flophemesyldiethylamine
flophemesyldisilazane >> flophemesylimidazole. Unlike trimethylsilyl-imidazole, flophemesylimidazole was found to be virtually unreactive and is of little value as a flophemesylating reagent. This unpredicted feature was presumably due to electron-withdrawal of the pentafluorophenyl ring, preventing the development of a negative charge on nitrogen in the transition between breaking a Si-N and formation of a Si-O bond.[80] The uncatalysed reactions of flophemesyldisilazane and flophemesyldiethyl-amine with cholesterol paralleled those of the trimethylsilyl reagents, giving 85% conversion in 24 hours at room temperature. Increasing the reaction temperature did not improve the yield. Flophemesyl chloride in chloroform reacted with cholesterol to form approximately 80% of the ether after heating at 60°C for 16 h., while in pyridine the reaction went to completion in 2-3 h. It would seem in this case that pyridine was acting as a catalyst, perhaps through the formation of an intermediate, such as a N-flophemesylpyrinidium salt.In the absence of base catalysis, flophemesyl chloride was not a strong flophemesylating reagent. The flophemesylamine reacted quantitatively with cholesterol, ergosterol, and 2β,3β-dihydroxy-5α-cholestane in a variety of solvents at room temperature. It did not react with tertiary hydroxyl groups such as the 17β-OH in 17α-methyl-17β-hydroxyandrost-4-en-3-one or hindered secondary hydroxyl groups such as the 11β-OH in 11β-hydroxyandrost-4-en-3,20-dione As an added advantage, it did not promote the formation of flophemesyl enol ethers from unprotected ketone groups. Flophemesylamine at room and elevated temperatures did not react with C-3, -6, -11, -17 steroid ketone groups, or 4-en-6-one, 7-en-6-one unsaturated ketones. It is thus the reagent of choice for the flophemesylation of unhindered secondary

hydroxyl groups. It also enables a distinction to be made between normal secondary hydroxyl groups and hindered and tertiary hydroxyl groups with which it is totally unreactive.

Catalysed Flophemesylation Reactions.

The addition of an acid catalyst to a flophemesyl reagent increased its reactivity, reducing the time required for complete reaction and causing hindered hydroxyl groups to react. This was not particularly marked with the flophemesylamine reagent, and since other flophemesyl reagents show a marked increase in activity with acid catalysis, the amine is more useful as an uncatalysed, selective protecting reagent.

All the Lewis type acid catalysts tried had catalytic properties but the most efficient catalyst was flophemesyl chloride or flophemesyl bromide. Boron trifluoride and p-toluenesulphonyl chloride or acid had an advantage in being soluble in organic solvents but small amounts of secondary peaks due to decomposition of the steroid were observed. Aluminium chloride was exceptional in producing a large number of secondary peaks. Ammonium sulphate was inefficient, probably because of its low solubility in the reaction mixture.

Using flophemesyl chloride as catalyst, the reactivity of the flophemesyldisilazane and particularly the flophemesyldiethylamine, increased markedly. The catalysed flophemesyldisilazane gave a quantitative yield of flophemesyl cholesterol in 3 h at 65°C and the flophemesyl-diethylamine in less than fifteen minutes at room temperature. The flophemesyldisilazane did not react with hindered secondary hydroxyl groups even with a catalyst.

Flophemesyldiethylamine when catalysed by flophemesyl chloride in pyridine as solvent was the strongest flophemesylating reagent mixture produced. The reactivity of the reagent combination was dependant on the

amount of catalyst employed. A 10:1 mixture of flophemesyldiethylamine and flophemesyl chloride reacted quantitatively with unhindered secondary hydroxyl groups of the cholesterol type and also tertiary hydroxyl groups such as the 17β-OH of 17α-methyl-17β-hydroxyandrost-4-en-3-one. It did not react with the hindered 11β-OH in 11β-hydroxyandrost-4-en-3,17-dione. Unprotected ketone groups were not enolized by this reagent combination. A 1:1 mixture of flophemesyldiethylamine and flophemesyl chloride reacted readily with ketone groups necessitating their protection, usually as the methoxime derivative, before reaction of the hydroxyl groups. In pyridine, this reagent combination completely converted the 11β-OH of 11β-hydroxy-androst-4-en-3,17-dimethoxime to its flophemesyl ether in 3 h at 60°C and the 17α-OH of 17α-hydroxypregn-4-en-3,20-dimethoxime to the flophemesyl ether in 6 h at 85°C or 1 h at 150°C. The 14α-OH of 2β,3β,14α-trihydroxy--5β-cholest-7-en-6-methoxime was unaffected by all reagents.

The reactivity of the flophemesyl reagents with various steroids is summarized in Tables (1.11) and (1.12).

TABLE (1.11)

CONDITIONS FOR THE FORMATION OF FLOPHEMESYL STEROID ETHERS

STEROID	FLOPHEMESYL CHLORIDE IN PYRIDINE	FLOPHEMESYL-AMINE	FLOPHEMESYL-DIETHYLAMINE: FLOPHEMESYL CHLORIDE 10:1	FLOPHEMESYL-DIETHYLAMINE FLOPHEMESYL CHLORIDE 1:1
Cholesterol	A	B	B	B
Ergosterol	A	B	B	B
Cholestanol	A	B	B	B
2β,3β-dihydroxy-5α-cholestane	A	B	B	B
2β,5α,6β-trihydroxycholestane	2β,6β A	2β,6β B	2β,6β B	2β,6β B
2β,5α-dihydroxycholestan-6-one	2β A *	2β B	2β B	NQ *
2β,3β,14α-trihydroxycholest-7-en-6-one	2β,3β A *	2β,3β B	2β,3β B	2β,3β B *
3α,20α-dihydroxy-5β-pregnane	A	B	B	B
17α-methyl-17β-hydroxy-androst-4-en-3-one	NQ *	NR	B	B *
11β-hydroxy-androst-4-en-3,17-dione	NR	NR	NR	A
17α-hydroxypregn-4-en-3,20-dione	NR	NR	NR	C
3α,17α,20α-trihydroxy-5β-pregnane	CP	CP	CP	CP
17α,21-dihydroxypregn-4-en-3,11,20-trione	IR	IR	IR	-
17α,11β,21-trihydroxypregn-4-en-3,20-dione	IR	IR	IR	NQ

A = 3 h. at 60°C
B = 0.25 h. at room temp.
C = 6 h. at 85°C

NR = no reaction
NQ = none quantitative
CP = cyclic product (see text)
IR = not all hydroxyl groups react

* Ketone protected as its methoxime.

TABLE (1.12)

REACTIVITY OF FLOPHEMESYL REAGENTS

REAGENT	HYDROXYL GROUP ENVIRONMENT
Flophemesyl chloride in pyridine	Unhindered secondary hydroxyl groups[1] when ketones are first protected.
Flophemesylamine	Selectively reacts with unhindered secondary hydroxyl groups[2] in the presence of ketone groups. Does not react with tertiary or hindered secondary hydroxyl groups.
Flophemesyldiethylamine Flophemesyl chloride (10:1)	Unhindered secondary hydroxyl groups and exposed tertiary hydroxyl groups[3] in the presence of ketones.
Flophemesyldiethylamine Flophemesyl chloride (1:1)	Unhindered and hindered secondary, and exposed tertiary hydroxyl groups. Very hindered tertiary hydroxyl groups[4] do not react completely. Ketone groups must be protected.

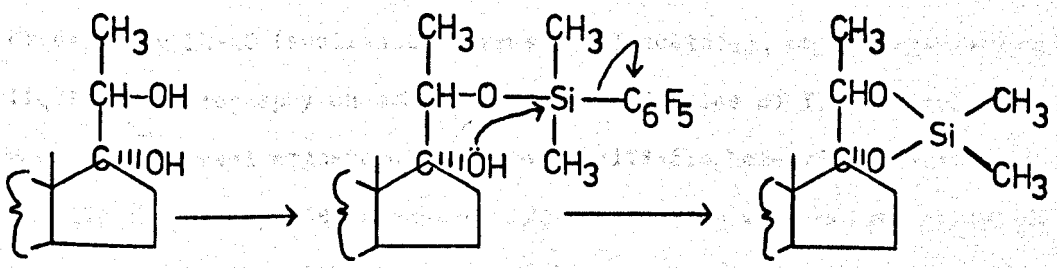
1 The 3β-OH of cholesterol is taken to be a typical unhindered secondary hydroxyl group.

2 The 11β-OH of 11β-hydroxyandrost-4-en-3,17-dione is taken to be a typical hindered secondary hydroxyl group.

3 The 17β-OH of 17α-hydroxy-17β-methylandrost-4-en-3-one is taken to be a typical exposed tertiary hydroxyl group

4 The 17α-OH of 17α,11β,21-trihydroxypregn-4-en-3,20-dione is taken to be a typical very hindered tertiary hydroxyl group.

The trimethylsilyl group, is a large group, more bulky than the tertiary butyl group. The flophemesyl group is larger than the trimethylsilyl group, so one would expect to see some limitations on reactivity for steric reasons. Two examples of this type have been observed. The 17α-OH in 17α-hydroxypregn-4-en-3,20-dimethoxime is converted efficiently to its flophemesyl ether with a C-20 methoxime as a near neighbour. When a 21-OH group was present as in 17α,11β,21-trihydroxypregn-4-en-3,20--dimethoxime, C-21 OH presumably reacted more quickly, hindering access to the 17α-OH, with the consequence that only 30% reaction of the latter group occurred. With a C-17,20-dihydroxy side chain as in 3α,17α,20-trihydroxy-5β-pregnane an unexpected product was obtained in quantitative yield. In this instance, reaction at the C-3 and C-20 positions is rapid and a cyclic product is formed by nucleophilic attack of the C-17 OH on the C-20 flophemesyl ether with the expulsion of pentafluorobenzene. This could take place either in solution or be the product of a thermal reaction when the steroid mixture is injected into the gas chromatograph.



Again, in a 3β,5α,6β-trihydroxycholestane, the 5-OH position is completely inhibited by more rapid ether formation at the secondary C-3 and C-6 hydroxyl groups. However, in the case of the 3β,5α-dihydroxycholestan--6-methoxime, partial reaction of the 5-OH group is achieved.

The flophemesyl ethers of steroids have excellent thermal stability. Gas chromatography temperatures in the range 230-320°C have been employed in this study. A crystalline sample of flophemesyl cholesterol was stable in the laboratory atmosphere for at least 48 h. It is stable to thin layer chromatography on silica gel. The pentafluorophenyl group absorbs moderately in the UV which allows detection of the flophemesyl ether. Visualization with iodine or 20% sulphuric acid with heating to 110°C was used to ensure that no decomposition of the ether occurred. Recovery of flophemesyl cholesterol from silica gel TLC plates by elution with diethyl ether, dichloromethane or ethyl acetate led to approximately 10% hydrolysis to cholesterol. Recovery by shaking silica gel with a mixture of benzene and water produced only 3% hydrolysis. A more detailed study is required to ascertain the feasibility of recovering flophemesyl ethers from silica gels of different types. Flophemesyl cholesterol could be chromatographed unchanged on columns of neutral alumina, eluting with hexane-ethyl acetate, or sephadex LH-20 (swollen in hexane-ethyl acetate), or by reversed-phase liquid chromatography on amberlite XAD-2. Samples of flophemesyl cholestanol, flophemesyl ergosterol, 17α-methyl-17β-flophemesylandrost-4-en-3-one and 17β-flophemesyl-5β-pregn-4-en-3,20-dimethoxime were column chromatographed on neutral alumina without decomposition, but a sample of 3α,20α-di-flophemesyl-5β-pregnane could only be recovered in low yield and the parent sterol was removed with more polar solvent indicating hydrolysis in this case.

A number of solvents were studied in both the catalysed and uncatalysed reaction. Unhindered secondary hydroxyl groups react smoothly

in a variety of solvents and their use is essential when the steroid shows low solubility in the flophemesylating medium. The effect of solvent on the rate of reaction of the 17-OH group in 17α-hydroxypregn-4-en-3,20-dimethoxime, with a 1:1 mixture of flophemesyldiethylamine and flophemesyl chloride, is illustrated in Table (1.13).

TABLE (1.13)

EFFECT OF SOLVENT ON THE EXTENT OF REACTION BETWEEN 17α-HYDROXYPREGN--4-EN-3,20-DIMETHOXIME AND A 1:1 MIXTURE OF FLOPHEMESYLDIETHYLAMINE AND FLOPHEMESYL CHLORIDE.

4 h at 120°C

SOLVENT	FLOPHESYL ETHER FORMATION (%)
Pyridine	100
Acetonitrile	85 (complete in 7 h)
Dimethylformamide	Decomposition
Dimethyl sulphoxide	35
Tetrahydrofuran	30
Ethyl acetate	25

Generally a more polar solvent is preferred. Dimethylformamide should be avoided as the decomposition of several flophemesyl ethers has been observed in this solvent. The solubility of flophemesyl reagents in dimethyl sulphoxide is low, resulting in the formation of two layers. Pyridine was generally observed to be the best solvent. The nature of the effect of pyridine is not clear, though it suggests some more specific interaction than solvation. The formation of an N-flophemesylpyridinium salt might be involved as a reactive intermediate. Flophemesyl chloride is more reactive in pyridine towards secondary hydroxyl groups than it is in chloroform, tetrahydrofuran or acetonitrile.

ELECTRON-CAPTURE PROPERTIES

The least detectable amounts (LDA) of the fluorocarbonsilyl ethers of cholesterol are given in table (1.14).

TABLE (1.14)

ELECTRON CAPTURE SENSITIVITIES OF CHOLESTEROL SILYL ETHERS.

COMPOUND	LEAST DETECTABLE AMOUNT x 10^{-9}	PULSE PERIOD μs
$CF_3(CH_2)_2Si(Me)_2$-cholesterol	1500	50
$C_6F_5(CH_2)_2Si(Me)_2$-cholesterol	200	50
$CF_3(CF_2)_2(CH_2)_2Si(Me)_2$-cholesterol	115	15
$ClCH_2Si(Me)_2$-cholesterol	75	5
$C_6F_5Si(Me)_2$-cholesterol	4	50
C_3F_7CO-cholesterol	Unstable	

For the fluoroalkylsilyl ethers, an increase from three to seven fluorine atoms resulted in a twelvefold improvement in sensitivity. Neither of the fluoroalkylsilyl ethers were as sensitive as the chloromethyldimethylsilyl ether which is available from the commercial reagent. The pentafluorophenyldimethylsilyl (flophemesyl) ether was nearly twenty-times more sensitive than the chloromethylsilyl ether. It is interesting that the pentafluorophenyl group when separated from silicon by an alkyl chain as in the 2'-pentafluorophenylethyldimethylsilyl ether, is fifty times less sensitive.[71] A possible explanation for this is that in the flophemesyl ether the captured electron is buried in the π-orbitals of the phenyl ring which is further stabilized by $(p \rightarrow d)\pi$ bonding with low energy d-orbitals of silicon. In the pentafluorophenylalkylsilyl ether, back bonding does not occur.[71]

The least detectable amount of seven flophemesyl steroid ethers is given in table (1.15).

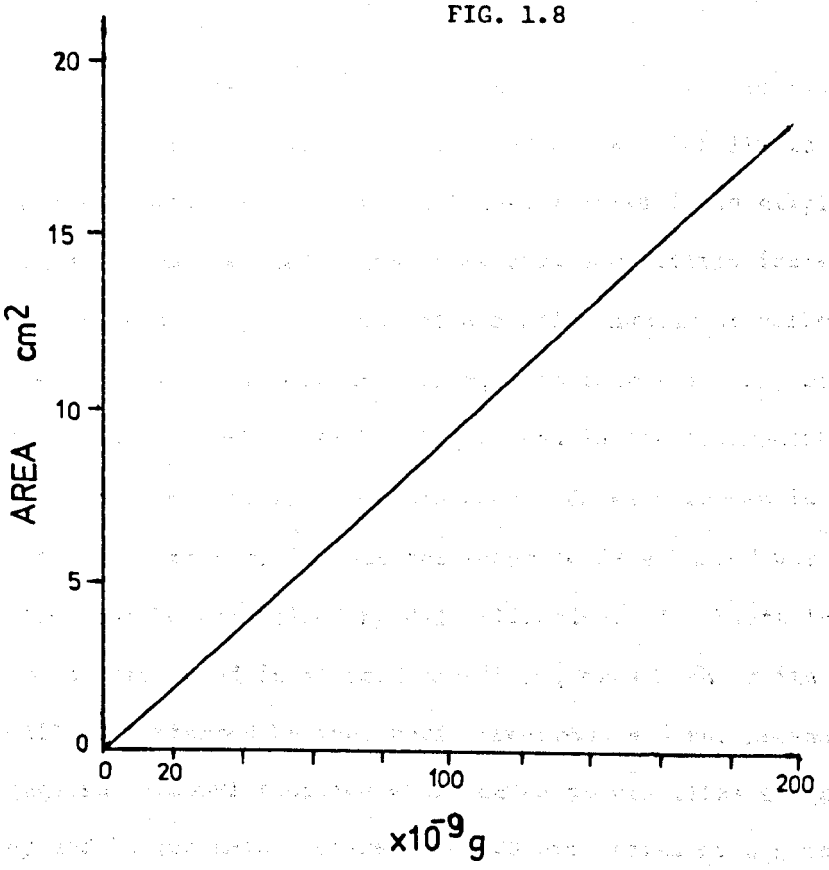
TABLE (1.15)

LIMIT OF DETECTION OF SOME STEROIDS BY ECD AS THEIR FLOPHEMESYL DERIVATIVES

STEROID	NUMBER OF PENTAFLUORO-PHENYL GROUPS	LEAST DETECTABLE AMOUNT (ng)
17α-hydroxypregn-4-en-3,20-dimethoxime	1	8.0
17α-methyl-17β-hydroxyandrost-4-en-3-one	1	5.0
Cholesterol	1	4.0
Ergosterol	1	1.0
3α,20α-dihydroxy-5β-pregnane	2	0.20
3β,5α,6β-trihydroxycholestane	2	0.07
2β,3β-dihydroxy-5α-cholestane	2	0.03

The presence of one flophemesyl group allows detection at the nanogram level, whereas two groups extends the range to the picogram level. The response of the electron capture detector to flophemesyl cholesterol is linear over the range 4-200 x 10^{-9} g (Fig. 1.8) which was the extent of the range investigated.

FIG. 1.8



One of the difficulties in designing reagents suitable for electron capture gas chromatography is a lack of knowledge of the capture coefficient of many potentially useful electrophores. When considering the introduction of an electrophore into a dimethylsilyl ether for the analysis of steroids, attention needs to be given to the chemistry of silicon and the expected volatility of the derivative formed. C.A. Clemons and A.P. Altshuller[81] in a series of halogenated compounds containing between one and six carbon atoms observed responses to the electron capture detector varying over seven orders of magnitude. Low responses were shown by saturated and vinyl-type fluorinated hydrocarbons including those containing one atom of chlorine. The response decreased in the order I>Br>Cl>F but increased markedly as the number of Cl, Br or I atoms in the molecule increases. They also noted that hexafluorobenzene was more sensitive by a factor of

approximately one hundred than pentafluorobenzene. Thus although iodine and bromine are more sensitive to electron capture than fluorine, their greater mass produces less volatile compounds which limits their use in steroid analysis. Closely bound fluorine atoms in an alkyl or aryl compound are remarkable in that they show very little increase in boiling-point compared to hydrocarbons of a similar number of carbon atoms in spite of the increase in molecular weight, this increase being offset by a decrease in intermolecular bonding forces in the fluorocarbon.[82] It was for this reason that fluorocarbon compounds were chosen in this study. A further point to note is that the response is enhanced whenever the captured electron can be stabilized by delocalization. The heptafluorobutyryl ester, which is often used in steroid chemistry, owes much of its popularity to the volatility conferred by the perfluoro-group and the presence of the conjugated carbonyl function which helps to stabilize the captured electron. Exley and Dutton have compared the ECD properties of a number of halogenated derivatives of 17β-oestradiol and oestrone.[84] Their results are in accord with the above discussion.

A useful source of information on the electron capture of thermal electrons by fluorocarbon molecules is provided by γ-radiolysis studies of perfluorocarbons in alkane solvents.[85] High energy electrons are reduced to thermal energies by interaction with solvent molecules and are then captured by fluorocarbon scavengers. With scavengers such as CF_4, C_2F_6, C_3F_8 and C_4F_{10}, their electron capture ability is weak and increases with the number of fluorine atoms.[86,87]

In the gas phase, electron capture by a molecule results in the formation of a negative ion with internal energy equal to the electron affinity of the compound formed. The excess energy of the ion may be dissipated by a dissociative process but in the case of perfluoroalkanes this reaction has been claimed to be thermodynamically unlikely because

of the high energy of the C-F bond, and the ion will more probably lose an electron again unless it is stabilized in some way. In small molecules such as CF_4, C_2F_6, and C_3F_8 this auto-ionization is apparently very rapid (less than 1μ sec).[87] It is this auto-ionization which explains the poor electron capture response of the heptafluoropentyl and the trifluoropropyl group. This would not apply to heptafluorobutyryl esters as the captured electron can be stabilized by carbonyl conjugation. Aromatic fluorocarbons have been shown to be much more effective electron scavengers than alkylfluorocarbons and in the case of hexafluorobenzene a nondissociative mechanism of electron capture was shown to be operative.[88]

Attempts have been made to gain a better understanding of the electron capture process by analysis of the products formed. In a plasma chromatograph, negative and positive ions are formed by electron bombardment from a ^{63}Ni source, the products separated and identified in an ion drift tube. Chloro- and bromoaromatic compounds have been investigated and shown to capture electrons dissociatively.[89] This is in agreement with the results of Durbin et al.[90] who used a corona discharge cell to create electrons of thermal energies and analysed the products by mass spectrometry. No results for fluorocarbon compounds have been obtained by either method. For the haloaromatics, other than fluorine, electron capture by a dissociative process would be expected as the electron affinity of the halogen atom exceeds the bond dissociation energy of the carbon-halogen bond.[91]

THERMAL INSTABILITY OF CHOLESTEROL HEPTAFLUOROBUTYRATE

For the purpose of making a comparison of the electron capture sensitivity of cholesterol heptafluorobutyrate and flophemesyl cholesterol, a sample of the former was synthesised. An initial GC-FID investigation of the heptafluorobutyrate gave a single peak the retention time of which

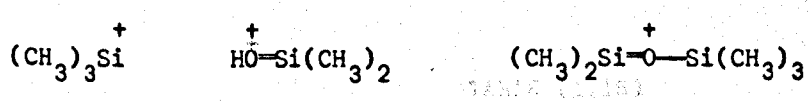
was less than that of cholesterol, as would be expected. Dual monitoring of the column effluent by FID and ECD produced two peaks of different retention time, the ECD giving a poor response to a compound eluting earlier than that shown by the FID. The formation of cholesterol heptafluorobutyrate and its subsequent GC has been widely reported.[6,13,19,23,92]

Variation of the temperature of the injection port heater of the gas chromatograph in the range 200-275°C, in all cases gave similar results. GC-MS indicated a molecular weight of 368 for the ester (molecular formula requires m^+ 582). Pyrolysis of cholesterol heptafluorobutyrate at 240°C/10 mm Hg for twenty minutes followed by TLC on silica gel eluting with light petroleum (40-60°C) gave two components, corresponding to the pyrolysed product (R_f=0.68) and unchanged heptafluorobutyrate (R_f=0.41) approximately in the ratio 10:1. The pyrolysis product was identified from its spectroscopic data and elemental composition as cholesta-3,5-diene. Its mass spectrum and retention time parameters were found to be identical with the FID trace for cholesterol heptafluorobutyrate. The ECD traces are either a small amount of cholesterol heptafluorobutyrate or alternatively a heptafluorobutyrate of an impurity present in the cholesterol.

MASS SPECTRAL PROPERTIES OF FLOPHEMESYL ETHERS OF STEROIDS.

The pentafluorophenyl ring is a strongly electron-attracting group which is able to influence the mode of fragmentation of steroid derivatives under electron impact in a way which led to diagnostic mass spectra often showing marked differences from those of TMS ethers. The mass spectrum is not complicated by abundant ions due to fragmentation of the pentafluorophenyl ring, neither is cleavage between silicon and the pentafluorophenyl ring a dominant process. A strong molecular ion is usually observed with enhanced sterol hydrocarbon fragments.

The general features of the mass spectra of TMS ethers of sterols[93-95] are usually characterised by the presence of ions m/e 73, m/e 75 and by the ion m/e 147 in the case of steroid diols. The ion m/e 73 is often so



m/e 73 m/e 75 m/e 147

abundant as to be the base peak of the mass spectrum and carries sufficient of the ion current to make much of the remaining spectrum undistinguished. The flophemesyl derivatives are characterised by ions m/e 58 and m/e 77. A flophemesyl equivalent of m/e 147 has not been observed in the spectra of several steroid diol flophemesyl ethers, when the TMS ethers of the same compounds produced a strong ion at m/e 147. The relative intensity of

m/e 58 m/e 77

m/e 58 and m/e 77 is variable, with m/e 77 generally the stronger. However, m/e 77 is rarely the base peak of the mass spectrum. The ion m/e 75 is observed in some flophemesyl spectra but its intensity is variable. Elimination of flophemesylanol (m - 242) is common, as is elimination of trimethylsilanol (m - 90), from TMS steroid ethers. A useful feature of the flophemesyl ethers is that they tend to produce stronger molecular ions than do the TMS ethers and much stronger ions than the halomethyldimethylsilyl ethers. The halomethyldimethylsilyl ethers are usually characterised by weak molecular ions due to the facile cleavage of CH_2X (X=Cl,Br,I).[96,97]

The mass spectra of TMS, chloromethyldimethylsilyl (CMDMS) and flophemesyl ethers of 5α-cholestanol show many common features. Table (1.16)

contains the % relative ion abundance of the fragment ions with respect to the base peak for the above derivatives, all measured under the same conditions.

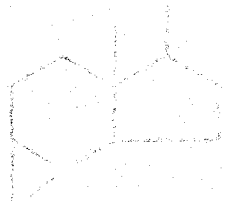
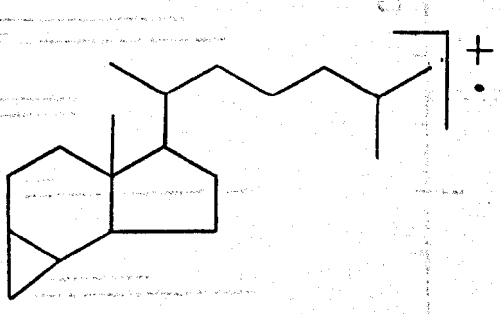


TABLE (1.16)

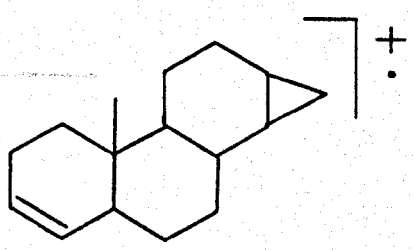
THE % RELATIVE ABUNDANCE OF IONS IN MASS SPECTRA OF CHOLESTANOL AND SOME OF ITS SILYL ETHERS MEASURED AT 80eV.

m/e	ASSIGNMENT	3β-ol,	3β-TMS,	3β-CMDMS	3β-FLO-PHEMESYL
	m^+	74	27	6	31
445	m-CH_2Cl	-	-	100	-
370	m-silanol	1*	21	10	8
355	m-silanol-CH_3	20*	49	13	39
285	m-silanol with scission of C_{20}-C_{22}	2*	6	-	7
262	$C_{19}H_{34}$	18	12	6	16
230	m-silanol-C_{16},C_{17} and side chain	17*	27	6	24
215	$C_{16}H_{23}$	87	100	44	100
201	215-CH_2	20	17	6	21
190	230-C_3H_4 from either ring A or C and D	19	8	7	29
175	190-CH_3	15	10	8	13
173	201-C_2H_4	12	12	4	16
161	190-C_2H_5	28	19	13	20
147	$C_{11}H_{15}$	14	38	17	50
121	C_9H_{13}	17	37	19	54
107	C_8H_{11}	69	68	29	91

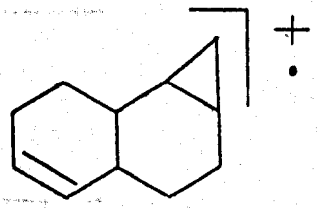
*for the parent sterol loss of water is equivalent to loss of silanol from ethers.



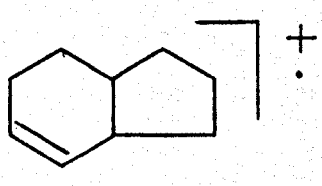
m/e 262



m/e 215



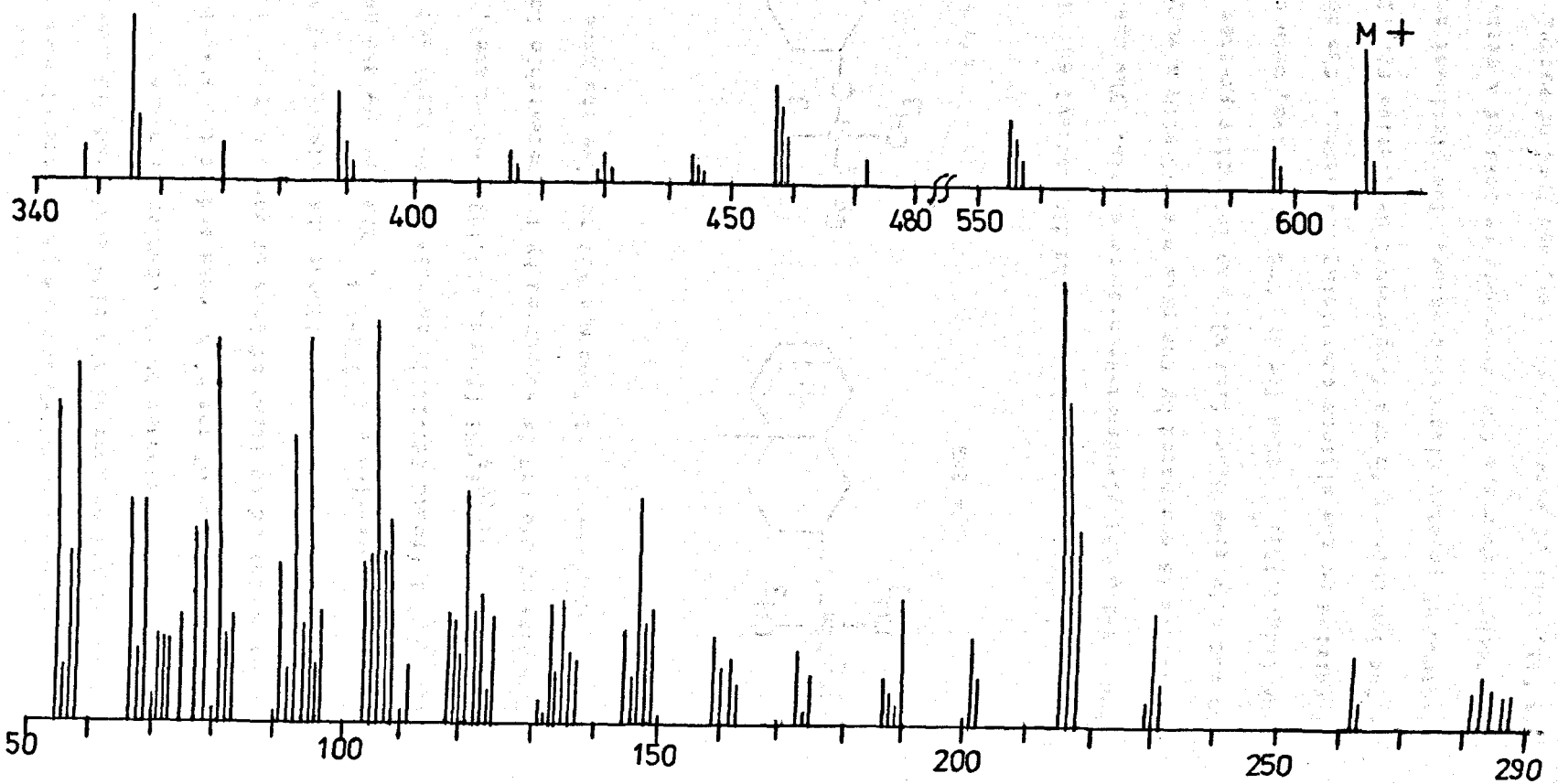
m/e 147



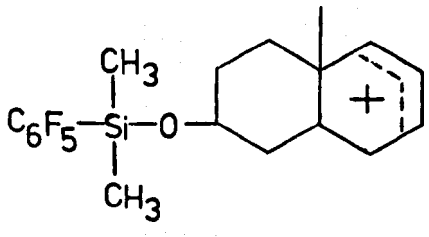
m/e 121

The CMDMS ethers are exceptional in their very facile cleavage of the chloromethyl group to produce the base peak m/e 445 (m - 49) with a consequent reduction in the intensity of the molecular ion and those masses containing the siloxy group in which the isotopic chlorine ratio might have been of much use in peak assignment. As less of the total ion current is carried by the silicon containing ions in flophemesyl cholestanol (Fig.1.9), the steroid hydrocarbon fragments are relatively more abundant. Elimination of the appropriate silanol to produce m/e 370 is not an especially favoured process. The parent sterol shows more ready cleavage of the C-19 methyl, followed by loss of water[99,100] as do the silyl derivatives for which m/e 355 is of greater abundance than m/e 370. The base peak of the TMS and flophemesyl derivative, m/e 215, arises from cleavage of 15,16- and 13,17-bonds.

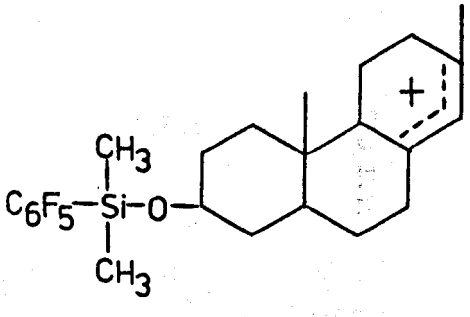
FIG. 1.9



It has been shown for the parent sterol that the base peak at m/e 233 arises by loss of the side chain and fragments of ring D in this way, and then decomposes to the abundant ion m/e 215 by loss of water.[99] The main features of the high mass end of the spectra of all the compounds can be explained in terms of loss of various fragments of the side chain and rings C and D, with and without the retention of the hydroxyl group or the silyl protecting group[101-103]. The ion m/e 389 has the composition $C_{19}H_{22}F_5OSi$ (found 389.1372; calculated 389.1360) and the ion m/e 457 the formula $C_{24}H_{30}F_5OSi$ (found, 457.1977; calculated, 457.1986). The structure of m/e 457 is supported by the metastable ion at m* 101.1 for loss of flophemesylanol from m/e 457 to give the base peak m/e 215.



m/e 389

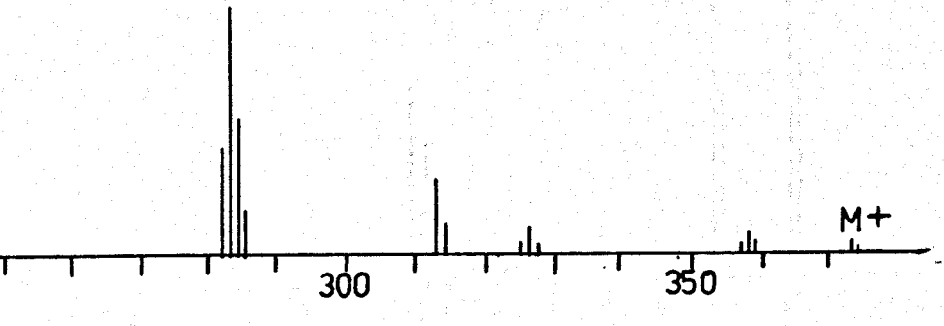


m/e 457

A sharp contrast between the flophemesyl and TMS ethers of 17β-hydroxy-17α-methylandrost-4-en-3-one exists. The spectrum of the TMS derivative is dominated by the peak m/e 143 with a very weak molecular ion and only five other ions with an intensity greater than 10% of the base peak (Fig.1.10).[98] Thus for the TMS derivative, much of the ion current is carried by the silicon containing fragments. The more even distribution of the ion current in the flophemesyl derivative Fig.(1.11) gives a mass spectrum of greater diagnostic value. The prominent molecular ion of the flophemesyl ether m/e 526, fragments by loss of a methyl radical to give m/e 511, loss of ketene to m/e 484, and loss of methyl and ketene to m/e 469.

60
100
150
240

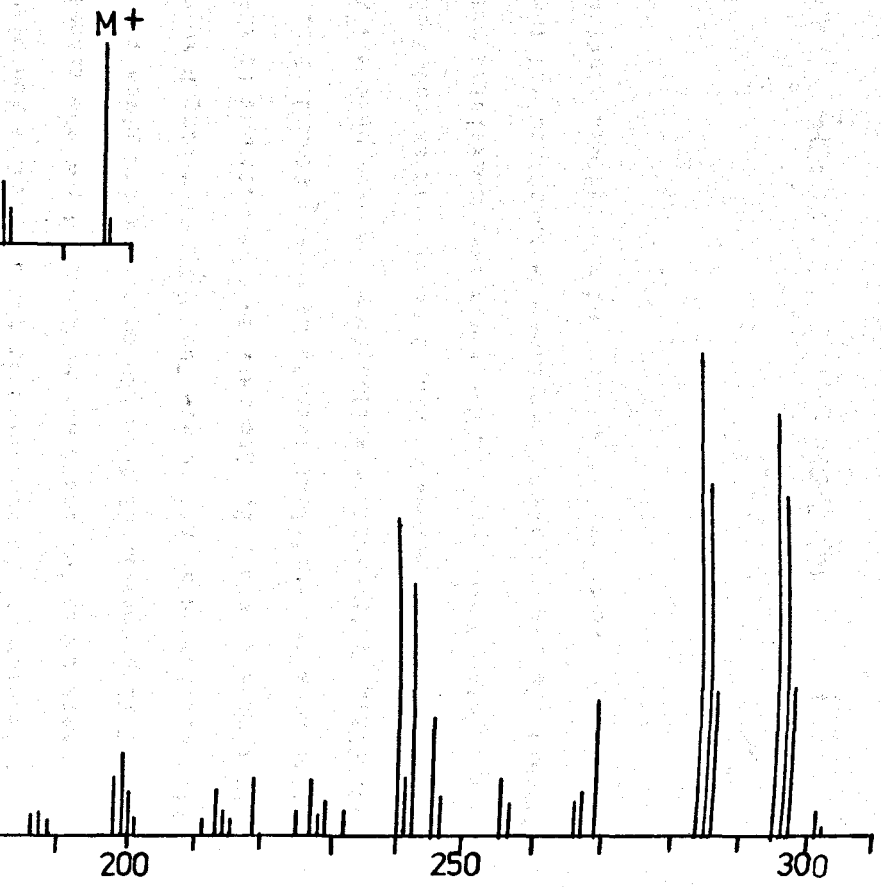
FIG 1.10



340 350 420
450
500

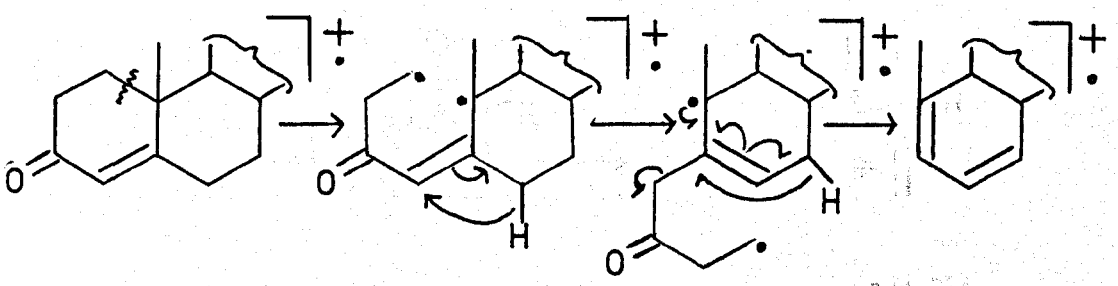
70
100
150

FIG 1.11



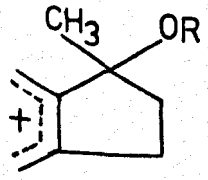
The abundant ion at m/e 456 probably arises by loss of C-1 to C-4 of ring A with migration of the double bond to Ring B, Scheme I.

SCHEME I

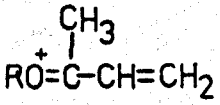


m/e 456

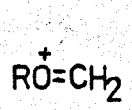
Thus the high mass end of the spectrum produces fragments which are characteristic of the unsaturated carbonyl at C-3. Cleavage of ketene from the molecular ion is supported by the metastable ion m*445.3 (m/e 526→484) but a metastable ion could not be observed for the transition (m/e 526→456). Loss of flophemesylanol from the molecular ion gives the base peak m/e 284, which eliminates methyl to m/e 269, C_3H_3 from ring D to m/e 245, and ketene from ring A to m/e 242. Accurate mass measurement of the ion m/e 240 establishes the empirical formula as $C_{18}H_{24}$ (found, 240.1880; calculated 240.1878). This probably arises from the base peak m/e 284 by loss of ketene and two hydrogens of ring B to give an extended conjugated system. An analysis of the spectrum for those ions containing the flophemesyl group reveals m/e 348 and m/e 255 of moderate abundance and the very abundant ion m/e 295. The elemental composition of m/e 295 was confirmed by accurate



m/e 348



m/e 295

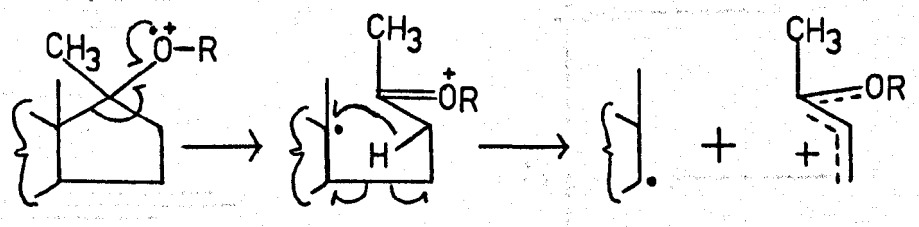


m/e 255

R = $C_6F_5Si(CH_3)_2$-

mass measurement as $C_{12}H_{12}F_5OSi$ (found, 295.0568; calculated 295.0577) and it probably arises from ring D as illustrated in Scheme II.

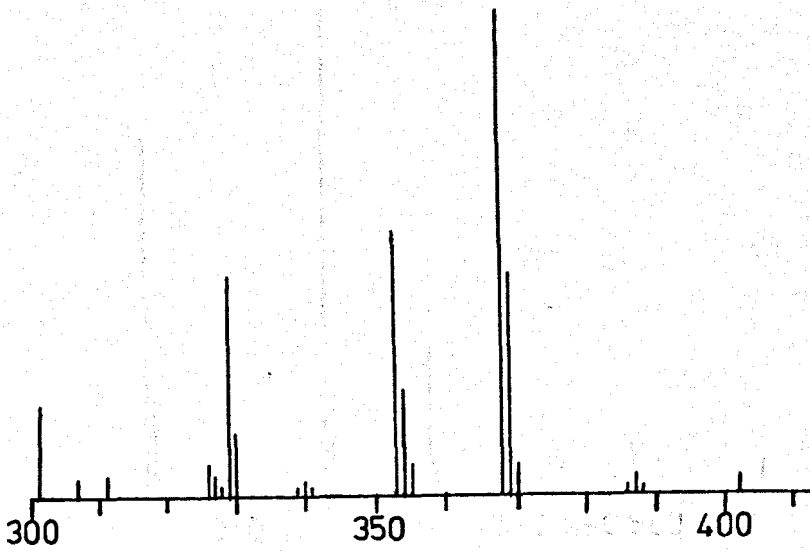
SCHEME II.



m/e 295

A metastable ion at m* 220.4 confirmed the transition m/e 295 ⟶ 255.

Probably the most studied examples of TMS ethers are those containing a 3β-hydroxyl group and a 5,6-double bond such as cholesterol and ergosterol, partly because of their availability but also because of their characteristic fragmentation[93,94,103-109] to produce ions of m/e 129 and $(M-129)^+$. The presence of a second double bond at C-7 in ring B favours the formation of an ion m/e 131, and $(M-131)^+$ is the second most abundant ion in the mass spectrum of the TMS ether of ergosterol. The ion m/e 129 is nearly as abundant as m/e 131 but $(M-129)^+$ is virtually absent. For the flophemesyl derivative of cholesterol (Fig.1.12), the ion m/e 281 (equivalent to m/e 129 in the TMS ether), is very weak although M-281 is of moderate intensity. In the flophemesyl derivative of ergosterol (Fig.1.13), m/e 281 is weak and M-281 absent. The ion m/e 283 is nearly eight times more abundant than m/e 281 and M-283 is the second most abundant ion in the mass spectrum. The ergosterol derivative might be expected to fragment differently because of the tendency to aromatize ring B. Scheme III.


300
350
400

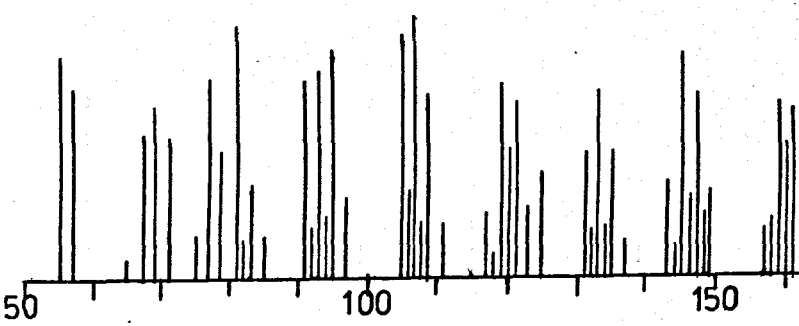
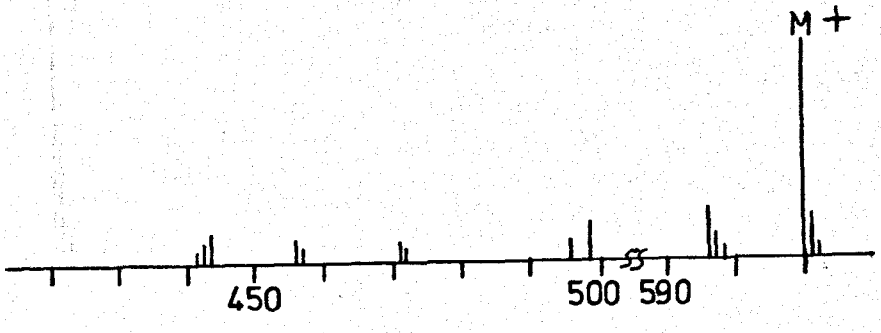
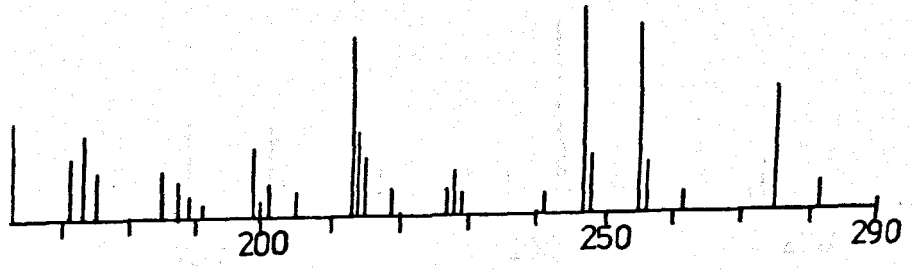
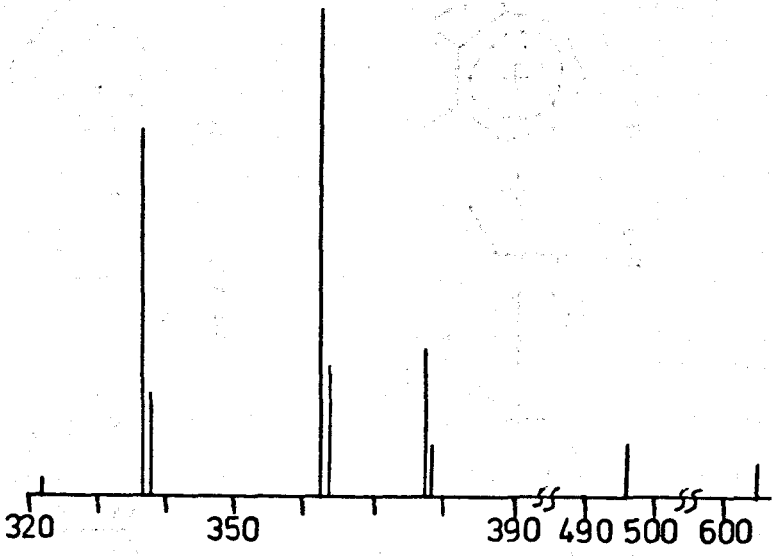

50
100
150

FIG 1.12






320
350
390
490
500
600

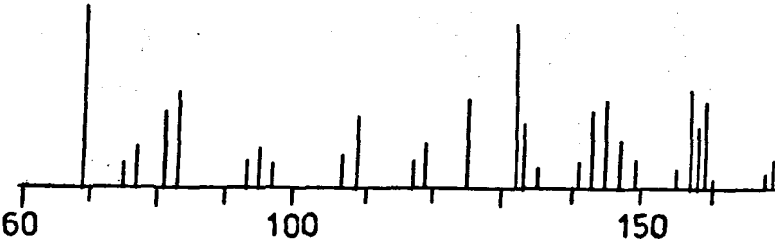
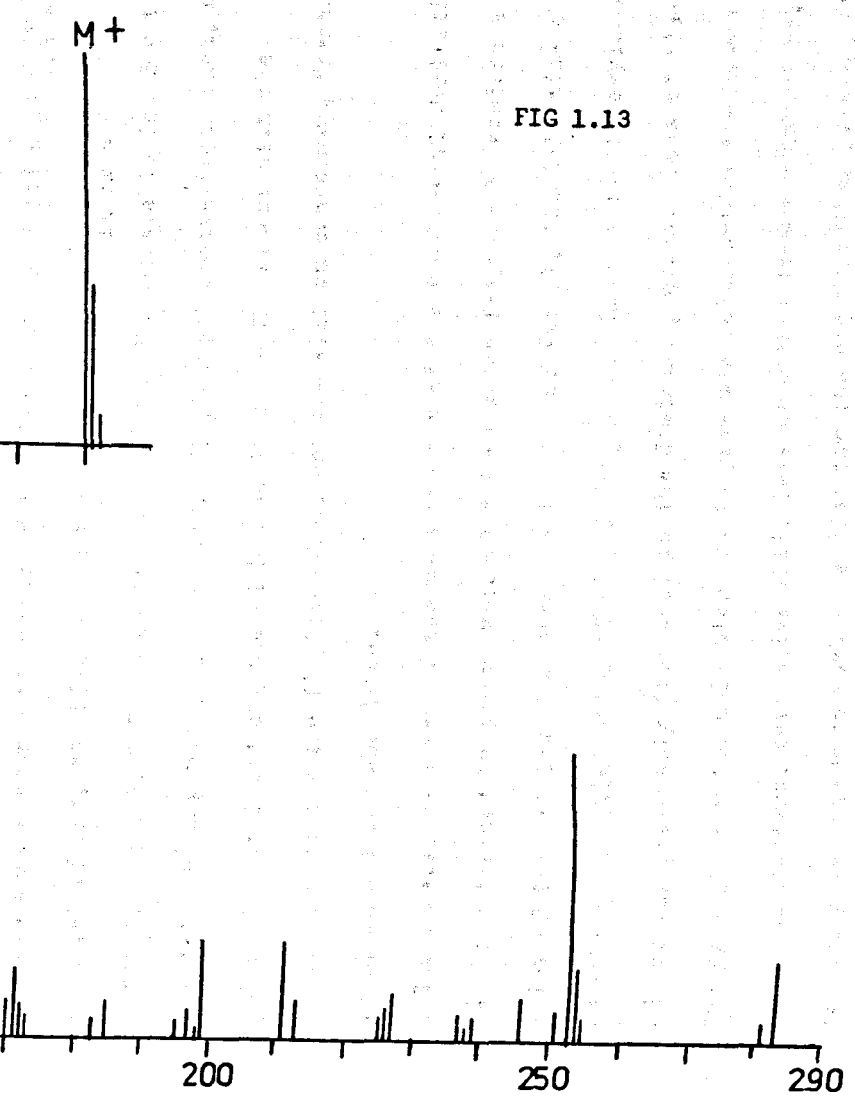
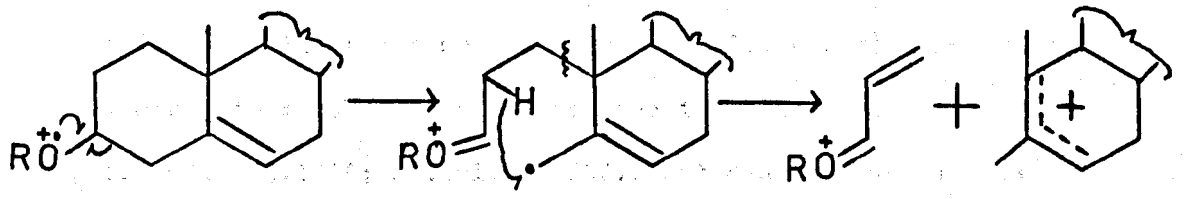
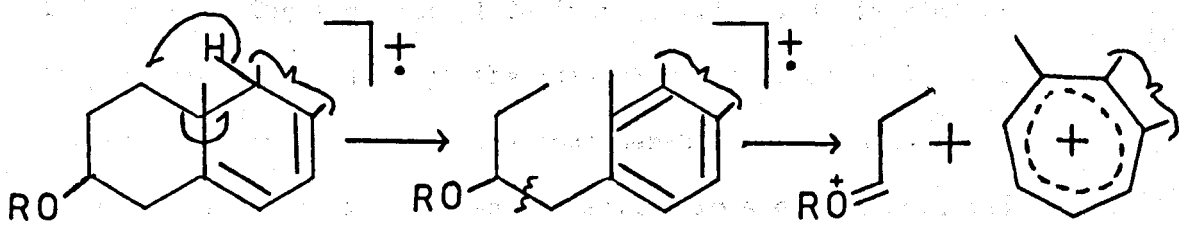

60
100
150

FIG 1.13



SCHEME III.





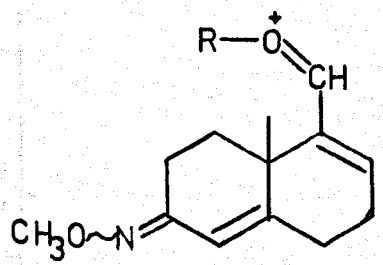
R = $C_6F_5Si(CH_3)_2$-

Accurate mass measurement confirmed the elemental composition as $C_{25}H_{37}$ for m-283 (found, 337.2893; calculated 337.2895) and $C_{11}H_{12}F_5OSi$ for m/e283 (found 283.0555; calculated 283.0572). No metastable ion for the transition $M^+ \longrightarrow$ M-283 was observed. Flophemesyl derivatives, then, of both cholesterol and ergosterol behave like the TMS ethers with the exception that for the flophemesyl ethers the silicon containing fragments are of much lower abundance.

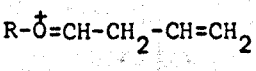
The general features of the mass spectra of the flophemesyl derivatives are characterised by strong molecular ions which fragment to produce a series of ions of low abundance due to loss of methyl, the C-17 side chain and subsequent elimination of ring D fragments. Loss of flophemesylanol and methyl, presumably from C-19 is the dominant process. Loss of flophemesylanol from the molecular ion to give m/e 368 provides the base peak for the cholesterol derivative while loss of methyl from the tetra-ene, m/e 378, gives the base peak (m/e 363) for the ergosterol derivative. The

abundant ion m/e 253 in the mass spectrum of flophemesyl ergosterol was shown by accurate mass measurement to have the elemental composition $C_{19}H_{25}$ (found, 253.1956; calculated 253.1956) and represents loss of flophemesylanol and the C-17 side chain from the molecular ion.

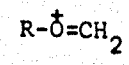
The 11β-TMS derivative of 11β-hydroxyandrost-4-en-3,17-dimethoxime has a weak molecular ion and the base peak is the familiar m/e 73 due to the silyl fragment. The flophemesyl derivative (Fig.1.14) is similar with a base peak at m/e77, although the molecular ion is appreciably stronger and the steroid ring fragments are considerably more abundant. The high mass end of the spectrum involves sequential loss of methoxyl and methyl radicals to give the ions m/e 569 (m-CH_3), m/e 553 (m-OCH_3), m/e 538 (m-OCH_3 and CH_3), m/e 522 (m-2 x OCH_3) and m/e 507 (m-2 x OCH_3 and CH_3) all of which are of low intensity and of lesser abundance than their equivalents in the TMS derivatives. Loss of flophemesylanol to give an ion m/e 342 and loss of flophemesylanol with a methoxyl radical to m/e 311 are facile fragmentations. The ions at m/e 444, 295 and 255 are presumed to contain the flophemesyl group, but with the exception of m/e 295 they are of low abundance.



m/e 444



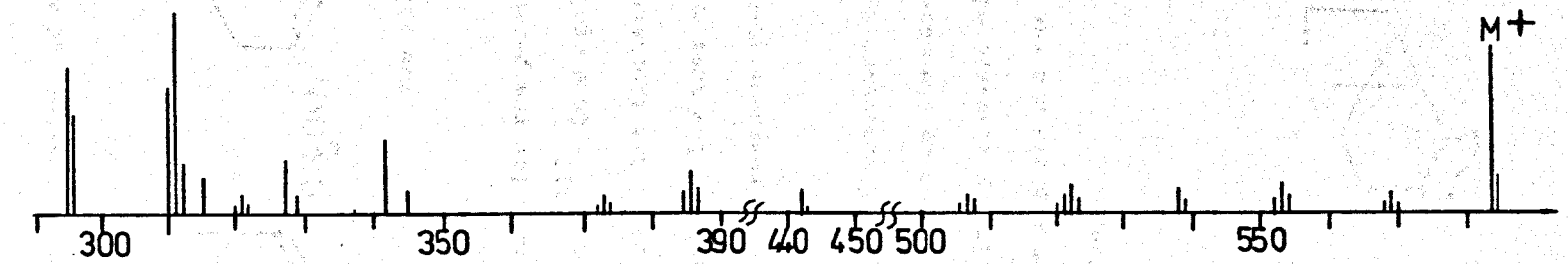
m/e 295

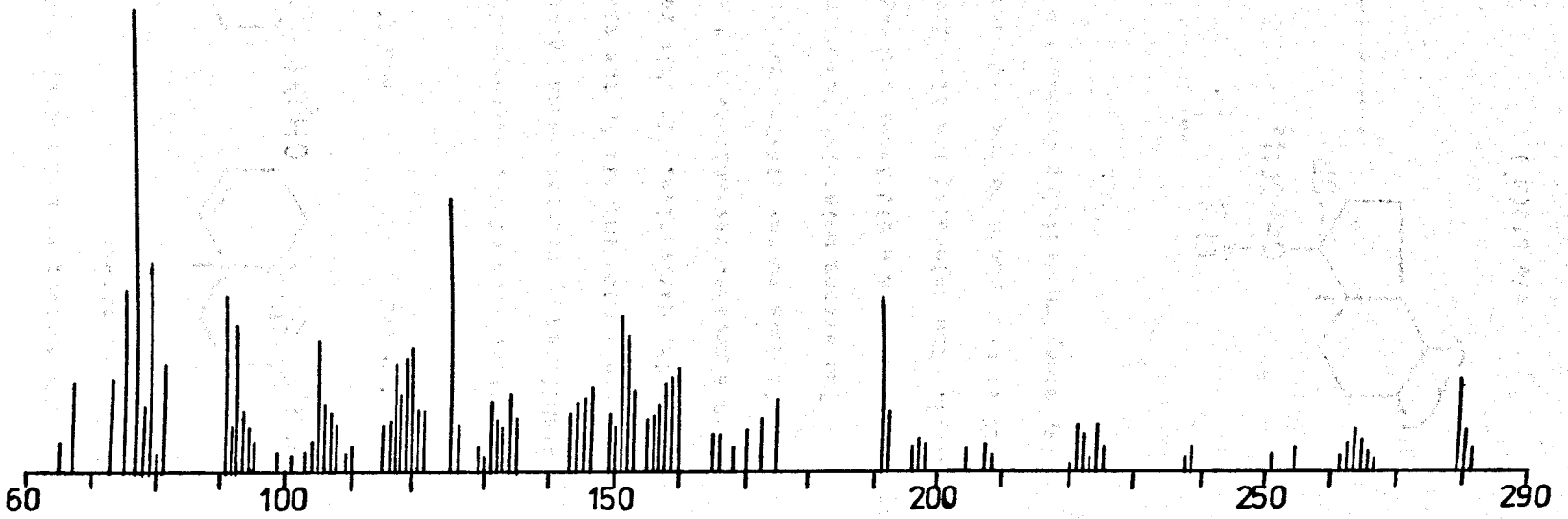


m/e 255

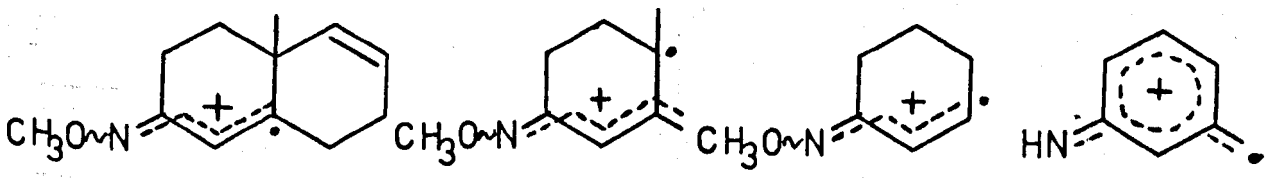
The TMS equivalents of the above ions have been recorded previously in the mass spectra of C-11 TMS androstanols.[104,109] The prominent ions at m/e 191, m/e 151, m/e 125 and m/e 105 are present in the parent sterol dimethoxime,

FIG 1.14





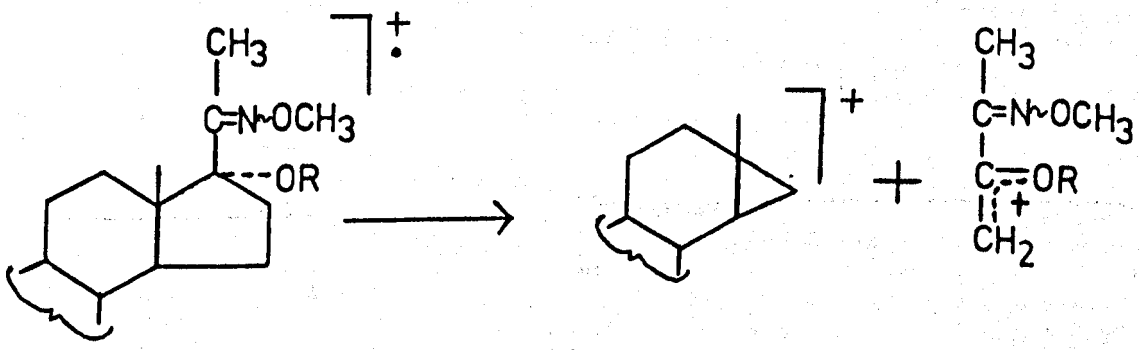
the TMS derivative and the flophemesyl derivative and are assigned the structures shown:



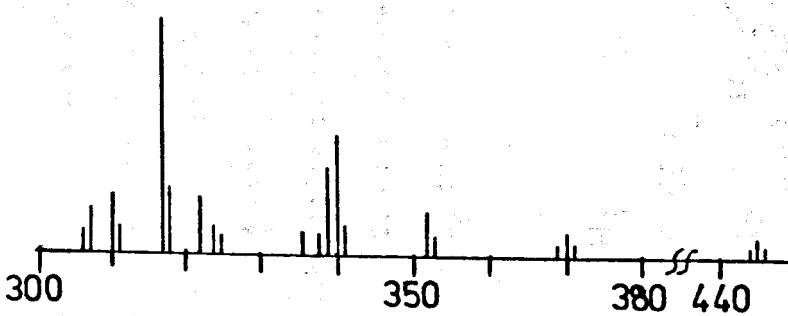
m/e 191 m/e 151 m/e 125 m/e 105

The composition of the corresponding ions for the parent ketones have been established by accurate mass measurement.[110] Once again, these ions are relatively more intense in the spectrum of the flophemesyl derivative than in the TMS derivative. In this respect the low mass end of the spectrum shows a greater resemblance to the 11β-hydroxyandrost-4-en-3,17-dimethoxime than it does to the 11β-TMS derivative.

The strong molecular ion of the 17β-flophemesyloxypregn-4-en-3,20-dimethoxime at m/e 612 loses methoxyl to produce the base peak m/e 581 (Fig. 1.15). The molecular ion also fragments by cleavage of C-13 to C-17 and C-15 to C-16 bonds to the abundant ion m/e 273 and the less abundant flophemesyl-containing fragment m/e 339.



m/e 612(M^+) m/e 273 m/e 339


300
350
380
440

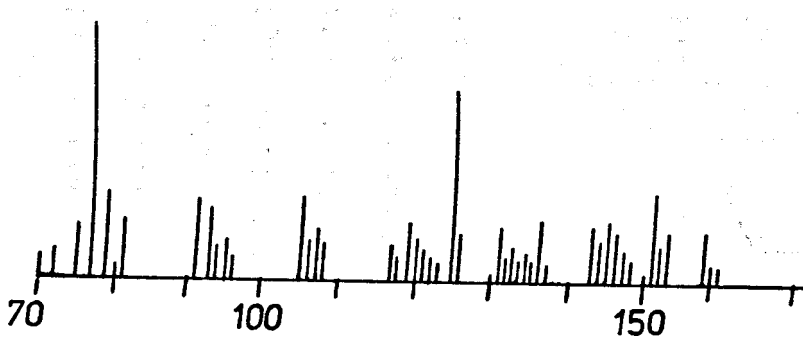
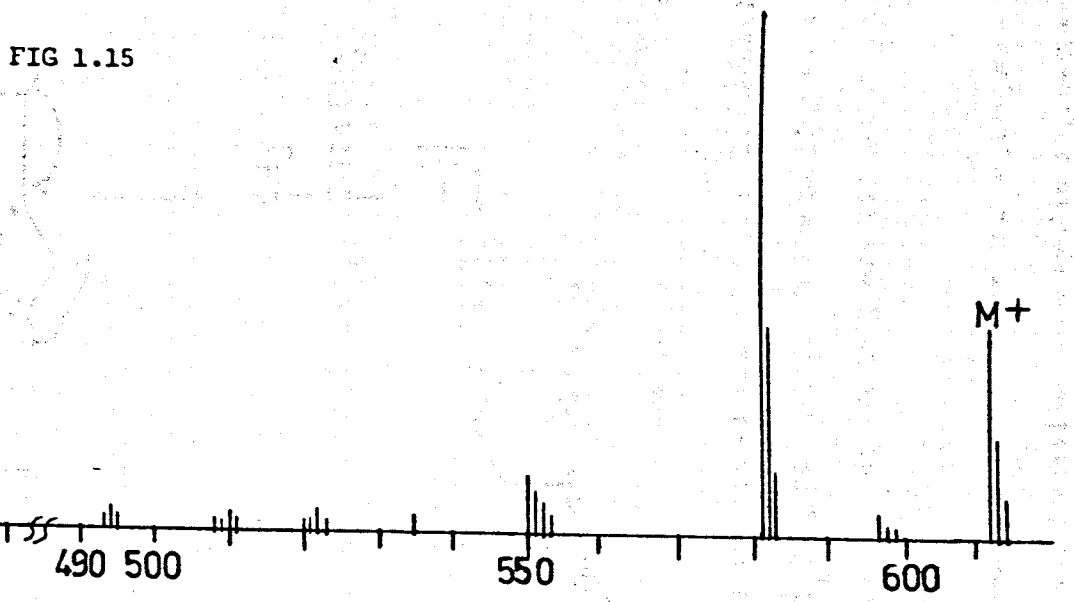
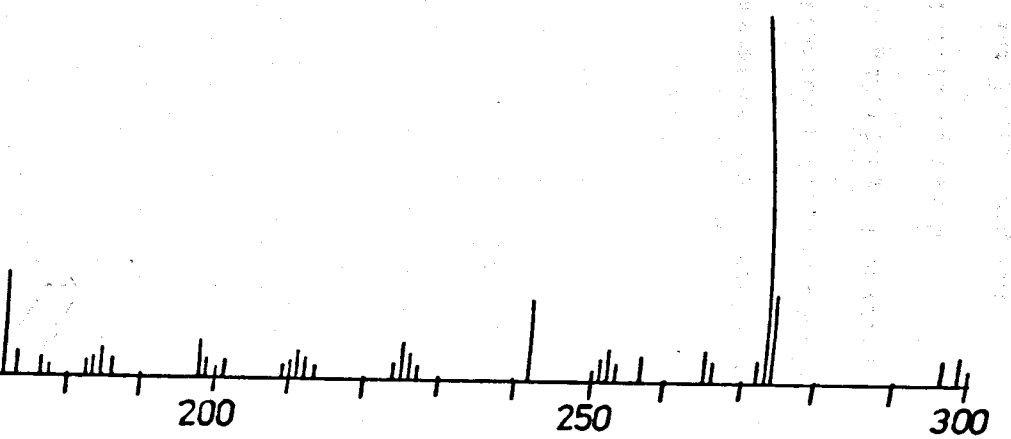
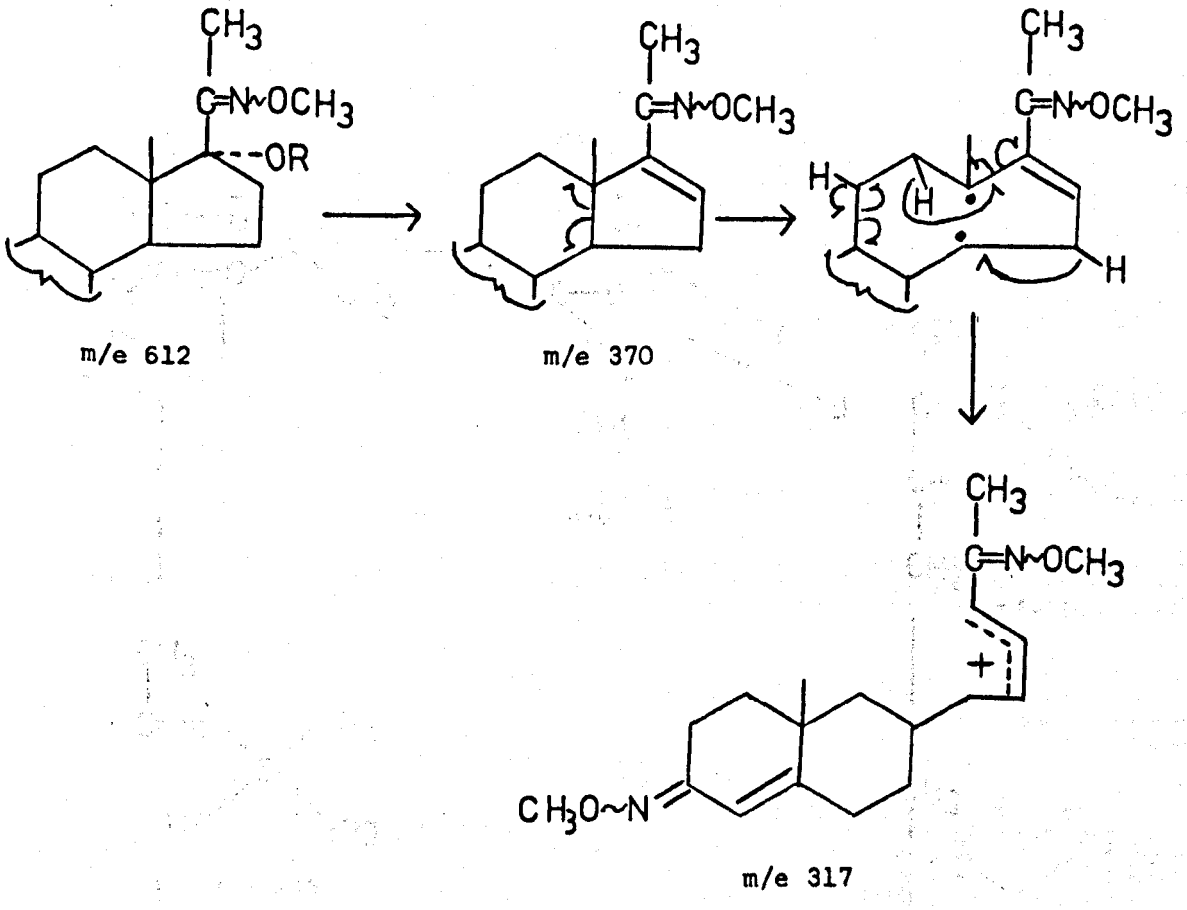

70
100
150

FIG 1.15





The ion m/e 339 is probably the precursor ion of m/e 172 for which accurate mass measurement established the elemental composition as $C_7H_{14}NO_2Si$ (found 172.0784; calculated 172.0794), corresponding to elimination of the pentafluorophenyl radical from m/e 339. A metastable ion was not observed for this transition. The elemental composition of the abundant ion m/e 317 was established as $C_{19}H_{29}O_2N_2$ (found 317.2230; calculated 317.2229) and represents loss of flophemesylanol and C_4H_5 from the molecular ion:



Accurate mass measurement establishes the ion at m/e 242 to be flophemesylanol (found 242.0159; calculated for $C_8H_7F_5OSi$, 242.0186). The remaining mass spectrum is undistinguished except for ions at m/e 151, m/e 125 and dimethylsilylfluoride, m/e 77.

The product of the reaction between flophemesylamine and 3α,17α,20α-trihydroxy-5β-pregnane (see page 49) was identified as a cyclic 17,20-di-

methylsilyl ether of 3α-flophemesyloxy-5β-pregnan-17α,20α-diol on the basis of its mass spectrum. The derivative (Fig. 1.16) has a strong molecular ion and the base peak, m/e 157 arises by cleavage of ring D with retention of the C-17 side chain. The base peak undergoes further fragmentation to produce the abundant ions at m/e 143, 129, 75 and possibly m/e 119 ($C_4H_{11}O_2Si$). The high mass end of the spectrum is characterised by ions formed by the fragmentation of ring A and loss of flophemesylanol, which are of low abundance.

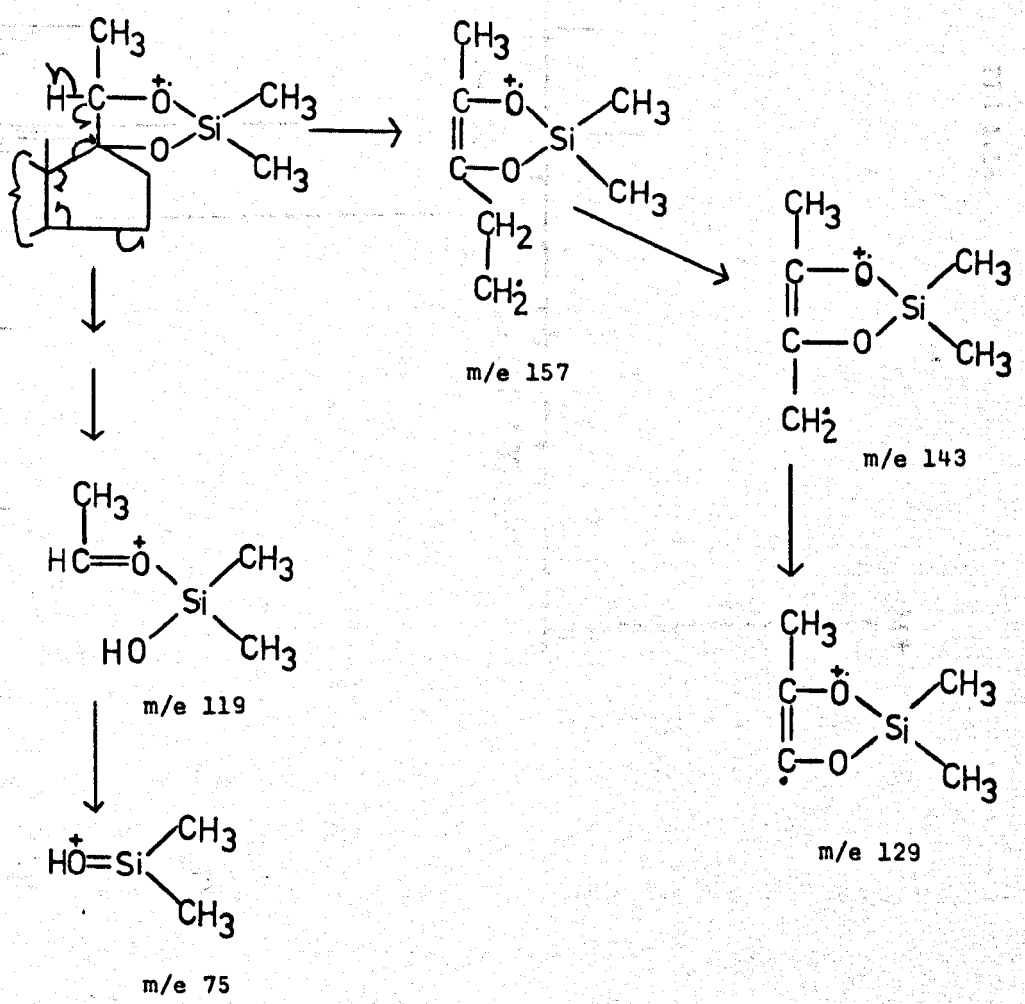
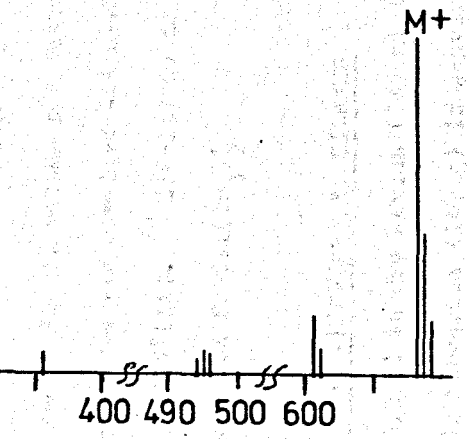
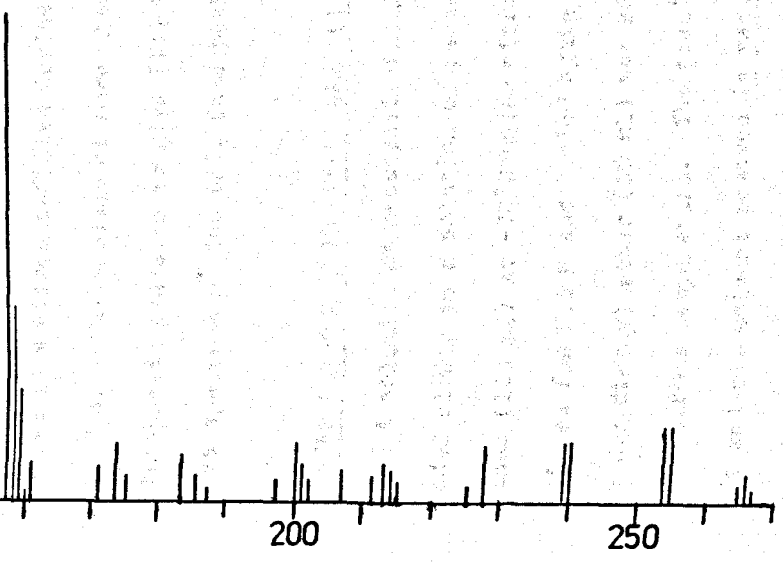


FIG 1.16


M+
400 490 500 600


200
250

EXPERIMENTAL

3,3,3,-TRIFLUOROPROPYLDIMETHYLCHLOROSILANE (I)

3,3,3-Trifluoropropene (2.0 g) was condensed into a Carius tube of 75 ml. volume, dimethylchlorosilane (2.0 g) and chloroplatinic acid (0.25 ml. of 0.1 m solution in isopropanol) were added under nitrogen. The tube was sealed and heated at 210°C for six hours, cooled, the products of more than one tube combined and distilled at atmospheric pressure to give (I) b.p. 117-119°C (lit.[31] 117°C) in 65% yield. Mass spectrum m^+ 190 (5), base peak m/e 93 $(CH_3)_2SiCl^+$.

3,3,4,4,5,5,5-HEPTAFLUOROPENTYLDIMETHYLCHLOROSILANE (II)

3,3,4,4,5,5,5-Heptafluoropentene (3.0 g), dimethylchlorosilane (1.68 g) and chloroplatinic acid (0.2 ml. of 0.1 m solution in isopropanol) were sealed in a nitrogen-filled Carius tube of 75 ml. volume and heated at 240°C for 7 h. The product of more than one tube was combined and distilled at atmospheric pressure to give (II) b.p. 115°(lit.[38] 116-117°C) in 55% yield. Mass spectrum m^+ 290 (6), base peak m/e 101 $FC{\equiv}CSi(CH_3)_2^+$.

PENTAFLUOROPHENYLDIMETHYLSILANE (III) AND CHLOROMETHYL SILANES

A solution of n-butyllithium (36.0 g) in dry hexane (250 ml.) was added slowly to a solution of pentafluorobenzene (100 g) in anhydrous diethyl ether (100 ml) at -70°C under nitrogen. The mixture was warmed to -10°C, stirred for 0.5 h and cooled again to -70°C. Dimethylchlorosilane (56.0 g) in dry diethyl ether (50 ml) was added slowly and the mixture allowed to attain room temperature. The precipitate of lithium chloride was filtered off and the solvent removed in vacuo. The remaining liquid was fractionated to give (III) 61.0 g b.p. 144°/575 mm. (lit.[51] 157°C/685 mm).

In an analogous procedure, the following were prepared:

	COMPOUND	CHLOROSILANE	b.p./mm	YIELD %
(III)	$C_6F_5Si(CH_3)_2$-H	Dimethylchlorosilane	144/575	61
(IV)	$C_6F_5Si(CH_3)_2$-CH_2Cl	Dimethylchloromethylchlorosilane	110/72	50
(V)	$C_6F_5Si(CH_3)_2$-$CHCl_2$	Dimethyldichloromethylchlorosilane	122/50	44

Note: It has been subsequently found that the yield of (III) can be increased to 84% if the pentafluorophenyllithium is not warmed above -20°C.

PENTAFLUOROPHENYLDIMETHYLCHLOROSILANE (VI)

Through a solution of (III) (50 g) in anhydrous carbon tetrachloride (200 ml) was bubbled chlorine, dried with magnesium perchlorate, into a reaction vessel covered with aluminium foil to exclude light and arranged so that it could be intermittently immersed in an ice-salt bath to maintain the temperature below 25°C. A buffer volume in the gas line prior to the reaction vessel and an auxillary supply of nitrogen connected to the chlorine line were used to avoid loss of material due to suck-back. The reaction was rapid and when complete ($\approx$0.75 h), excess chlorine was purged with nitrogen, solvent removed *in vacuo* and the remaining liquid fractionated by distillation to give (VI) (52.0 g) b.p. 96°C/30 mm.

PENTAFLUOROPHENYLDIMETHYLBROMOSILANE (VII)

To (III), (10 g) in dry hexane (25 ml), was added slowly a solution of bromine (6.9 g) in the same solvent (40 ml) under nitrogen. After stirring for 1 h., the bromine colour had virtually disappeared, the solvent was removed under vacuum and the remaining liquid fractionated to give

(VII) (10.1 g) b.p. 79°C/150 mm (lit.[52] 56°C (1.0 mm).

PENTAFLUOROPHENYLDIMETHYLSILYLAMINE (VIII)

Dry ammonia was passed over the surface of a stirred solution of (VI) (20 g) in anhydrous toluene (200 ml) for 6 h. Ammonium chloride was filtered off under nitrogen, toluene removed under reduced pressure and the less volatile liquid fractionated to give (VIII) (12.2 g) b.p. 52°C/16 mm.

1,3-BIS(PENTAFLUOROPHENYL)-1,1,3,3-TETRAMETHYLDISILAZANE (IX)

Hexamethyldisilazane, (6.2 g), and (VI) (20 g), were refluxed for 1 h. in the presence of a crystal of aluminium chloride under nitrogen. Fractionation of the mixture gave starting material and (IX) (9.8 g) b.p. 151°C/125 mm.

PENTAFLUOROPHENYLDIMETHYLSILYLDIETHYLAMINE (X)

Diethylamine (11.0 g) in dry hexane (50 ml) at -70°C was added slowly to (VI) (20 g) in hexane (50 ml) stirred under nitrogen. The mixture was allowed to reach room temperature, solids were filtered off under nitrogen, and then solvent and diethylamine removed *in vacuo* and the remaining liquid fractionated to give (X) (15.3 g) b.p. 81°C/10 mm.

PENTAFLUOROPHENYLDIMETHYLIODOMETHYLSILANE (XI)

To a solution of (IV), (1.25 g) in acetone (4.0 ml) was added dry sodium iodide (0.8 g) and the mixture refluxed under nitrogen for 0.5 h, the precipitate of sodium chloride filtered off and acetone removed with a stream of nitrogen at 50°C. The residual liquid was identified as (XI), from its mass spectrum, and shown to be uncontaminated by GC.

PENTAFLUOROPHENYLDIMETHYLSILYLIMIDAZOLE (XII)

To (V) (20 g) in dimethylformamide (50 ml) at -10°C was added dropwise a solution of imidazoyllithium, (7.4 g) in the same solvent (20 ml) under nitrogen, stirred for 0.5 h, solvent reduced by distillation in vacuo, solids were removed by filtration and the yield of (XII) estimated as 30% by GC. Compound (XII) was identified by its mass spectrum.

PREPARATION OF FLOPHEMESYL STEROL ETHERS

For cholesterol, ergosterol, and cholestanol, to the sterol (0.3 g) was added flophemesylamine (0.5 ml) and after 30 min., the reagent removed in vacuo.

To 17β-hydroxy-17α-methylandrost-4-en-3-one (0.3 g) in pyridine (0.5 ml) was added flophemesyldiethylamine (0.4 ml) and flophemesyl chloride (0.04 ml). After 30 min., solvent was removed with a stream of nitrogen at 50°C.

To 17β-hydroxypregn-4-en-3,20-dimethoxime, (0.3 g) in pyridine (0.5 ml) was added flophemesyldiethylamine (0.4 ml) and flophemesyl chloride (0.4 ml) and the mixture heated at 85°C for 6 h. Solvent was removed with a stream of nitrogen at 50°C. In each case the crude reaction products were passed down a column of neutral alumina (Grade 0), eluted with hexane-ethyl acetate (9:1) and recrystallized to give the following:

Cholestanol flophemesyl ether (acetonitrile-benzene) 81-83°C

Cholesterol flophemesyl ether (acetonitrile-benzene) 81.5-82.5°C

Ergosterol flophemesyl ether (acetonitrile-ethyl acetate) 86.5-87.5°C

17α-methyl-17β-flophemesyloxy-androst-4-en-3-one (acetonitrile-acetone) 81.5-82.5°C

17β-flophemesyl-5β-pregn-4-en-3,20-dimethoxime a colourless oil.

Flophemesyl cholesterol (Found: C, 68.8, H, 8.3; $C_{35}H_{51}OSiF_5$ requires C, 68.8; H, 8.4%) gave λ_{max} (cyclohexane) 262, 264, 268 nm. ε_{max} = 1, 500,

IR ν(C-H) 2980-2840 s cm^{-1}, ν(Si-C_6F_5) 1639 m, ν(C-F) 1510 m, 1460 s and 1088 s cm^{-1}.

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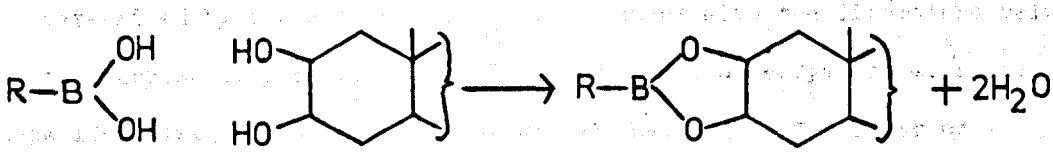
CHAPTER 2

SELECTIVE DETECTION OF STEROID BORONATES

Cyclic boronates have been used to improve the thermal stability and reduce the polarity of a wide range of bifunctional compounds for gas chromatography. The bifunctional compounds include 1,2-diols; 1,3-diols; 1,2-enediols; α-,β-, and γ-hydroxy acids and β- and γ-hydroxy amines.[1] In a typical example a boronic acid reacts with a steroid diol to form a cyclic boronate (boronic ester).



The boronic esters in which R = methyl, propyl, n-butyl, t-butyl, cyclohexyl and phenyl have all been used.[2-3] These derivatives have excellent gas chromatographic properties but are limited to detection by the flame ionization detector. The introduction of an electrophore with detection by electron capture or the selective determination of boron by an element-selective detector might offer much greater sensitivity to detection at low levels. The determination of ecdysones in biological tissue might then be possible, as one or two diol functions are usually present in the steroid skeleton.

HETERO-ATOM SELECTIVE DETECTION.

Element selective detectors perform two functions as gas chromatographic detectors. They are sensitive detectors with improved detection limits by

comparison with non-specific detectors such as flame ionization and thermal conductivity. They also show selectivity to a particular element and have a high discrimination factor for compounds containing that element compared to similar compounds in which the element is absent. A combination of good detection limits and compound selectivity has made them popular in analytical chemistry as reliable results from complex samples can be obtained with the minimum of sample purification. In this respect, the two most popular detectors are the alkali flame ionization detector (AFID) and the flame photometric detector (FPD).

The relative response factors of element selective detectors for several common hetero-atoms are shown in Table 2.1. The value for boron was determined by the author and is in agreement with the literature value[4]. For the purpose of predicting the detection limit of ecdysterone, a peak shape identical to that obtained on GC for the hexakis-TMS ether of ecdysterone was assumed. This is a reasonable approximation for comparative purposes as only small molecular weight and polarity changes are being considered.

TABLE 2.1

RESPONSE OF GC ELEMENT SELECTIVE DETECTORS.

ELEMENT	RESPONSE FACTOR (g/sec^{-1})	DETECTOR	REFERENCE	MOLE FRACTION	LEAST DETECTABLE AMOUNT ng
Boron	6.6×10^{-12}	AFID		0.033[1]	11.0*
Nitrogen	1.0×10^{-13}	AFID	5,6	0.029[2]	18.0*
				0.015[3]	37.0*
Phosphorus	5.0×10^{-14}	AFID	5	-	-
	1.3×10^{-13}	FPD	6	-	-
Sulphur	1.0×10^{-12}	AFID	7	0.033[4]	16.0*
	9.0×10^{-12}	FPD	6	0.033	14.0*
Hydrocarbon[5]	1.0×10^{-9}	FID		1.0	50.0

1. 2β,3β,20,22-bis(methyl boronate)bis(trimethylsiloxy)ecdysterone.
2. hexakis(trimethylsiloxy)ecdysterone dimethylhydrazone
3. hexakis(trimethylsiloxy)ecdysterone methoxime
4. hexakis(trimethylsiloxy)ecdysterone ethylenethioketal
5. hexakis(trimethylsiloxy)ecdysterone

* Calculated from response factors obtained from the references quoted.

Phosphorus is the element most sensitively detected by either AFID or FPD. Unfortunately its introduction into the steroid skeleton is not easy. Phosphinic esters have been prepared for the GC of monohydroxy steroids[8], but it is unlikely that this procedure could be extended to polyhydroxy steroids. The introduction of sulphur is attractive as this can be achieved by the formation of ethylenethioketal derivatives of keto steroids[9]. For the case of ecdysones, the model steroid 2β,3β,14α-trihydroxy-5β-cholestan-7-en-6-one, produces several peaks when reacted with ethanedithiol using boron trifluoride etherate or p-toluenesulphonyl chloride as catalysts. Milder acid catalysts do not promote complete derivatization in cholestan-4-en-3-one or 5α-cholestan-6-one. Nitrogen can be introduced into steroids containing ketone groups by the formation of dimethylhydrazones or methoximes. Dimethylhydrazone formation would allow the introduction of two nitrogen atoms which has sensitivity advantages. It has been shown that hydrazines produce steroid derivatives which are both air and light sensitive with limited thermal stability.[10] Ecdysones have been shown to form methoxime derivatives, but these are normally separated into two peaks by GC as their syn- and anti- methoxime isomers.[11] This limits their usefulness. The AFID has been evaluated for the analysis of steroid methoxime derivatives.[10] In the light of the above discussion, boron was considered worthy of further investigation.

Boron Selective Detectors.

Boron hydrides after GC have been detected by their characteristic light emission in a flame employing the photometric principle (MELPAR detector).[12] The AFID has also been shown to be selective for boron and has been used to detect the boronic esters of simple sugars.[4] The sensitivity of the two detectors have been shown to be essentially equal for boron.[4] As an AFID detector was available it was chosen for evaluation.

Operation of the AFID.

The alkali flame ionization detector (AFID) is described by the manufacturers as a nitrogen or phosphorus selective detector. The range of elements to which it shows a selective response is much wider and includes sulphur, arsenic, antimony, lead, tin, the halogens and boron.[13] The chemical nature of the selective response is not well understood. Thermal energy is the source of the ionization energy while the ionization current (signal) is the sum of a number of processes, the substantial part coming from the alkali metal ionizations. The detection mechanism is affected by specific interactions of hetero-atoms in organic compounds, among which are especially important electron capture, formation of thermally stable compounds and the formation of products with a low ionization potential.[14]

Selectivity for a particular hetero-atom is achieved by careful adjustment of the operating parameters such as flame shape, size, temperature and the composition of the salt tip. For this reason maximum response for the selected hetero-atom is achieved over a narrow range of carrier gas, hydrogen and air flow rates as well as salt tip composition, position and detector oven temperature. Accurate metering of flame gases is essential to maintain selectivity in the region of high flame background currents in which the AFID is operated. The setting for the hydrogen flow rate is correct for one setting of the carrier gas and changes in the hydrogen-air ratio alter the flame temperature. At higher than optimum hydrogen flow rates, detector sensitivity increases but signal intensity is reduced due to the substantial increase in the noise level of the flame. Any attempt to reduce the noise level by decreasing the hydrogen flow rate below optimum results in a drop in ionization efficiency. The air flow rate should be regulated so that a proper burning process is maintained and combustion products are removed. With a low air flow rate the

sensitivity of the detector appreciably decreases owing to a lack of oxygen. If the air flow rate is too high, a loss of sensitivity of the detector is observed due to cooling of the salt tip of the detector by the large volume of uncombusted cool air and at the same time the background noise of the flame increases as a result of turbulence. Operational problems with the AFID are discussed in references [4,13-15].

Two recent innovations have improved the stability, sensitivity, selectivity and working life of the AFID. The use of three electrode geometry increases sensitivity and selectivity by separation of those ionization processes due to the flame background and hydrocarbon compounds from the ions produced by interaction between the hetero-atom and alkali ions.[13] The two ion-production processes are detected in different circuits which relieves the interference of background on signal found with two electrode geometry. The average working life of an alkali electrode was normally quite short and required frequent optimization to allow for the physical changes induced in the alkali electrode by the flame with time. This has been overcome by using a glass bead containing the alkali metal as a non-volatile rubidium silicate. The bead is maintained at a slight negative potential and since glass is a good electrical conductor at flame temperatures, atomic alkali metal ions are generated and vapourized into the flame. After ionization the resulting positive alkali ions are captured again by the negative bead and thus the characteristics of the bead are not changed with time[5]. Low background currents and noise levels similar to that of a FID are achieved.

DETERMINATION OF STEROID DIOLS WITH AFID

The operating conditions for the Pye Series 104 model 154 nitrogen thermal detector are given in table (2.2). This instrument employs three electrode geometry with a conventional alkali metal annulus and was fitted

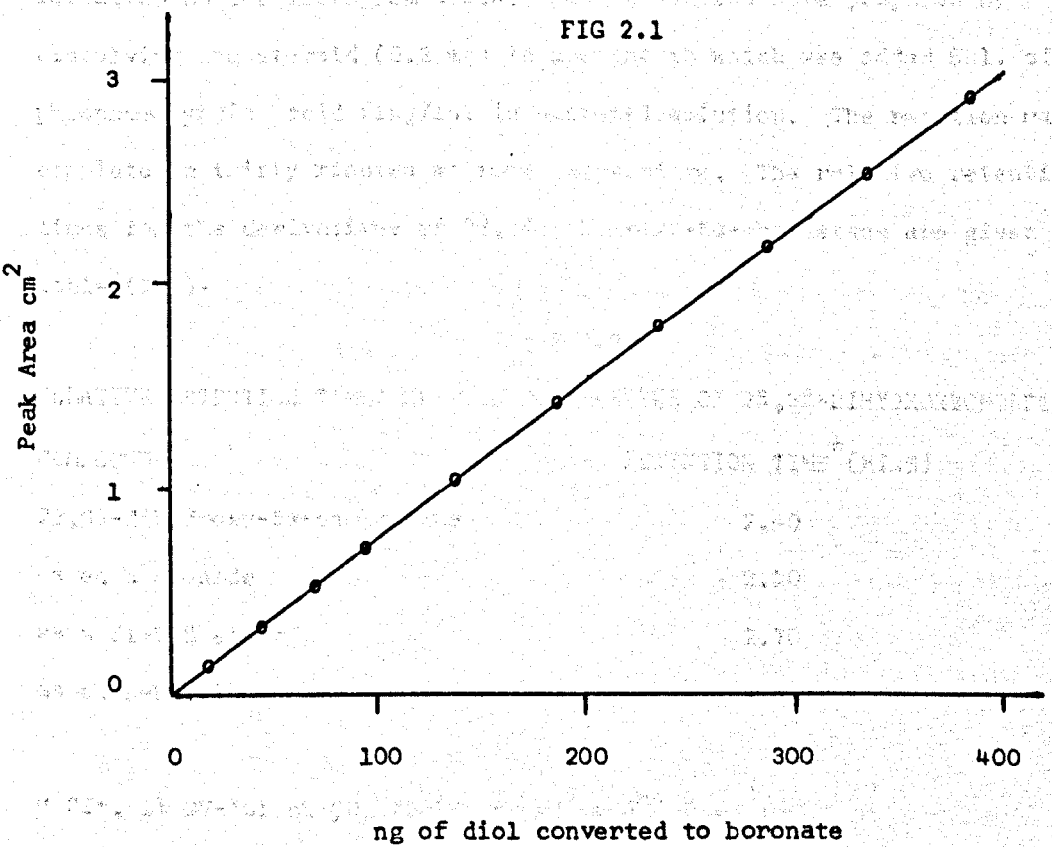
VARIABLE	RbCl tip	CsBr tip
Salt tip height	0.008 in.	0.000
Hydrogen flow rate	37 ml min^{-1}	37 ml min^{-1}
Carrier gas flow rate	40 ml min^{-1}	40 ml min^{-1}
Air flow rate	375 ml min^{-1}	500 ml min^{-1}
Detector Oven Temperature	300°C	300°C
Least Detectable amount	50 x 10^{-9}g	10 x 10^{-9}g
G.L.C. Conditions		

TABLE 2.2

COMMENTS
Response with RbCl varies markedly with height above the flame, having a maximum at the position indicated. With CsBr height is less critical and maximum response is observed at 0.000 in.
Some tolerance in setting for RbCl but it is critical for CsBr.
Convenient for both resolution and optimum detector response.
The flow rate is optimum for RbCl and values either side of this show a poorer response. A maximum response with CsBr is reached at 400 ml min^{-1} and increases beyond this have little effect.
Sufficiently high to prevent condensation of sample material.
Expressed as g. of 2β,3β-dihydroxy-5α-cholestane. With RbCl tip base line stability was very good and an attenuation setting of x20 could be used. Base line stability of CsBr was good but the maximum attenuation factor which could be used was x100.
1.5 ft. column of 1% OV-101 on CQ, 250°C phenylboronic anhydride Rt=0.75 min (used to establish the original detector profile) 2β,3β-dihydroxy-5α-cholestane n-butyl boronate Rt=3.90 mins.

with a "phosphorus type" jet. The detector was optimized by repeated injections of phenylboronic anhydride with sequential changes in detector variables. The least detectable amount of boron in 2β,3β-dihydroxy--5α-cholestane n-butyl boronate was found to be 6.6×10^{-12} g. B sec^{-1}, which agrees favourably with the value of 9.2×10^{-12} g. B sec^{-1} found by Greenhalgh and Wood.[4] This shows that the detector is indeed selective to boron and largely independent of the hydrocarbon content of the molecule. A linear response was obtained over the range investigated 10-400 x 10^{-9} g of steroid boronate Fig. (2.1).

FIG 2.1



Linearity of response and sensitivity of the NTD to boron in the n-butyl boronate of 2β,3β-dihydroxy-5α-cholestane

THE STABILITY OF STEROID DIOL DERIVATIVES TO OTHER REAGENTS.

The reaction of ecdysterone with a selective reagent for diol functions would leave two hydroxyl groups unprotected at C-14 and C-25. Although it might prove possible to chromatograph such a derivative; there are advantages in using a fully protected derivative. Boronic esters have been shown to be stable to further treatment with methoxyamine hydrochloride, bis-trimethylsilylacetamide (BSA) and acetic anhydride.[1] To form boronic esters, equimolar amounts of steroid and n-butylboronic or phenylboronic acid were allowed to stand for fifteen minutes at room temperature in pyridine. As well as boronic esters, used to selectively protect steroid diols, acetonides were investigated and a procedure developed for their formation on the microgram scale. The acetonides were prepared by dissolving the steroid (0.2 mg) in acetone to which was added 6µl. of phosphomolybdic acid (1µg/1µl in acetone) solution. The reaction was complete in thirty minutes at room temperature. The relative retention times for the derivatives of 2β,3β-dihydroxy-5α-cholestane are given in table (2.3).

TABLE 2.3

RELATIVE RETENTION TIMES OF SOME DERIVATIVES OF 2β,3β-DIHYDROXYCHOLESTANE

COMPOUND	RETENTION TIME* (MINS)
2β,3β-dihydroxy-5α-cholestane	2.40
as an acetonide	2.10
as a di-TMS ether	2.70
as an n-butylboronate	3.60

* 3ft, 1% OV-101 on CQ, 260°C, 80 ml. min^{-1} N_2.

The C-14 hydroxyl group in ecdysones is not converted to a silyl ether by BSA.[11] Catalysis by trimethylchlorosilane (TMCS) causes partial formation of the enol ether of the ketone group at C-6. Trimethylsilylimidazole (TMSIm) is the reagent of choice to overcome both of these problems. Although the n-butylboronate of the 2β,3β-dihydroxy-5α-cholestane is stable to BSA and BSA-TMCS mixtures it is readily cleaved by TMSIm under mild conditions, see Table (2.4).

TABLE 2.4.

DISPLACEMENT OF BORONIC ESTERS BY SILYL ETHER WITH TMSIm

CONDITIONS	n-butylboronate	di-TMS ether
30 min. at room temp.	86%	14%
40-45°C for 30 min.	80%	20%
40-45°C for 4 hr.	50%	50%

The acetonide derivative was also cleaved at room temperature by TMSIm (70% di-TMS ether after 30 min. at 22°C). Also the n-butylboronate of 11β,17α,21-trihydroxypregn-4-en-3,20-dione was cleaved by TMSIm at room temperature and more readily at elevated temperatures to form the appropriate TMS derivative. The above observations were confirmed by simultaneous GC and TLC of samples with standards and by mass spectrometric identification of all products.

BORONATES CONTAINING AN ELECTROPHORE.

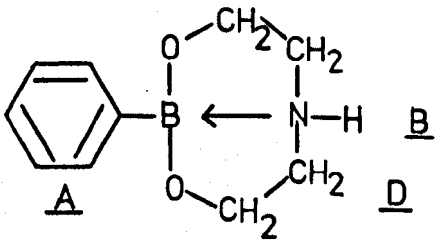
The introduction of an electrophore into an alkyl or arylboronic ester with detection by electron capture is an alternative to the selective detection of boron by an AFID. Organoboron compounds with electronegative

substituents α or β to boron in an aliphatic chain undergo ready thermal elimination[16-17]. A γ-chlorine atom in an aliphatic chain would lack sensitivity to electron capture and is hydrolytically unstable under basic conditions[18]. The γ-Bromopropylboronic acid is thermally and hydrolytically stable but would lack sufficient volatility on gas chromatography as a steroid derivative to be useful with ecdysones.

The pentafluorophenyl group is sensitive to electron capture detection and volatile on GC when incorporated into a silyl ether. So pentafluorophenylboronic acid was synthesized for evalution. Pinacol and 2β,3β-dihydroxy-5α-cholestane were used as model diols. Esterification was carried out in the usual way by mixing approximately equi-molar amounts of the boronic acid and diol in an anhydrous solvent. The reaction was monitored by GC and TLC. Under these conditions, no ester was formed and analysis of the reaction mixture indicated the presence of pentafluorobenzene, which was identified by comparison of its I.R. spectrum with that of an authentic sample and by mass spectrometry. Chambers and Chivers[19] indicated that the stability of the boronic acid to nucleophiles is dependent on the acidity of the medium and that hydrolytic cleavage of the pentafluorophenyl group is likely in basic or neutral medium. The esterification procedure was modified to produce an acid medium to which drying agents were added to remove the water which is produced during the reaction and is the most likely source of nucleophiles. The presence of acid (1-10% v/v) glacial acetic or dissolving dry hydrogen chloride gas in the solvent, drying agents (magnesium sulphate, molecular sieves) by themselves or in acid medium, or acidic drying agents such as phosphorus pentoxide were all unsuccessful. In all cases pentafluorobenzene was observed in the reaction mixture. The unusual susceptibility of pentafluorophenylboronates to nucleophilic cleavage even in acid medium makes them of little value as derivatives of steroid diols.

SYNTHESIS OF ARYL BORONIC ACIDS

The chemistry of boronic acids has been reviewed by Torsell.[20] Pentafluorophenylboronic acid has been prepared by Chambers and Chivers[19] from pentafluorophenylboron dichloride by careful addition of the calculated weight of water in acetone at -70°C but neither the dichloride nor the starting material for its preparation (trimethylpentafluorophenyltin) are readily available.[19] A more direct route seemed possible by the reaction of the Grignard reagent formed from pentafluorobromobenzene[21] and trimethyl borate. The experimental procedure was modelled on the general synthesis of boronic acids described by Snyder et al.[22], modified in the light of the work by Morlyan and Rostomyan[23]. By equal portions and in increments, the Grignard reagent and trimethyl borate were added to a volume of solvent which was rapidly stirred and cooled to -70°C. The reagent solutions were added in nitrogen equalized dropping funnels whose ends were bent inwards towards the stirrer shaft to avoid excessive precipitation of solid on the walls of the reaction vessel. Phenylboronic acid prepared by this method was characterised as its diethanolamine ester, the N.M.R. spectra of which, in d_6-DMSO was very distinct;



Diethanolamine phenylboronate

A = 7.5-7.0 ppm (multiplet)

B = 7.0-6.7 ppm (broad)

C = 3.75 ppm (multiplet)

D = 2.80 ppm (multiplet)

on addition of D_2O

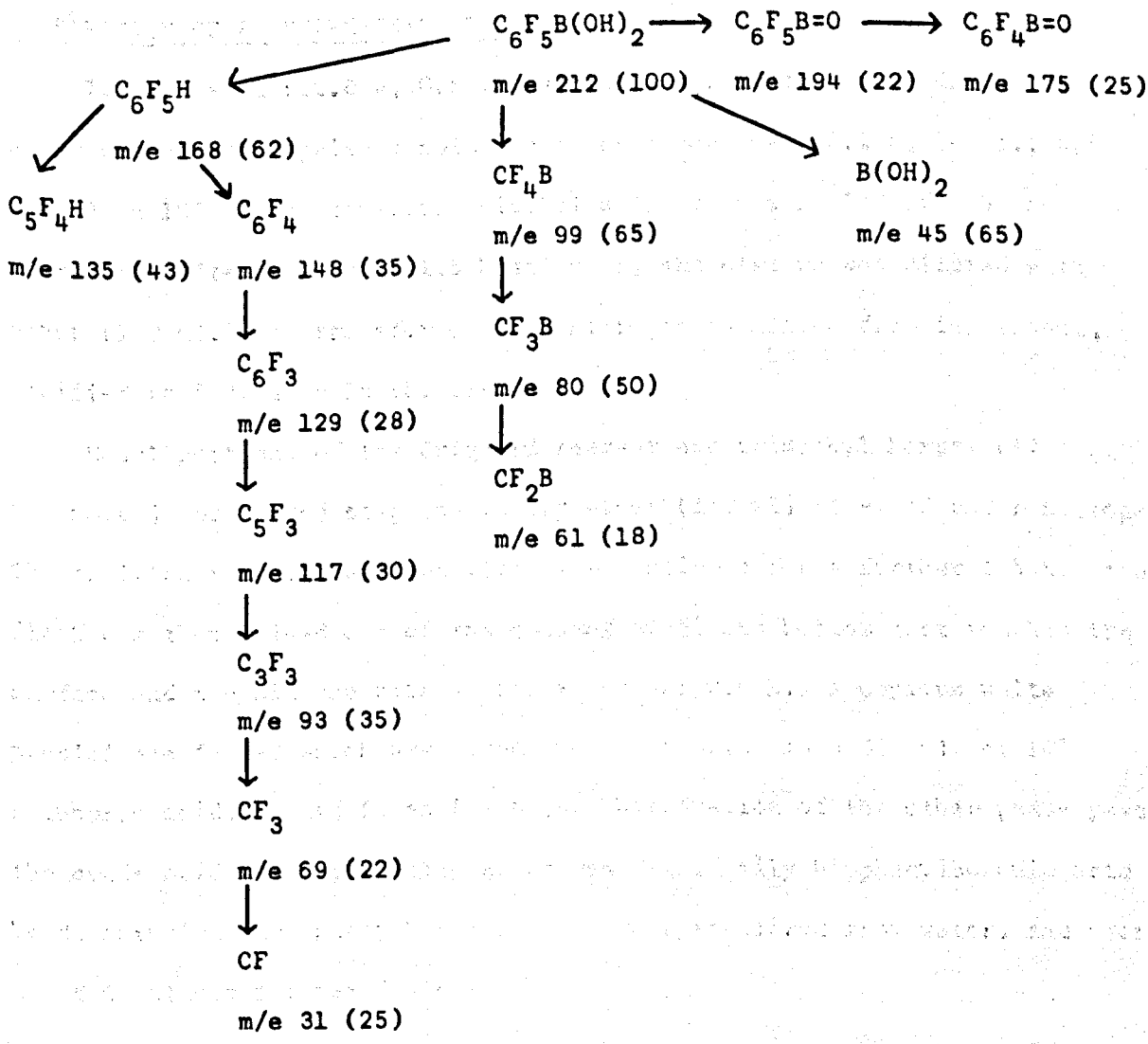
(1) 7.0-6.7 B disappears

(2) 2.80 D collapses to a triplet.

The boronic acids are difficult to characterize by their melting-points and suitable esters are usually prepared for this purpose. This is because the acids melt sharply with conversion to the anhydride which solidifies and melts again a few degrees higher (e.g. phenylboronic acid 215° C, anhydride 219°C).[20] Boronic acids are readily dehydrated which makes drying difficult without anhydride formation. It has been suggested that 65% sulphuric acid under vacuum is a suitable drying agent.[22] With phenylboronic acid, it was always found, that some anhydride formation occurred.

Pentafluorophenylboronic acid was prepared in an analogous manner to that described for phenylboronic acid. Its melting-point was considerably lower than the literature value, even after vacuum sublimation and zone refining. The mass spectrum of the compound is completely interpretable in terms of the expected fragmentation of pentafluorophenylboronic acid with no fragment ions of greater m/e than the molecular ion.

Mass Spectrum (80 eV, 150°C)



Those masses containing boron can be identified from the relative isotope abundance ratio of ^{11}B and ^{10}B (i.e. approximately 4:1). Analysis of the acid by GLC and GC-MS gave a single peak which was identified as the anhydride.

EXPERIMENTAL

PREPARATION OF PHENYLBORONIC ACID.

To magnesium (12.0 g, 0.5 moles) in 30 ml. of anhydrous diethyl ether was added dropwise a solution of bromobenzene (80.1 g, 54 ml., 0.5 moles) in 100 ml. of anhydrous diethyl ether over a period of 2 h under nitrogen. After a further 1.5 h stirring, the mixture was diluted with ether (100 ml.) and transferred to a nitrogen equalized dropping funnel, modified as indicated in the discussion.

Equal portions of the Grignard reagent and trimethyl borate (52.0 g, 0.5 moles) were added stepwise to dry ether (150 ml) at -70°C under nitrogen. The addition took 1h. and the mixture was stirred for a further 1.5 h., the flask was then raised out of the coolant until its bottom just touched the surface and the mixture stirred for a further 0.5 h. A copious white precipitate formed which was decomposed by pouring into 300 ml. of 10% sulphuric acid, cooled in an ice bath. Evaporation of the ether phase gave the crude acid (42.0 g), which after removal of oily bisphenylboronic acid by decantation gave phenylboronic acid, recrystallized from water, and stored at -8°C without further drying.

PREPARATION OF DIETHANOLAMINE PHENYLBORONATE.

The damp acid (4.0 g) and diethanolamine (3.5 g, freshly distilled) in toluene (80 ml.) were refluxed in a Dean and Stark apparatus to remove water. The reaction was considered complete when no further water separated from the azeotrope. The toluene was removed under vacuum and the sample purified by recrystallization from chloroform and sublimed at 250°C, 0.2 - 0.3 Torr, to give diethanolamine phenylboronate m.p. 209.5 - 210.5°C (lit.[24] mp. 209-210°C).

Found; C, 62.7; H, 7.3; N, 7.5 calculated for $C_{10}H_{14}BNO_2$ C, 62.8; H, 7.3; N, 7.3. %

I.R. ν(N-H) kBr disc 3,400 cm^{-1} broad.

PREPARATION OF PHENYLBORONIC ANHYDRIDE.

A sample of the damp acid (5.0 g) when vigorously dried at 100°C under vacuum in a drying pistol over phosphorus pentoxide for 12h. gave phenylboronic anhydride (4.7 g), mp 219.0-219.5°C from hexane-n-butyl ether (lit[20] mp 219°C). Found; C, 69.3; H, 5.1 calculated for $C_{18}H_{15}B_3O_3$ C, 69.3; H, 5.1. %

Mass Spectrum (m/e, relative intensity)

312 (m^+,100), 267 (6), 235 (10), 208 (15), 164 (25), 156 (9), 131 (12), 104 (25), 87 (5), 77 (8), 67 (19), 57 (5), 41 (15), 31 (15).

GLC. (3 ft., 1% OV-17, 236°C,68 ml. min^{-1}) R_t = 1.3 min.

PREPARATION OF PENTAFLUOROPHENYLBORONIC ACID.

To magnesium (1.0 g) in anhydrous ether (10 ml) under nitrogen was added a solution of ethyl bromide (4.5 g) in ether (10 ml) at a rate sufficient to maintain a gentle reflux. When the reaction subsided a solution of bromopentafluorobenzene (10 g) in 10 ml. of ether was added slowly over 0.5 h. When the reflux ceased, the mixture was diluted with 25 ml. of ether and transferred under nitrogen to a pressure equalized dropping funnel.

Using the general procedure described in the synthesis of phenylboronic acid, the Grignard reagent and a solution of trimethyl borate (4.2 g) in 20 ml. of ether were added to 50 ml. of ether at -70°C. After the addition, the mixture was stirred at -70°C for 1 h., the flask raised until its bottom just touched the coolant and stirred for a further 1.5 h. 40 ml. of 10% hydrochloric acid and 15 ml. of ether were cooled by adding

small lumps of solid carbon dioxide. The reaction mixture was poured into it and the ether layer rapidly separated and evaporated to dryness, the residue recrystallized from a large volume of anhydrous toluene. A small amount of insoluble material was removed by filtration to give pentafluorophenylboronic acid mp. 127-132°C, 6.6 g yield 76% sublimed to constant mp. 131-132°C zone refined for 100 hr., mp. 131-132°C (lit[19], 290°C).

SUMMARY

It was shown that steroid diols form boronic esters under mild conditions in a quantitative fashion. The selective detection of boron in such derivatives with an AFID is feasible. When unreacted hydroxyl groups remain and mixed derivative formation is desirable, then TMS ethers cannot be formed using TMSIm as this leads to cleavage, even under mild conditions. Substituted boronic acids sensitive to electron capture were not found to be practical due to the unusual hydrolytic instability of pentafluorophenylboronic esters and the poor volatility to be expected from γ-bromopropylboronic esters of steroids.

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CHAPTER 3

PENTAFLUOROPHENYLHYDRAZONES AS STEROID DERIVATIVES

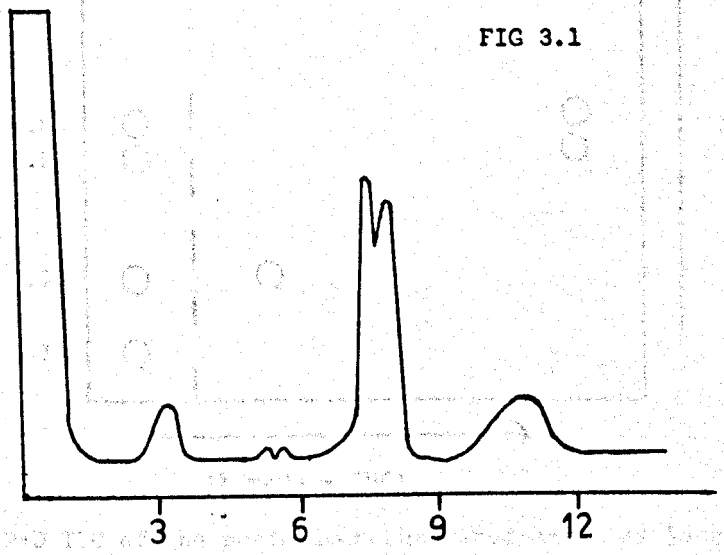
Steroid ketones have been conveniently identified as their 2,4-dinitrophenylhydrazones, which are normally highly crystalline derivatives of sharp melting point. More recently pentafluorophenylhydrazones have been prepared and used in the analysis of oestrone and oestradiol by GC-ECD at levels down to 0.08 ng.[1,2] To asses the possibility of forming a mixed trimethylsilyl - pentafluorophenylhydrazone derivative of ecdysone, for the determination of the hormone at sub-nanogram levels; the formation and properties of pentafluorophenylhydrazones of 5α-cholestan-3-one, 5α-cholestan-6-one and cholest-4-en-3,6-dione were investigated.

The method of Mead et al.[2], in which pentafluorophenylhydrazine in methanol-acetic acid (9:1) is used as the reagent, did not give a quantitative reaction, and unreacted steroid was identified in all cases. For the formation of 2,4-dinitrophenylhydrazones of steroid ketones, acetic acid[3], acetic acid-sodium acetate buffer[4], phosphoric acid[5], hydrochloric acid[6-8] and oxalic acid[9] have all been used as catalysts. The formation of 2,4--dinitrophenylhydrazone derivatives of steroids has been reviewed[7-8]; the general conditions for favourable reaction can be identified as acid catalysis with polar solvents. Using ethanol as solvent, hydrochloric acid was found to be the best catalyst as indicated by complete reaction of 5α-cholestan-3-one, determined by GC and TLC. The reaction with acetic acid, phosphoric acid and oxalic acid in ethanol, dimethyl sulphoxide, dimethylformamide, tetrahydrofuran and dioxane was not quantitative as witnessed by a peak for the ketone on GC.

The product of the reaction between 5α-cholestan-3-one and pentafluoro-

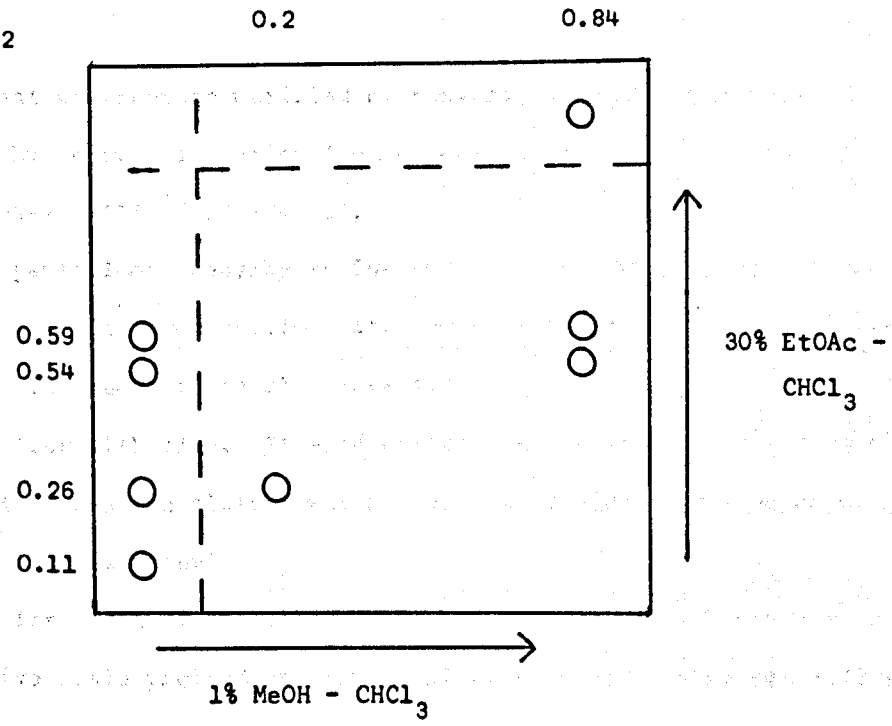
phenylhydrazine was shown to contain a minimum of four components by GC (Fig. 3.1).

FIG 3.1



The conditions for the analysis (1.5 ft. column of 1% OV-101, 285°C, 60 ml. min^{-1}) indicate the poor volatility of the derivative. TLC of the reaction mixture in 30% ethyl acetate-chloroform showed a minimum of four components. Spraying with an acid solution of stannous chloride[10] and heating at 110°C for fifteen minutes, spotting with authentic 5α--cholestan-3-one and developing in 1% methanol-chloroform at right-angles to the first direction indicated the presence of the keto-steroid in the two faster-moving components. The plate was visualized with both uv light and iodine (Fig. 3.2).

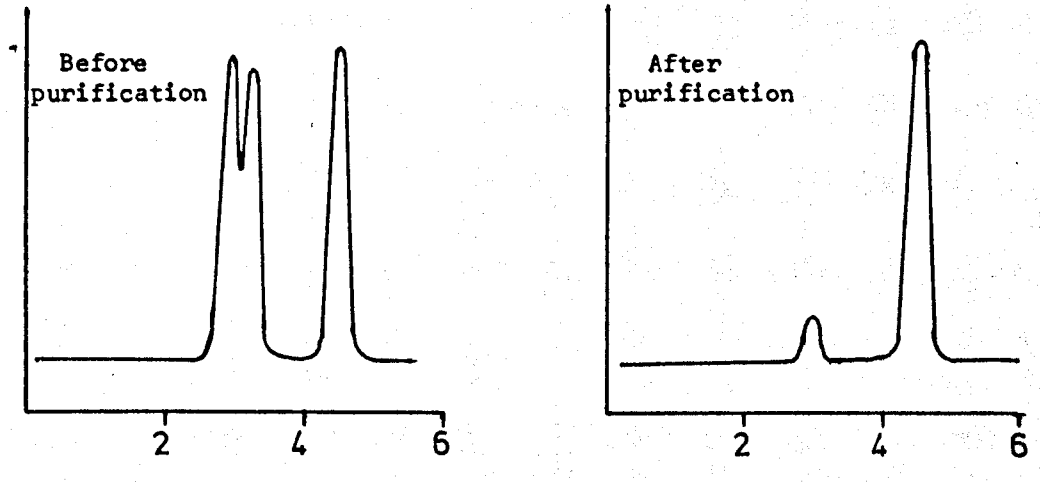
FIG 3.2



2-D TLC of the pentafluorophenylhydrazone of 5α-cholestan-3-one

The pentafluorophenylhydrazine used in this study was suspected of being impure. The commercial reagent was treated with charcoal and recrystallized from light petroleum (bp 100-120°) three times followed by chromatography on Davison silica gel, eluting with chloroform and monitoring the effluent by high pressure liquid chromatography (HPLC) using Porasil A (500 mm, 37-75μ, mobile phase 80% chloroform in iso-octane, 1 ml min^{-1})(Fig. 3.3).

FIG 3.3

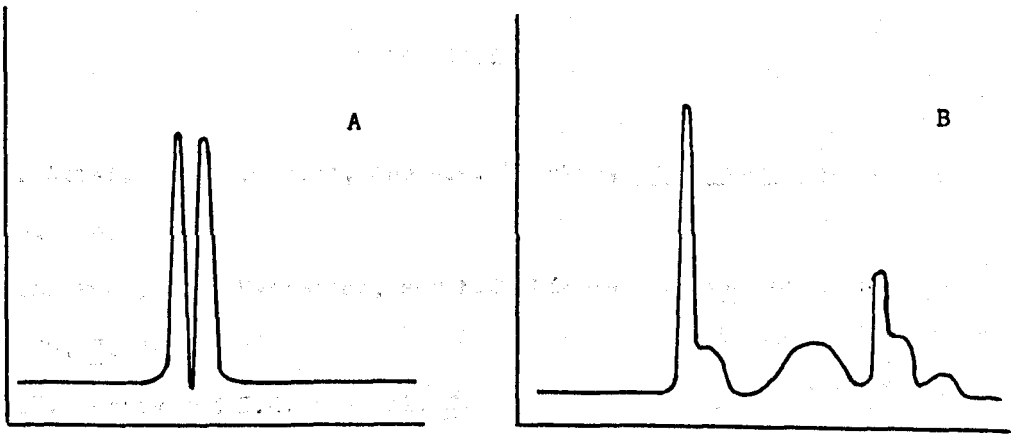


The reagent was further purified by conversion to its hydrochloride followed by recrystallization from ether-ethanol and sublimation to constant m.p. 239-242°C (lit[10] 238-240°C).

The pentafluorophenylhydrazine hydrochloride was a useful reagent allowing quantitative reaction with 5α-cholestan-3-one in ethanol for 2h. at 55°C. The reagent was also more stable as its hydrochloride and did not discolour with time. It also obviates the necessity of adding mineral acid to the reaction mixture which would be of advantage when working with acid labile material.

The formation of cholestan-3-one pentafluorophenylhydrazone on a preparative scale yielded an orange oil which would not recrystallize after removal of solvent. Chromatography of the oil on bentonite-Kieselguhr (4:1)[8] or silica gel showed decomposition of the derivative and all fractions that contained the derivative were contaminated with the keto-steroid. On preparative layer plates, two closely eluting bands were removed and the crude material from each band had a similar mass spectrum and an ion required for the molecular weight of the keto derivative. It is likely that these two components are syn- and anti- isomers of the keto-derivative. If the derivatives were allowed to stand for some time at room temperature, their colour darkened noticeably and analysis produced complex chromatograms (Fig 3.4).

FIG 3.4



HPLC analysis of the pentafluorophenylhydrazone derivative of 5α-cholestan-3-one. A, freshly prepared. B, after standing at room temperature for 6 h.: (500mm PORASIL A, isoctane - chloroform 1 : 4, 0.5 ml. min^{-1}.)

The information obtained suggested that the pentafluorophenylhydrazone derivatives of ecdysones would not be suitable due to their limited volatility, poor hydrolytic stability and potential instability when exposed to the atmosphere and sunlight.

In summary, it can be concluded that pentafluorophenylhydrazone derivatives of ecdysones would be unsuitable for their analysis at low levels.

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CHAPTER 4

THE MASS SPECTROMETER AS A GAS CHROMATOGRAPHIC DETECTOR

The technique of combined gas chromatography-mass spectrometry (GC-MS) has become indispensable for the analytical characterisation of small samples of organic compounds. By comparison, the use of the mass spectrometer as a selective, sensitive and quantitative detector is relatively recent. Sweeley *et al.*[1] described its use in the identification of the components of unresolved gas chromatographic peaks. The construction of an accelerating voltage alternator allowed ions of different m/e to be focused at the detector by rapidly switching the accelerating voltage at constant magnetic field strength. For a magnetic sector instrument, ions of mass m/e are focused at the detector according to the relationship,

$$m/e = \frac{R^2H^2}{2V}$$

R = radius of ion beam
H = magnetic field
V = accelerating voltage

so that at constant magnetic field H, a higher accelerating voltage will bring a lower mass into focus. Rapid switching of the accelerating voltage between V and V + ΔV allows the simultaneous monitoring of more than one ion. Sweeley was able to quantify the components of a binary mixture having characteristic fragment ions (m/e) within 10% of each other, even though they were not resolved by the gas chromatograph. The quantification of drug metabolites by an improved instrumental technique was described by Hammar *et al.*[2]. They coined the phrase mass fragmentography for the process now more generally known as multiple ion detection (M.I.D.). In a later paper, Hammar and Hessling[3] described a much improved voltage alternator and peak matcher which is the forerunner of the commercial systems now available. Three fragment ions could be simultaneously

focused with an expanded mass range of 20%. Greater sensitivity in each channel was achieved by the provision of base-line and gain controls as well as efficient filtering of the signal and fast switching of the accelerating voltage. Simultaneous recording of the selected fragments and total ion current was possible and the provision of a novel peak matching device allowed high resolution mass data to be obtained with a medium resolution single focusing mass spectrometer. As many as eight fragments can be monitored simultaneously in eight separate channels on modern instruments. The use of the multiple ion technique and a high resolution mass spectrometer allows the direct analysis of crude biological material by direct insertion without prior separation by gas chromatography.[4]

Single Ion Monitoring.

It is possible to use a mass spectrometer as a gas chromatographic detector without a voltage alternator. This is achieved by the selection of one characteristic ion and monitoring this fragment throughout a gas chromatographic run.[5-6] The above process is known as single ion monitoring (S.I.M.). As only one ion is monitored it is obviously less specific than the M.I.D. technique and is more affected by extraneous background material with the same m/e value.

SAMPLE REQUIREMENTS.

Any organic compound which is thermally stable, sufficiently volatile for GC and produces a mass spectrum under electron impact is a suitable sample. The best results are obtained with those compounds which fragment to produce relatively few intense ions. Selection of one or more of these ions ensures that maximum sensitivity is achieved. The selected ion should also appear in a part of the mass spectrum which is free from interference due to background contributions. Occasionally the ion current of the selected ion is maximized at lower than normal ionizing energies which can

be very useful in reducing background contributions and thus substantially increase the signal-to-background ratio.

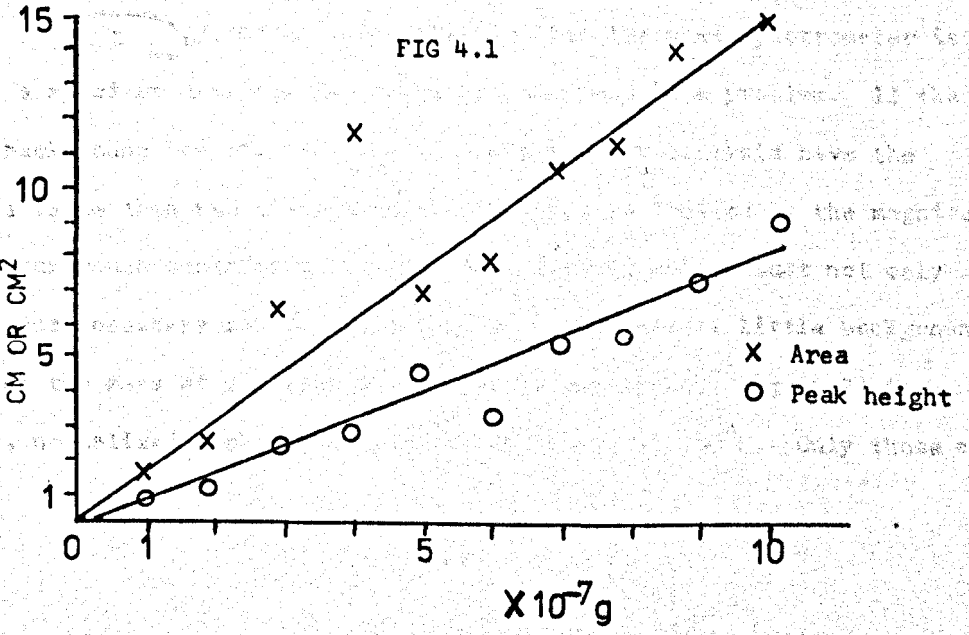
For quantitative analysis by either S.I.M. or M.I.D. an internal standard is required, to permit the analyst to make allowances due to losses caused by variation in injected sample volume, column adsorption effects and all those variations in ion current or voltage which influence the intensity, focusing or measurement of the ion beam. For S.I.M. the internal standard must fulfil the following requirements;

(i) have the same m/e value as the selected fragment. This ion must arise by a similar fragmentation process as the compound under study to allow for changes in ionizing beam energies. Implied in the above is molecular similarity between sample and standard.

(ii) the internal standard and the compound of interest should have similar retention times but be separable by GC. Then, the only variation in detector sensitivity which will affect the results are those occurring between the elution of the two peaks.

(iii) the internal standard should not differ greatly in mass to the compound to be measured otherwise effusion discrimination in the separator will become important.

FIG 4.1



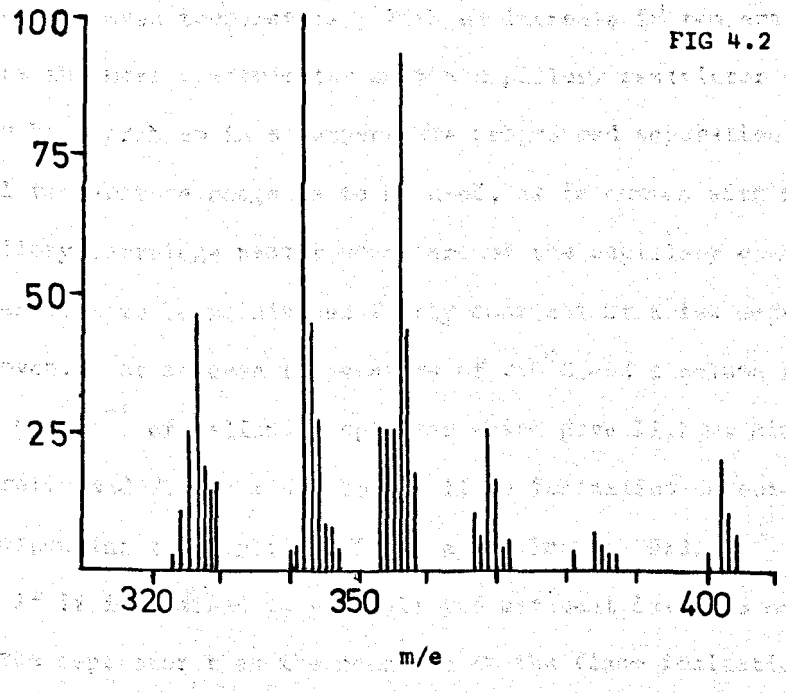
The precision which can be expected when an internal standard is not used is illustrated in (Fig. 4.1). The ion m/e 368^{+} in trimethylsilyl-cholesterol was monitored at increasing concentration of the steroid. When the technique of M.I.D. is used, there is no need for any chemical differences, and mass differences alone are sufficient to discriminate between the compound being analysed and the standard. This allows the use of the ideal internal standard, namely the actual compound under study modified to contain an increased mass by the introduction of stable isotopes (e.g. D, N^{15}).

OPTIMIZATION OF GC CONDITIONS

The range of carrier gas flow rates is governed by separator design and more critically by the maximum operating pressure of the mass spectrometer. For the RMU-6E this means a range of 10-20 ml. min^{-1} of helium, with the Watson-Biemann separator described later. For optimum performance, columns of small diameter (0.32 cm), low liquid phase loadings and short lengths are used as far as this is compatible with the required resolution of sample components. Some compensation for low flow rates is achieved by operation at higher temperatures. For steroid analysis, this limits the number of useful GC phases to those which are most thermally stable (e.g. OV silicone oils). When the mass spectrometer is used as a specific detector excessive column bleed is a problem. If the column background and the fragment ion selected for analysis have the same m/e value then the minimum detection limit is limited by the magnitude of the background contribution. Thus the selected column must not only provide the necessary sample resolution but also produce little background signal at the mass of the fragment ion to be monitored. Fig (4.2) is a partial, normalized background spectra for OV-101 at 250°C. Only those mass

regions of low background can be used analytically for single ion monitoring.

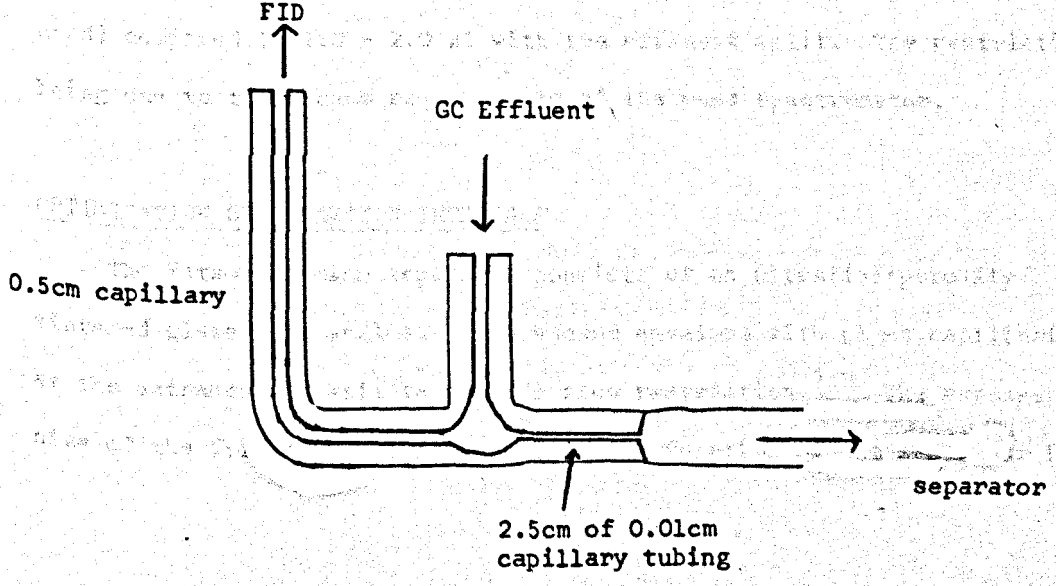
FIG 4.2



T-Piece Splitter.

A T-piece splitter (Fig (4.3) apportions the effluent from the gas chromatographic column between the mass spectrometer and flame ionization detector.

FIG 4.3



A short length of capillary tubing acts as a flow restrictor to the mass spectrometer. As it is positioned in the GC oven its dimensions are dependent on the oven temperature. With an increase in temperature more effluent enters the mass spectrometer as the capillary restrictor expands. This could be a problem in a temperature programmed separation. When only a small temperature range is to be used, as is common with steroids, an auxiliary cartridge heater wound around the capillary enables its temperature to be maintained fairly constant at a few degrees higher than the oven. At an oven temperature of 260°C and a column flow rate of 18.6 ml min^{-1} of helium, a splitter which gave 11.1 ml min^{-1} to the separator and 7.5 ml min^{-1} to the flame ionization detector was used, corresponding to a split ratio of approximately 2:3.

If it is desired to pass all the effluent into the mass spectrometer via the separator then the coupling to the flame ionization detector can be capped off and made leak-tight with only a few cm^3 of dead volume. This small volume, due to the short length of glass tubing making up the unused part of the T, does not have a marked effect on separator or mass spectrometer performance. In this configuration the maximum column flow is limited to 12 ml min^{-1}. As well as this, the total volume of sample which can be injected is limited to less than 0.5 µl (0.3 µl was normally used) compared to 1.0 - 2.0 µl with the effluent split. The restriction being due to the vacuum requirements of the mass spectrometer.

OPTIMIZATION OF SEPARATOR INTERFACE

The Watson-Biemann separator consists of an ultrafine-porosity sintered glass tube enclosed in a vacuum envelope with glass capillaries at the entrance and exit to provide flow restriction.[7-8] The average pore size of the frit is approximately 1µm. The function of the separator is to

achieve an enrichment of sample in the carrier gas while simultaneously producing a pressure drop from atmospheric at the column exit to the working pressure of the vacuum system of the mass spectrometer. The diameter of the entrance capillary is important in effecting a pressure drop sufficient to satisfy the conditions of molecular flow in the pressure reduction system. For the condition of molecular flow to apply the mean free path of the gas must be large (>10X) in comparison with the diameters of the pores. Thus the corresponding pressure in the glass frit is of the order of a few torr (typically 1-10 torr). Under these conditions the rate at which a gas effuses to the exhaust vacuum will be inversely proportional to the square root of the molecular weight and directly proportional to the partial pressure of each component. The quantity of any gas Q going through the porous glass is given by:

$$Q = K.p.\sqrt{1/M}$$

K = constant depending on the conductance of the porous tube

p = partial pressure of the component

M = molecular weight

The ratio of the quantity of sample Qs to the quantity of helium carrier gas V_{He} that goes through the frit is:

$$\left(\frac{Q_s}{V_{He}}\right)_{frit} = \frac{P_s}{P_{He}}\sqrt{\frac{M_{He}}{M_s}}$$

Consequently a fractionation of sample to carrier gas is obtained which depends upon the inverse ratio of the square root of the molecular weights. The exit constriction diameter controls the sensitivity of the apparatus, the diameter is limited only by the maximum pressure that can be tolerated in the ion source of the mass spectrometer. If fractionation of the sample

entering the mass spectrometer through the exit capillary is to be avoided then the mean free path of the gas must be small compared with the diameter of the exit capillary. Thus under conditions of viscous flow, the flow is independent of molecular weight, and all components are transported with equal velocity. The quantity of a given component entering the mass spectrometer is given by:

$$\left(\frac{Q_s}{V_{He}}\right)_{ms} = \frac{P_s}{P_{He}}$$

and the ratio of the quantity of sample to the quantity of helium that enters the mass spectrometer depends on the partial pressure of each component in the fritted tube. The diameter of the entrance and exit capillary tubing is set by trial and error. Once optimized it is not normally altered.

The performance of any type of separator is characterised in terms of its separation factor (enrichment) N and separator yield (efficiency) Y.[9] The separator yield is defined as the ratio of the amount of sample entering the spectrometer (Q_{ms}) to that entering the separator (Q_{GC}), usually expressed as a percentage.

$$Y = \frac{(Q_{ms})}{(Q_{GC})} \times 100\ \%$$

It represents the ability of the particular device to allow organic material to pass into the ion source of the mass spectrometer. The separation factor is defined as the ratio of sample concentration in the carrier gas entering the mass spectrometer to the sample concentration in the carrier gas entering the separator.

$$N = \frac{Q_{ms}/V_{ms}}{Q_{GC}/V_{GC}} = \frac{Q_{ms} \cdot V_{GC}}{Q_{GC} \cdot V_{ms}} = \frac{(P_s/P_{He})_{ms}}{(P_s/P_{He})_{GC}}$$

The separation factor varies a great deal depending on the type of separator, the gas chromatographic flow, the vacuum system efficiency and the molecular weight of the sample. The separation factor N and the separator yield Y are algebraically related to each other by the expression

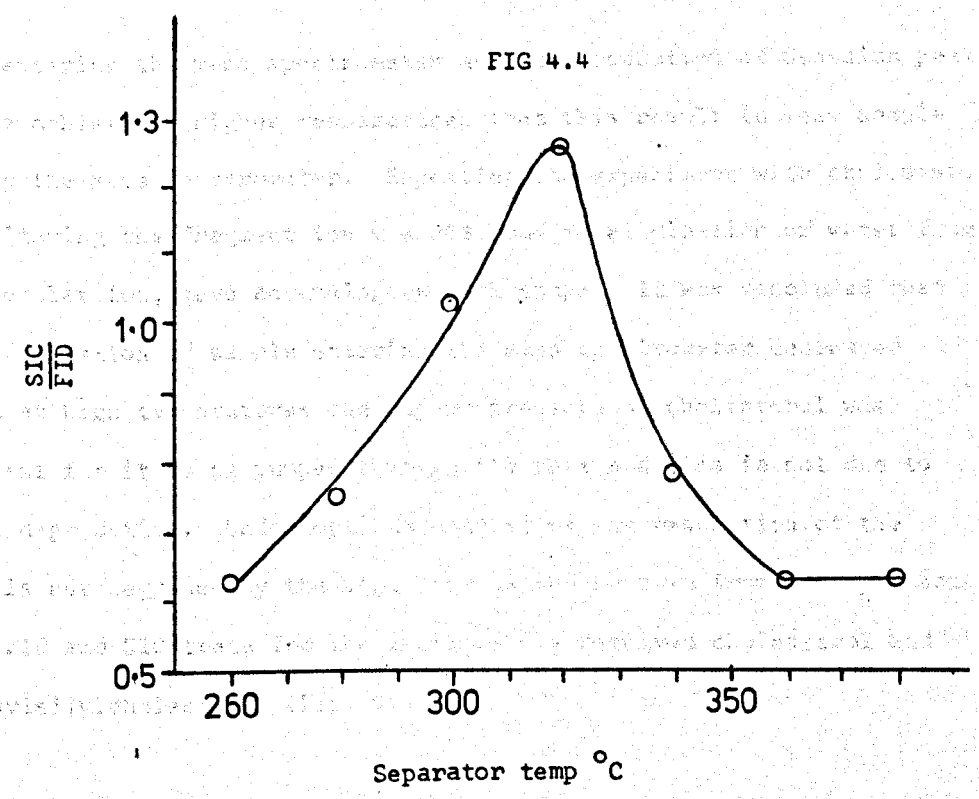
$$N = \frac{Y}{100} \times \frac{V_{GC}}{V_{ms}}$$

With all parameters optimized for steroid analysis, values of Y = 18% and N = 6-10 were obtained using trimethylsilylcholesterol as representative steroid. The separator yield Y, is good, but the separation factor N is lower than one would like. This is probably a function of the diameter of the entrance capillary and the low pumping speed of the source of the mass spectrometer.

The variable parameters of the Watson-Biemann separator are optimized by experiment. Cholesterol and its silylated derivatives were chosen as being typical of the type of steroid on which mass spectral data would be required. Cholesterol is known to be sensitive to thermal dehydration and was considered to be suitable as a model for a labile steroid.

Effect of Temperature: With the GC flow rate (18 ml. min^{-1}) set to the maximum that the mass spectrometer would accept after splitting and passage through the separator, the integrated signal strength for a constant weight of cholesterol monitored as its molecular ion m/e 386 was plotted as a function of separator temperature (Fig. 4.4).

FIG 4.4



The ratio of the peak area of the single ion chromatogram (SIC) to that of the flame ionization detector (FID) was selected as the function for the y-axis as this made allowance for small differences in amount of steroid injected or adsorbed in the apparatus possible. It also allowed comparison of peak shape to be made. If good peak shape is observed on the FID trace but skew peaks on the SIC then the cause lies in the separator and associated glass lines and not with the gas chromatograph. A very definite maximum in efficiency with temperature exists. At temperatures less than 230°C no peaks are observed on the SIC for cholesterol although the FID indicates that sample is entering the mass spectrometer. With temperatures between 230° and 290°C increasing amounts of sample enters the mass spectrometer. The peak shape of the SIC by comparison with FID is poor but tailing is diminished with higher temperature. An optimum in temperature is reached between 300-320°C when both a maximum in

sample entering the mass spectrometer and the production of Guassian peak shape is achieved. Higher temperatures than this result in less sample entering the mass spectrometer. Repeating the experiment with cholesterol, but monitoring the fragment ion m/e 368, due to elimination of water from the molecular ion, gave an analogous peak shape. It was concluded that the concentration of sample entering the mass spectrometer decreased because at high temperatures the vapour pressure of cholesterol was sufficient for it to be pumped through the frit and loss is not due to thermal degradation. Under optimum conditions the resolution of the column is not degraded by the separator as can be seen from a comparison of the FID and SIC trace for the incompletely resolved cholesterol and trimethylsilylcholesterol (Fig. 4.5).

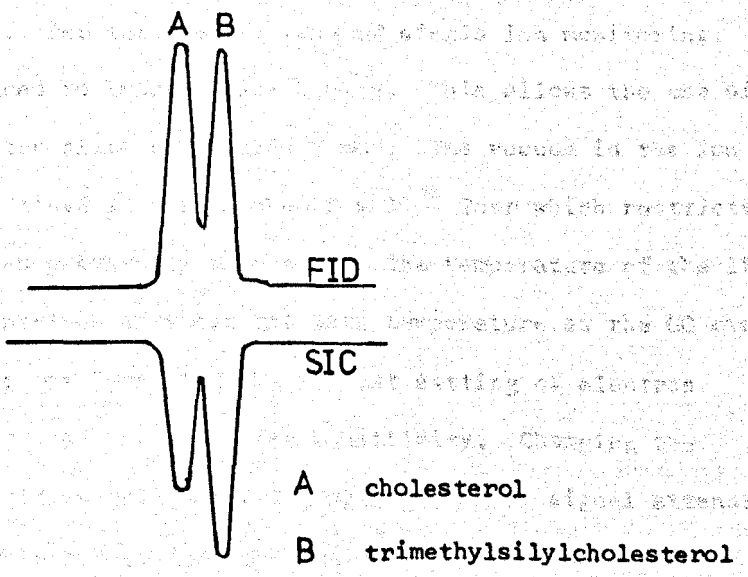


FIG 4.5

Carrier Gas Flow Rate: The helium flow rate affects the quality of the gas chromatographic separation, the efficiency of the separator and the performance of the mass spectrometer. The conductance of the ion source of the mass spectrometer will accept flow rates of the order 0.2 - 0.3 ml. min^{-1}. It is this flow restriction which is the principal limiting criteria,

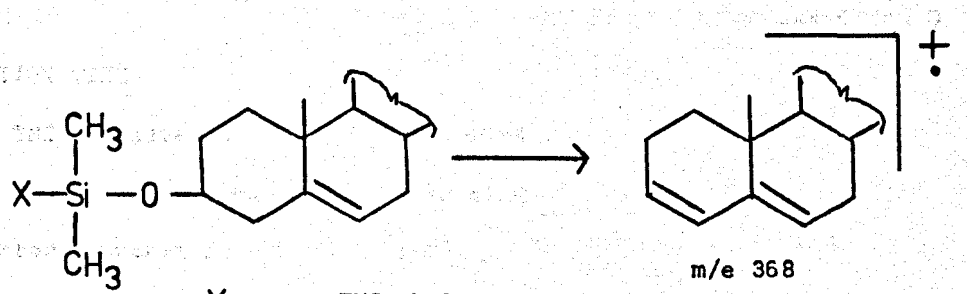
although separator performance is important. At low flow rates peak shape and spectrometer response are poor. Increasing the flow rate improves peak shape and response until a maximum is reached. This flow rate, approximately 11 ml min^{-1} going to the separator, is very close to the upper limit at which the mass spectrometer will operate. For the gas chromatography of steroids the maximum possible flow rate is required to maintain good peak shape at moderate temperatures.

OPTIMIZATION OF THE MASS SPECTROMETER.

The Hitachi Perkin-Elmer RMU-6E is a single focusing 20 cm magnetic sector mass spectrometer with a 90° deflection angle. Rapid scanning and high sensitivity is achieved by a ten stage electron multiplier detector. Resolution as high as M/ΔM (50% valley) 2,500 is obtainable under the most favourable conditions. For the special case of single ion monitoring, resolution is sacrificed to improve sensitivity. This allows the use of wide source and detector slits (typically 2 mm). The vacuum in the ion source has to be maintained at better than 5×10^{-6} Torr which restricts the helium flow rate as previously discussed. The temperature of the lines and ion source is maintained at about the same temperature as the GC oven. Maximum sensitivity is obtained with the highest setting of electron multiplier voltage and head pre-amplifier sensitivity. Changing the multiplier voltage in steps provides a convenient form of signal attenuation. The output from the detector is displayed on a 1mV chart recorder for convenience and permanence. The amplified signal from the ion detector when fed directly to the pen recorder gave an unstable base line due to excessive short term high frequency noise. This was removed with a 2,200 μfd capacitance. Lower rated capacitors filtered too little noise whereas larger capacitors caused severe damping of the recorder pen giving a sluggish response and asymmetric peaks.

ANALYSIS OF TRIMETHYLSILYLCHOLESTEROL BY SINGLE ION MONITORING.

The ion m/e 368, due to the formation of the ionized diene from the molecular ion in trimethylsilylcholesterol (TMS cholesterol) was selected for analysis and chloromethyldimethylsilylcholesterol (CMDMS cholesterol) was used as an internal standard. An evaluation of several columns showed that OV-101 gave the best separation of sample and standard with an acceptable, although not insignificant, background contribution (see Fig. 4.2).



X = CH_3 TMS cholesterol
X = $CHCl_2$ CMDMS cholesterol

The experimental conditions are given in Table 4.1. A minimum detection limit of 10ng. was established and the response for TMS cholesterol was linear over the investigated range 10⟶100ng. (Fig 4.6).

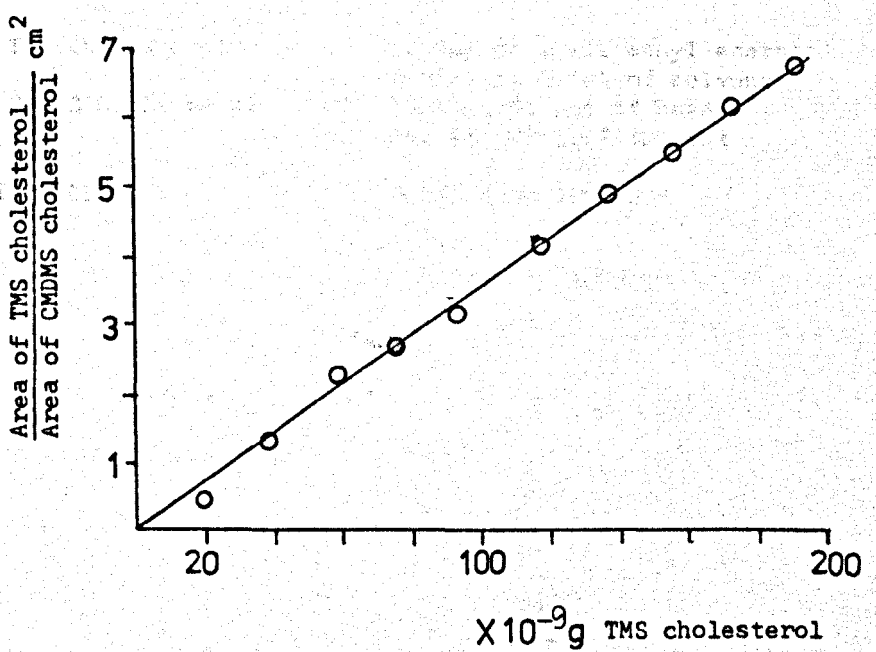


Table 4.1

OPTIMUM EXPERIMENTAL CONDITIONS FOR SIM

PARAMETER	VALUE
SELECTED ION	m/e 368
FLOW RATE	11 ml. min^{-1}
COLUMN TEMPERATURE	260°C
COLUMN	45 x 0.32cm, 1% OV-101 on Gas Chrom Q
RETENTION TIME	
(i) TMS Cholesterol	1.26 mins
(ii) CMDMS Cholesterol	2.83 mins
Separator temperature	310°C
Ionization Energy	70 eV
SLIT WIDTHS	
(i) Sample	2mm
(ii) Detector	2mm
ACCELERATING VOLTAGE	3.0 kV
SAMPLE DILUTIONS	
(i) TMS Cholesterol	1.03mg in 10ml. ethyl acetate 0-95µl in 100µl of solvent
(ii) CMDMS Cholesterol	10.05mg in 2ml of hexane 5µl in 100µl of solvent
INJECTED VOLUME	0.2µl from 100µl.

A more favourable detection limit would be expected for a compound in which a greater percentage of the ion current was carried by the selected ion and a lower background contribution from the column obtained.

ANALYSIS OF ECDYSTERONE BY SINGLE ION MONITORING.

The determination of ecdysones by multiple ion detection of their trimethylsilyl derivatives has been described by Miyazaki et al.[10]. Quantitative estimation of the hormones at the 10^{-10}g level was possible by monitoring the very prominent ions at m/e 561 for an ecdysone with a C-20 trimethylsilyl group or m/e 564 for ecdysone. The optimum conditions established, the ion m/e 561 was monitored for the hexakis TMS ecdysterone using pentakis TMS cyasterone as an internal standard. The successful separation of the two compounds in a reasonable time by GC on a 45 x 0.32cm column of 1% OV-101 required temperatures in excess of 330°C. At lower temperatures retention times were long and peak shape was poor. This was particularly true of cyasterone, which with its lactone group, is noticeably less volatile than the other ecdysones. The high column temperature produced excessive column bleed in the ion region under study which had a marked effect on the sensitivity of the analysis. Quantities of the order of micrograms were required to give a minimum response at 70 eVs ionizing energy. Electrons of lower ionizing energy gave a marked increase in sensitivity, largely due to a reduction in the background contribution. Several columns were tried but a high background in the analytical region was always obtained. It was found that an increase in the overall sensitivity could only be achieved by an increase in instrument sensitivity and a decrease in the extraneous background contribution. This will be dealt with in the next section.

SUMMARY

The reported study illustrates that the Hitachi Perkin-Elmer RMU-6E is not ideally suited for use as a sensitive gas chromatographic detector of high molecular weight, ecdysones. A further disadvantage with the instrument is that facilities for multiple ion detection are not available.

The mass spectral properties of the ecdysones are well suited to the technique of single or multiple ion monitoring. The low volatility of their TMS derivatives necessitates a reasonably high column flow rate so that a compromise temperature can be chosen at which the column background becomes negligible. This is achieved by the use of a differentially pumped mass spectrometer. For a single pumped instrument, like the RMU-6E, one pump provides the vacuum for the entire mass spectrometer housing. In differentially pumped instruments, the source housing is pumped separately from the analyzer unit, the two being connected by a small hole of sufficient diameter to allow passage of the ion beam. This simple expedient allows an increase in flow rate into the mass spectrometer as well as increasing the overall sensitivity. Flow rates at the entry to the mass spectrometer of approximately 2 ml. min^{-1} can be tolerated so that after separator enrichment, column flow rates of 40-80 ml. min^{-1} become possible. Such an instrument would enable column temperatures of 250-270^{o}C to be used for ecdysones. At such temperatures the background contribution to the fragment ions selected for analysis is negligible.

The RMU-6E is fitted with a ten stage electron multiplier amplifier. A further increase in minimum detection limits could be expected with a more sensitive amplifier. Modern commercial instruments are now fitted with 16-20 stage amplifiers capable of providing higher gain characteristics. This is further improved by operating at a higher multiplier voltage than

is provided by the supply to the present electron multiplier amplifier. An increase in sensitivity of a factor of ten would seem in keeping with the above discussion on amplifiers.

A mass spectrometer combining the features of a high sensitivity electron multiplier and a differentially pumped source and analyser units would make a sensitive and quantitative gas chromatographic detector for ecdysones. When multiple ion detection is employed a high degree of specificity is built into compound identification at levels close to the detection limit of the substance under study.

References

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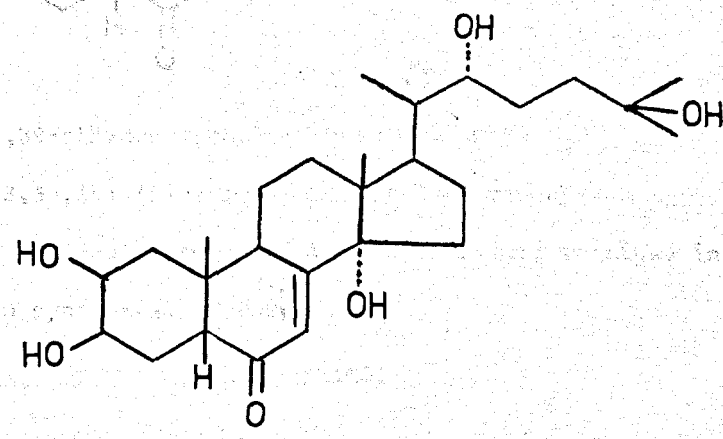
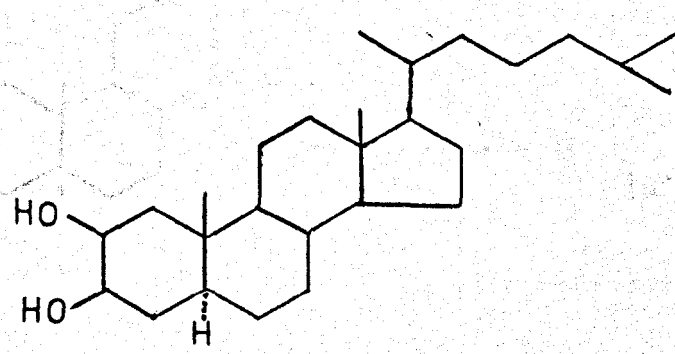
CHAPTER 5

THE SYNTHESIS OF MODEL STEROIDS

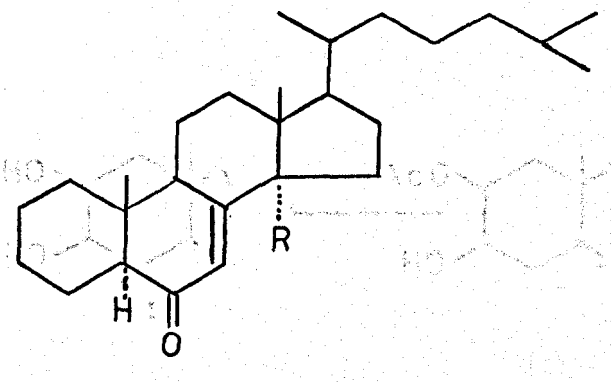
Model steroids, containing some of the features of the ecdysone molecule, were required in the development of analytical techniques and for spectroscopic studies. This was due to the limited availability of ecdysones which could only be obtained commercially in expensive mg. quantities. Also, in working with such complex molecules, much useful information can be found by looking at models embodying a portion of its functional groups, to gain a better understanding of their relationship to the whole. Those features which were of particular interest concern the tetracyclic nucleus and the five ecdysone analogues (I)→(V) were synthesized.



ECDYSONE

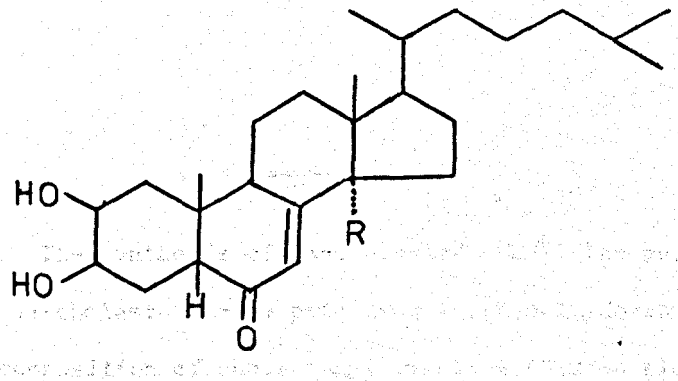


2β,3β-Dihydroxy-5α-cholestane(I)



R = H 5α-cholest-7-en-6-one(II)

R = OH 14α-hydroxy-5α-cholest-7-en-6-one(III)

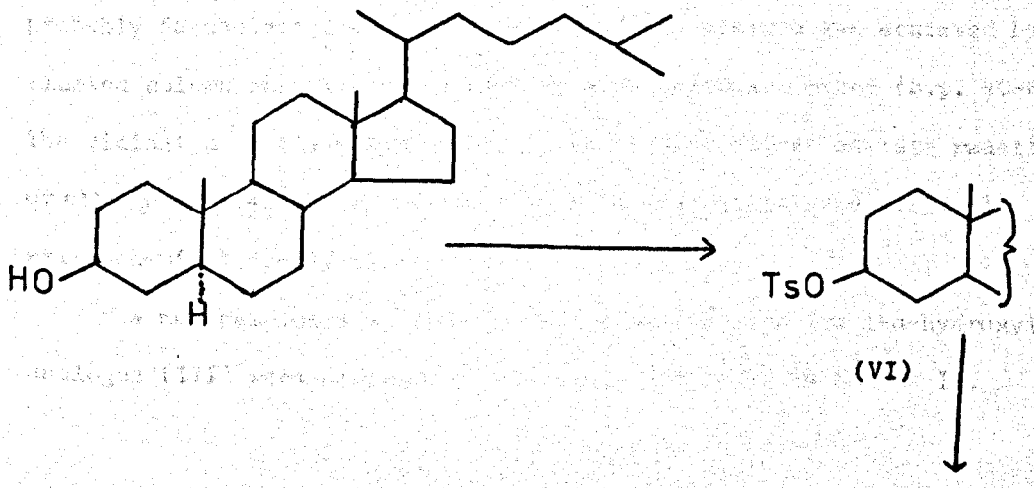


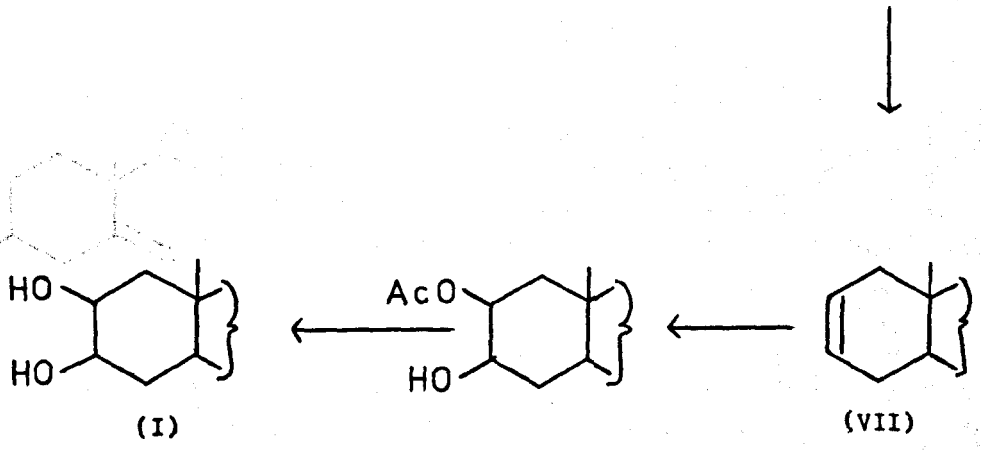
R = H 2β,3β-dihydroxy-cholest-7-en-6-one(IV)

R = OH 2β,3β,14α-trihydroxy-cholest-7-en-6-one(V)

The 2β,3β-dihydroxy-5α-cholestane(I) was obtained in good yield by a four step synthesis. Scheme I.

SCHEME I





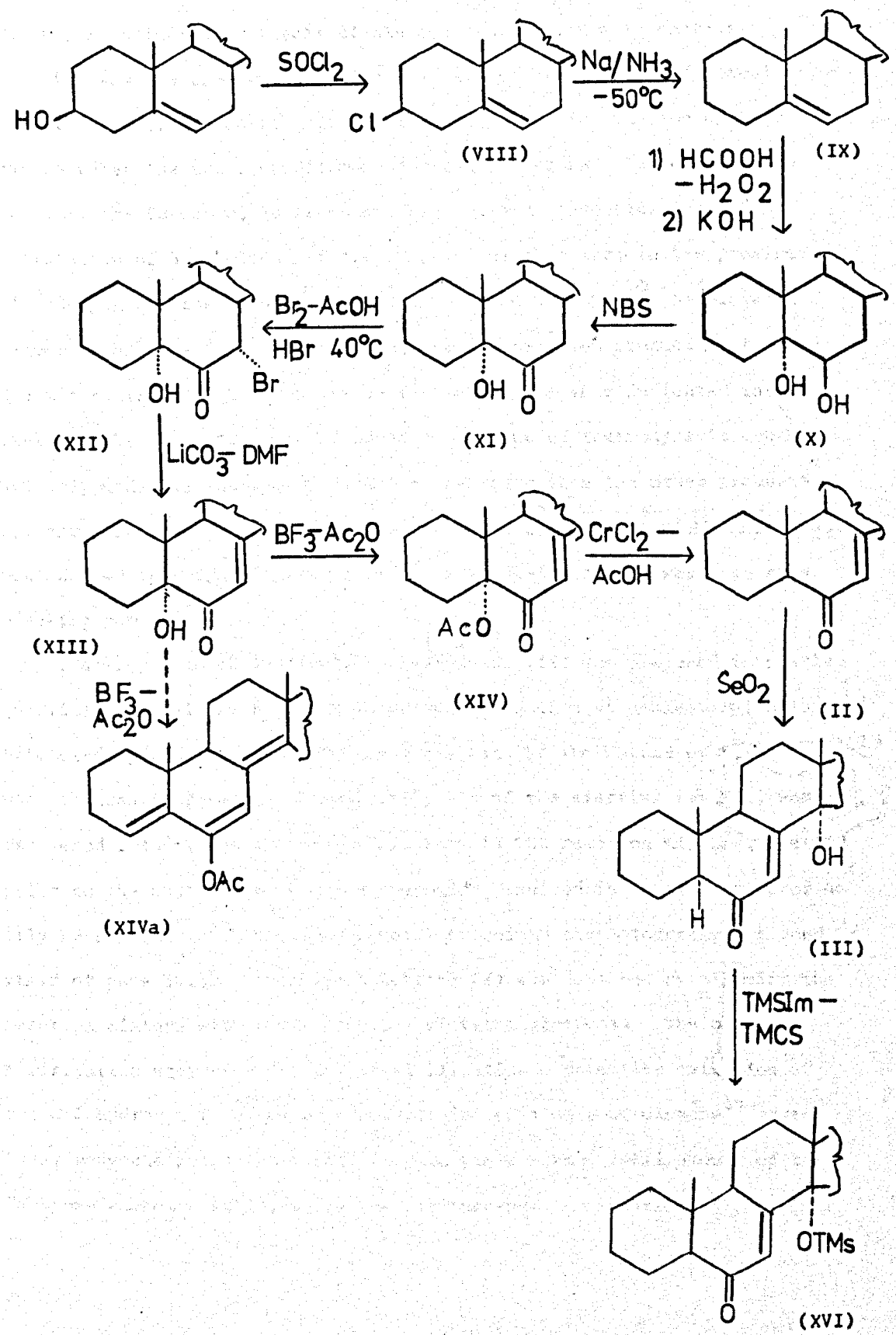
(I) (VII)

The synthesis of 5α-cholest-2-ene (VII) by sodium hydroxide treatment of 5α-cholestan-3-one p-toluene sulphonylhydrazone[1] and also by the decomposition of cholestanyl tosylate (VI) on alumina[2] has been reported.

The route adopted here, made use of the lithium bromide and lithium carbonate detosylation, a method favoured in the synthesis of ecdysones and their analogues[3-5]. The reaction mixture on silica gel TLC eluting with toluene cyclohexane (1:1) indicated two components; one at R_f=0.62, identified as (VII), and a trace of a second component R_f=0.55 which was probably 5α-cholest-3-ene. Separation of the mixture was achieved by alumina column chromatography eluting with petroleum ether (b.p. 40-60°C). The vicinal diol (I) was prepared by the iodine-silver acetate reaction of Ellington et.al.[6] which yields the 2β,3β product in good yield with stereochemical specificity.

The new compounds 5α-cholest-7-en-6-one (II) and its 14α-hydroxyl analogue (III) were prepared by the route indicated in Scheme II.

SCHEME II


HO
SOCl2
Cl
(VIII)
Na/NH3
-50°C
1) HCOOH
-H2O2
2) KOH
(IX)
(X)
NBS
(XI)
Br2-AcOH
HBr 40°C
OH
Br
O
(XII)
LiCO3 DMF
BF3-Ac2O
(XIII)
AcO
(XIV)
CrCl2 —
AcOH
SeO2
(II)
H
(III)
TMSIm —
TMCS
OTMs
(XVI)
BF3 —
Ac2O
OAc
(XIVa)

The key step in the synthesis of steroids containing a -7-en-6-one function was the bromination of the C-6 ketone which by dehydrobromination gave the required product. It was found that the bromination of 5α-cholestan--6-one under the usual conditions produced a mixture of products which included the 5α-bromo, 7α-bromo and the 5α,7α-dibromo steroids.[7-9] Bromination of 5α-cholestan-6-one under kinetic control in the presence of hydrogen bromide catalyst gave the 5α-bromo steroid.[10] At elevated temperatures, the 7α-bromo compound, was the expected product by thermodynamic control.[11] The results of several experiments indicated that the yield of the 7α-bromo compound under conditions of thermodynamic control was only moderate and was difficult to separate from the other products by column chromatography. For this reason, the procedure of Siddall et al.[5] was adopted in which a hydroxyl group in the C-5 position was used as a blocking group.

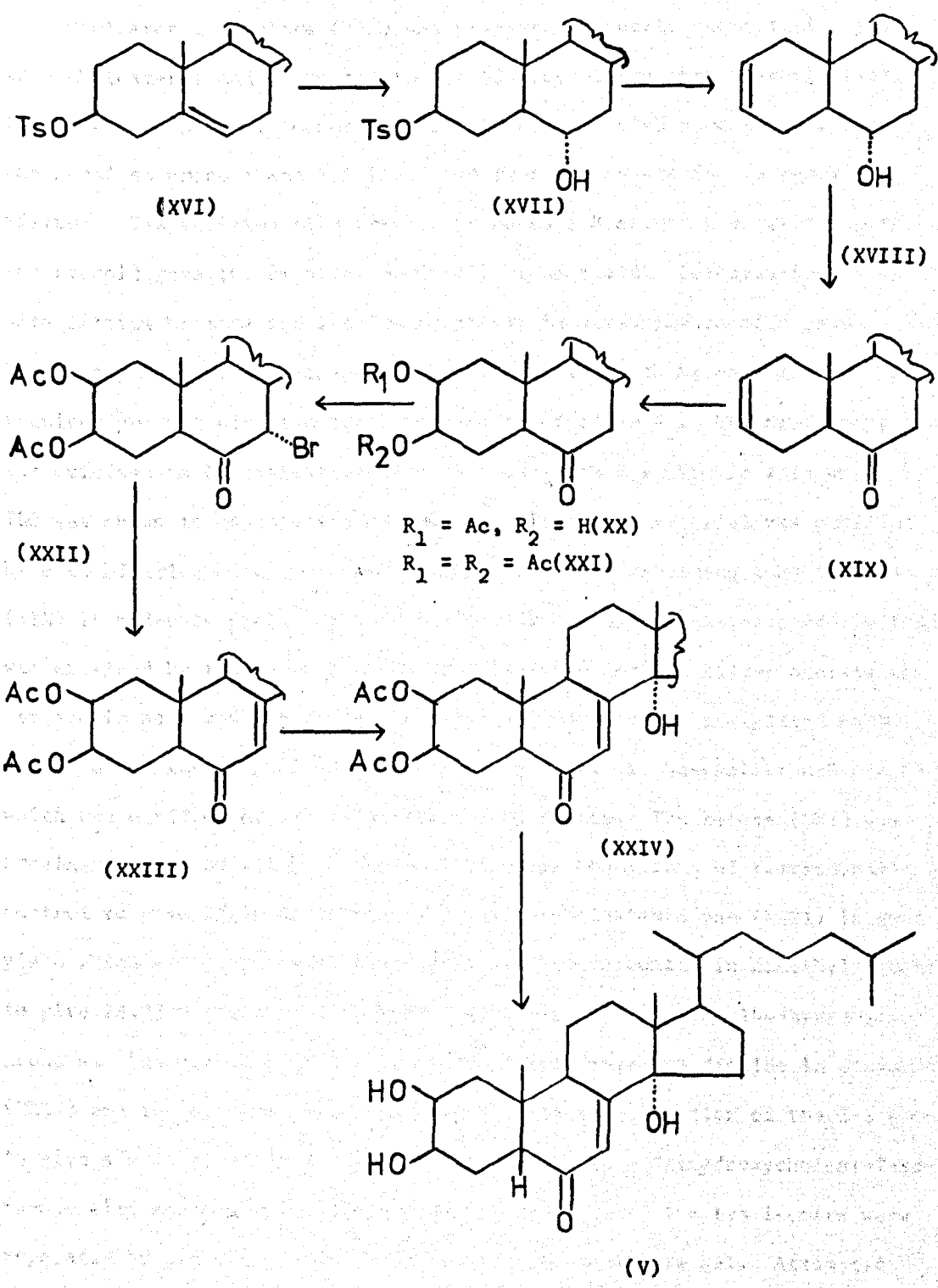
Starting from cholesterol, cholest-5-ene (IX) was prepared in nearly quantitative yield by a sodium in ammonia reduction of cholesteryl chloride.[12] Attempted hydroxylation of cholest-5-ene (IX) by the method of Reich et al.[13] was non-quantitative and approximately 40% of the starting material was recovered intact. To promote homogeneity in the reaction mixture, heating prior to the addition of hydrogen peroxide resulted in the formation of an oily by-product which required removal by column chromatography. A good yield of pure 5α,6β-dihydroxy-cholestane (X) was obtained by diluting the reaction mixture with a large volume of tetrahydrofuran. The only purification step required was crystallization. Selective oxidation of the C-6 hydroxyl group by N-bromosuccinimide in aqueous dioxane[14] gave 5α-hydroxy-cholestan-6-one (XI) in good yield with a small amount of the 7α-bromo compound (XII), which was not removed. Bromination of (XI) in

acetic acid with hydrogen bromide catalyst gave the 7α-bromo compound (XII), which was dehydrobrominated with lithium carbonate in dimethyl formamide to give 5α-hydroxy-cholest-7-en-6-one (XIII) in good yield after alumina column chromatography.[3-5,15] Acetylation of the 5α-hydroxyl group and removal of the acetate by chromous chloride in an inert atmosphere gave 5α-cholest-7-en-6-one. The hindered 5α-hydroxyl group could not be acetylated under mild conditions and acid catalysis was required. Boron trifluoride etherate in catalytic quantities gave a reasonable yield of 5α-acetoxylcholest-7-en-6-one (XIV) and an appreciable amount of 6-acetoxy--cholest-4,6,8(14)-triene which were easily separated by column chromatography on Davison silica gel. Removal of the tertiary acetate group with chromous chloride was virtually quantitative and the product 5α-cholest-7-en-6-one purified by recrystallization.[16] The 14α-hydroxyl group was selectively introduced by selenium dioxide oxidation in dioxane to give 14α-hydroxy-5α-cholest-7-en-6-one (III). The 14α-Tms ether was prepared by heating (II) in trimethylsilylimidazole containing 1% trimethylchlorosilane for 4 h @ 100°C.

All the reaction steps were conveniently monitored by a combination of gas chromatography and thin layer chromatography (TLC). A solvent system of 10% ethyl acetate in toluene was found to give the best resolution of components on silica gel. Compounds (XII) and XIII) are unstable to gas chromatography using the general conditions of a 3ft column of 1% OV-101, nitrogen flow rate of 80 ml. min^{-1} and column oven temperature 220-270°C. It has been observed that the 5α-hydroxy-7-en-6-one combination in a series of compounds was unstable to GC unless the C-5 hydroxyl group was protected either as the acetate or the trimethylsilyl ether.

Starting from cholesterol, 2β,3β,14α-trihydroxycholest-7-en-6-one (V) was synthesised by the method of Thompson *et al.*[3] as indicated in Scheme III.

SCHEME III


TsO
OH
(XVI)
(XVII)
(XVIII)
AcO
Br
O
R_1O
R_2O
R_1 = Ac, R_2 = H(XX)
R_1 = R_2 = Ac(XXI)
(XIX)
(XXII)
(XXIII)
(XXIV)
HO
H
(V)

Cholesteryl tosylate (XVI) was prepared in nearly quantitative yield from cholesterol and converted to the 3β-tosyl-6α-hydroxy-5α-cholestane (XVII) with diborane. Generation of diborane in situ gave a low yield of the required product and TLC indicated four components in the reaction mixture. The addition of a freshly prepared 1 M solution of diborane to the steroid gave the required product in good yield. Detosylation of (XVII) with lithium bromide and lithium carbonate in dimethylformamide gave 6α-hydroxy-5α-cholest-2-ene which by TLC was shown to be essentially the required product with two minor components of lower R_f. The crude material was oxidised to 5α-cholest-2-en-6-one (XIX) with 8 N chromic acid which by TLC was shown to contain six components. The crude material was purified by a combination of alumina and silica gel column chromatography to give (XIX) in moderate yield. The 2β-acetoxy-3β-hydroxy-5α-cholestan-6-one (XX) was obtained by the hydroxylation of (XIX) with iodine, silver acetate and wet acetic acid and the crude semi-crystalline material acetylated with acetic anhydride in pyridine to give 2β,3β-diacetoxy-5α-cholestan-6-one (XXI) which was purified by crystallization from hexane. The ketone (XXI) was brominated in a solution of acetic acid under conditions of thermodynamic control to give 2β,3β-diacetoxy-7α-bromo-5α-cholestan-6-one (XXII) in good yield which was dehydrobrominated with lithium carbonate in dimethylformamide to give 2β,3β-diacetoxy-5α-cholest-7-en-6-one (XXII). The 14α-hydroxyl group was introduced by allylic oxidation with selenium dioxide in dioxane (XXIV) and the acetate groups hydrolysed with equilibration of the C-5 proton to give a mixture of 5α and 5β epimers of 2β,3β,14α-trihydroxycholest-7-en--6-one with aqueous methanolic potassium carbonate. The two isomers were separated by preparative layer chromatography on silica gel. Attempted separation on a column of undeactivated Davison silica gel eluting with chloroform and chloroform-ethanol (1-10%) was unsuccessful. A sample of

2β,3β-dihydroxycholest-7-en-6-one (IV) was obtained by hydrolysis of (XXIII) with aqueous methanolic potassium carbonate and separation of the 5α and 5β epimers by preparative layer chromatography.

EXPERIMENTAL

Solvents were dried and purified by standard procedures. Glacial acetic acid was heated just below its boiling-point with a small amount of acetic anhydride and chromic oxide (2.0g/100 ml.) before fractional distillation. To prepare silver acetate, glacial acetic acid (8 ml.) was neutralized with 35% aqueous ammonia (using phenolphthalein) and added to a chilled solution of silver nitrate (10 g.) in water (20 ml), the precipitate of silver acetate was filtered off, washed rapidly with water followed by acetone and stored overnight in a darkened evacuated desiccator over phosphorus pentoxide.[6] The acetate was used within 36 h. of preparation. An 8N solution of chromic acid was prepared by dissolving chromium trioxide (26.72 g) in a mixture of concentrated sulphuric acid (23 ml) and enough water to make the total volume 100 ml.[18] Commercial selenium dioxide was purified by dissolution in concentrated nitric acid, followed by evaporation to dryness and sublimation of the residue in an atmosphere of oxygen. Lithium bromide and lithium carbonate were dried *in vacuo* at 50°C over phosphorus pentoxide before use. A solution of 500 ml of 1 M diborane was prepared by the addition of sodium borohydride (38.3 g) in diglyme to boron trifluoride etherate (270 g) in diglyme with collection of the evolved diborane in chilled tetrahydrofuran.[19]

Cholestanyl Tosylate (VI)

To a stirred solution of 5α-cholestanol, (10g), in pyridine (75 ml) at 5-15°C was added dropwise a solution of p-toluene sulphonyl chloride,

(10 g) in pyridine (50 ml). The mixture was allowed to stand overnight, poured into ice and water, the solids filtered off, air dried and then dried *in vacuo* at 65°C over phosphorus pentoxide to give (VI) (13.5 g). A sample recrystallized from ethyl acetate-methanol had m.p. 135-136°C (Found: C, 75.6; H, 9.9. $C_{34}H_{54}O_3S$ requires C, 75.2; H, 10.0%). NMR (CH_3-aromatic S) 2.45 ppm.

Cholest-2-ene(VII)

To (VI) (13.0 g) in dimethylformamide (80 ml) was added lithium carbonate (7 g) and lithium bromide (7 g) and the mixture refluxed under nitrogen for 1h. The mixture was filtered while hot, the filtrate allowed to cool, poured into ice-water, the precipitate filtered off, dried in air and then *in vacuo* at 50°C. The residue was chromatographed on alumina (Grade II), 300 g, eluting with light petroleum (b.p. 40-60°C) and collecting 50 ml. fractions. Fractions 3-6 contained (VII) (7.2 g) and fractions 6-9, a mixture (3.5 g) of (VII) contaminated with a further component which was probably cholest-3-ene. Recrystallization of (VII) from ether-methanol gave m.p. 69.5-71.5°C (lit[6] 71-72°C)(Found: C, 87.4; H, 12.5. $C_{27}H_{46}$ requires C,87.6; H, 12.4%).

2β,3β-Dihydroxy-5α-cholestane (I).

To (VII) (5.0 g) and freshly prepared silver acetate (6.75 g) in acetic acid (250 ml) under nitrogen in a reaction vessel protected from light was added, in portions at room temperature, over a twenty minute period, iodine (5.0 g). After 30 min. water, (2.5 ml), was added and the mixture stirred for 15 h., the product was extracted with ether, washed with water, aqueous sodium bicarbonate, water and dried over magnesium sulphate. The residue was dissolved in anhydrous ether (200 ml) and

refluxed for 1 h under nitrogen with lithium aluminium hydride (1.25 g), the mixture filtered, the ether layer washed with 1.0 M aqueous sodium hydroxide, water, dried over magnesium sulphate and the residue recrystallized twice from methanol to give (I) (3.6 g, 35%). m.p. 175-177°C (lit[6] 177-179°C) (Found: C, 80.3; H, 12.1. $C_{27}H_{48}O_2$ requires C, 80.0; H, 11.9%). IR ν(O-H) 3460 cm^{-1}(broad). NMR O-H 3.7 ppm (broad).

Cholesteryl Chloride (VIII)

Powdered cholesterol (100 g) was added slowly to thionyl chloride (100 ml) at a temperature between 0-10°C with stirring. The mixture was allowed to reach room temperature and stirred for a further 20 h. Excess thionyl chloride was removed in vacuo and the residue recrystallized from acetone to give (VIII) (96.0 g) m.p. 96°C (lit[20] 95°C) (Found: C, 80.1; H, 10.9 $C_{27}H_{45}Cl$ requires C, 80.0; H, 11.0%.)

Cholest-5-ene (IX)

To cholesteryl chloride (96 g) in diethyl ether (2 l) and anhydrous liquid ammonia (250 ml) at -50°C was added in portions sodium (14.0 g) with stirring. After 4 h., the solution was allowed to reach room temperature, ammonium chloride (10 g) added, followed by methanol (50 ml) to destroy excess sodium. The mixture was acidified, poured into water and extracted with ether. Evaporation of the dried ether solution gave (IX), (87.0 g) which had, after recrystallization from chloroform-methanol, m.p. 92-93°C (lit[12] 91-93°C) (Found: C, 90.1; H, 9.9. $C_{27}H_{46}$ requires C, 90.0; H, 10.0%).

5α,6β-Dihydroxycholestane (X)

To (IX) (87.0 g) in tetrahydrofuran (1.5 l) was added 90% formic acid (850 ml) and 30% hydrogen peroxide (85 ml) at 0-10°C with stirring.

The solution was allowed to reach room temperature, stirred for a further 20 h., the volume reduced *in vacuo*, diluted with water and extracted with ether. The ether solution was washed with 2 M aqueous sodium hydroxide and water, dried and evaporated to a residue. The residue was refluxed for 10 min with 2.5% methanolic potassium hydroxide (225 ml), acidified, concentrated *in vacuo*, diluted with water, and extracted with ether, the ether extract was washed with sodium carbonate, water and dried over magnesium sulphate. The residue after removal of solvent, was recrystallized from acetone-methanol to give (X) (82.0 g) m.p. 124-125°C (lit[13] 123.5-126.5°C) (Found: C 79.8; H 12.1. $C_{27}H_{48}O_2$ requires C, 80.0; H, 11.9%).

5α-Hydroxycholestan-6-one (XI)

To (X) (82 g) in dioxane (800 ml) and water (8.5 ml) was added N-bromo-succinimide (44.2 g) and the solution stirred for 1.5 h in the dark at 25°C. Bromine was destroyed with aqueous sodium sulphite and the mixture diluted with ice and water. The solid was filtered and dried *in vacuo* at 50°C to give material (78.2 g) which on TLC on silica gel (10% ethyl acetate-Toluene) showed two spots. The major spot corresponding to V (R_f=0.50) and the second spot to a small amount of (XII) (R_f=0.77). For an analytical sample, recrystallized twice from methanol gave pure (XI), m.p.152-153°C (lit[13] 153--154°C) (Found: C, 80.3; H, 11.6. $C_{27}H_{46}O$ requires C, 80.5; H, 11.4%).

7α-bromo-5α-hydroxy-cholestan-6-one (XII)

To crude (XI) (78.2 g) in glacial acetic acid (750 ml) and 32% hydrogen bromide in acetic acid (9.2 ml) at 40°C was added dropwise with stirring bromine (20 g) in glacial acetic acid (250 ml) over a period of 1 h. The solution was stirred for a further 1 h., cooled and poured into ice and water. The solid was filtered off and dried *in vacuo* at 50°C to give a solid (58.3 g). TLC on silica gel (10% ethyl acetate - Toluene)

indicated the absence of starting material and four spots. The major component (R_f=0.77) corresponding to (XII). An analytical sample recrystallized twice from acetone gave pure (XII) m.p. 141-142°C. (Found: C, 67.8; H, 9.7. $C_{27}H_{45}BrO_2$ requires C, 67.5; H = 9.4%). IR ν(O-H), 3570 cm^{-1} ν(C=O), 1710 cm^{-1}. NMR (7-H), 4.2 ppm.

5α-Hydroxy-cholest-7-en-6-one (XIII).

To crude (XII), (58.3 g) in dimethylformamide (500 ml) was added lithium carbonate (58.3 g) and the mixture refluxed under nitrogen for 1 h. The solution was filtered while hot, cooled and poured into ice water. The solid was filtered off, air dried *in vacuo* at 50°C. The residue was chromatographed on alumina Grade IV (1.2 kg) eluting with a benzene-ethyl acetate gradient to give (XIII) (35.2 g) m.p. 112-114°C. (Found: C, 81.0; H, 11.2. $C_{27}H_{44}O_2$ requires C, 81.0; H, 11.0%). IR ν(O-H) 3590, 3480 cm^{-1} (broad) ν(C=O) 1675 cm^{-1}, ν(C=C) 1620 cm^{-1}. NMR (7-H) 5.4 ppm. UV λ_{max} 249nm. (methanol) $\log_{10} \varepsilon_{max}$ = 4.109.

5α-Acetoxycholest-7-en-6-one (XIV) and 6-Acetoxycholest-4,6,8(14)-triene (XIVa)

To (XIII) (35.2 g) in acetic anhydride (200 ml) in a stoppered flask was added six drops of 48% boron trifluoride diethyletherate. The mixture was warmed gently to promote dissolution and allowed to stand at room temperature for 1 h., poured into ice-water, extracted with ether, the ether washed with sodium bicarbonate and water, dried and evaporated to give a solid residue, which on TLC on silica gel (toluene - 10% ethyl acetate) showed two components (XIV) R_f=0.34 and (XIVa) R_f=0.60. The material was chromatographed on Davison silica gel (1 kg. deactivated with 10% water). Eluting with toluene gave (XIVa) (14.8 g) and with

toluene - 50% ethyl acetate gave (XIV) (18.5 g). For 5α-Acetoxycholest--7-en-6-one (XIV) m.p. 110-110.5°C. (Found: C, 78.8; H, 10.7. $C_{29}H_{46}O_3$ requires C, 78.7; 10.4%), and had ν(acetate) 1745 cm^{-1}, ν(C=O) 1695 cm^{-1} and ν(C=C) 1625 cm^{-1}, NMR; acetate 1.88 ppm, (7-H) 5.40 ppm. UV ε_{max} 248 nm (methanol), $\log_{10} \varepsilon_{max}$=4.08. The 6-Acetoxycholest-4,6,8(14)-triene (XIVa) was obtained as an oil which could not be recrystallized. Distillation at 10^{-4} Torr gave a light red oil which darkened on standing. (Found; C, 81.0; H, 10.7. $C_{29}H_{44}O_2$ requires C, 81.7; H, 10.8%). Its mass spectrum at 80 eV, 150°C gave m/e (relative abundance) 424 m^+ (12%), 383 (49), 367 (96), 311 (7), 269 (42), 243 (57), 215 (41), 169 (40), 155 (49) and 132 (100); NMR (acetate) 2.09 ppm, (4-H) 5.50 ppm unresolved triplet and (7-H) 5.72 ppm singlet. IR ν(acetate) 1760 cm^{-1} and ν(C=C) 1520-1550 cm^{-1} (broad). UV λ_{max} 285 (ethanol).

5α-cholest-7-en-6-one (II).

To (XIV) (18.5 g) in glacial acetic acid (250 ml) was added 2 M aqueous chromous chloride* (170 ml) and the mixture shaken for 0.5 h under nitrogen. Solvents were degassed with nitrogen to remove all traces of oxygen. The mixture was poured into ice and water, the solid removed by filtration and recrystallized twice from methanol to give (II) (15.6 g) after drying in vacuo at 50°C, m.p. 117-118°C (Found: C, 84.4; H, 11.7 $C_{27}H_{44}O$ requires C, 84.4; H, 11.5%). IR ν(C=O) 1675 cm^{-1} and ν(C=C) 1625 cm^{-1}. NMR (7-H) 5.60 ppm. UV λ_{max} 235 (cyclohexane), $\log_{10} \varepsilon_{max}$ = 4.170 and λ_{max} = 246 (methanol) $\log_{10} \varepsilon_{max}$ = 4.203.

* Obtained from Lancaster Synthesis, St. Leonardgate, Lancaster, England.

14α-hydroxy-5α-cholest-7-en-6-one(III)

To (II) (7.0 g) in dry dioxane (200 ml) at 85°C was added in one portion selenium dioxide (7.0 g) under nitrogen. After 1 h., the mixture

was filtered hot, cooled and poured into ice - water, the solid was filtered off, recrystallized twice from aqueous methanol and dried in vacuo at 50° C to give (III) (4.2 g) m.p. 208-209°C (Found: C, 80.9: H, 11.2. $C_{27}H_{44}O_2$ requires C, 81.0; H, 11.0%) IR ν(O-H) 3580, 3460 cm^{-1} (broad ν(C=O) 1678, 1655 unresolved and ν(C=C) 1622 cm^{-1}. NMR (7-H) = 5.98 ppm and (O-H) = 2.70 ppm. UV λ_{max} = 242 (methanol) $\log_{10} \varepsilon_{max}$ = 4.134.

14α-Trimethylsiloxy-5α-cholest-7-en-6-one(XVI)

A sample of (III) (1.0 g) in trimethylsilylimidazole (4.0 ml) containing 1% of trimethylchlorosilane was heated at 100°C for 4h., cooled, poured into water, extracted immediately with toluene and dried with molecular sieves (Linde 4A). Removal of toluene in vacuo and recrystallization of the residue from aqueous acetone gave on drying at 50°C (XVI) (0.9 g) m.p. 89-90°C (Found: C, 75.9; H, 11.3. $C_{30}H_{52}O_2Si$ requires C, 76.3; H, 11.0%). IR ν(C=O) 1678 cm^{-1} and ν(C=C) = 1622 cm^{-1}. UV. λ_{max} = 242 (methanol), $\log_{10} \varepsilon_{max}$ = 3.996.

Cholesteryl Tosylate (XVI)

To cholesterol (100 g) in pyridine (300 ml) was added dropwise with stirring a chilled solution of p-toluene sulphonyl chloride (100 g) in pyridine (300 ml) under nitrogen and the temperature controlled between 5 and 15°C. The amber solution was stored over-night at 0-5°C, poured into ice-water and allowed to stand for 3h. with occasional stirring. The precipitate was filtered off, air dried and dried in vacuo at 65°C over phosphorus pentoxide to give (XVI) (100 g) m.p. 133-134°C (lit[3] 133-135°C), on TLC silica gel, eluting with 10% ethyl acetate-toluene it had R_f=0.6.

6α-Hydroxy-3β-Tosyl-5α-cholestane (XVII)

To (XVI) (100 g) in tetrahydrofuran (600 ml) at 5°C stirred under

nitrogen was added 1.0 M diborane in tetrahydrofuran (185 ml) over a 10 to 15 min period. The solution was kept at 5°C for 30 min, allowed to reach room temperature for 2.5 h, cooled again to 5°C and cold water (15 ml) added cautiously. When the effervescence ceased, 5% aqueous sodium bicarbonate (165 ml) and 30% hydrogen peroxide (70 ml) were added and after standing for 45 min. the mixture was poured into ice - water, the precipitate collected, air-dried and dried *in vacuo* at 65°C over phosphorus pentoxide to give (XVII) (99 g) m.p. 131-133°C (lit[3] 132-134°C). TLC on silica gel eluting with 10% ethyl acetate-toluene gave R_f=0.30.

6α-Hydroxy-5α-cholest-2-ene(XVIII)

To (XVII) (99 g) in dimethylformamide (700 ml) was added in one portion lithium bromide (60 g) and lithium carbonate (60 g) and the mixture refluxed under nitrogen for 1h. The solution was filtered while hot, cooled and poured into ice-water, the precipitate collected, air-dried and dried *in vacuo* at 50°C over phosphorus pentoxide to give crude (XVIII) (73.0 g).

5α-cholest-2-en-6-one(XIX)

To (XVIII) (73 g) in acetone (1 ℓ) at 20°C was added rapidly, dropwise a solution of 8N. chromic acid to a persistent orange-brown end-point. The mixture was poured into ice - water and the tacky precipitate collected. This material could not be induced to recrystallize and TLC indicated the presence of a minimum of six components. The crude material was column chromatographed on alumina (Grade 0, 1 Kg.), collecting 50 ml. fractions and monitoring by TLC and IR. Fractions 1-44 were eluted with petroleum ether (b.p. 40-60°C), fractions 45-50 with 10% toluene-pet.

ether, 51-57 with 50% toluene-pet. ether, 58-72 with toluene and 72-79 with 20% methanol-chloroform. Fractions 21-71 all showed absorption in the carbonyl region and were combined to give 62.0 g of crude oil which could not be induced to crystallize. The crude oil was re-chromatographed on a column of Biosil A (800 g), collecting 100 ml. fractions. Fractions 1-19 were eluted with 20% toluene-pet. ether, fractions 20-50 with 50% toluene-pet. ether, 50-55 with toluene, 55-61 with 20% chloroform-toluene, 62-70 with 50% chloroform-toluene and the column stripped with methanol. Fractions 28-58 were combined and recrystallized from aqueous acetone to give (XIX) (29.5 g) m.p. 109-110°C (lit[3] 109-110°C). IR ν(C=O) 1711 cm^{-1}.

2β-Acetoxy-3β-hydroxy-5α-cholestan-6-one(XX)

To (XIX) (29.5 g) and freshly prepared silver acetate (25.5 g) in acetic acid (1 ℓ) in the dark under nitrogen was added in portions iodine (19.7 g) over a period of 2h. After all the iodine had been consumed, water (3.0 ml) in acetic acid (25 ml) was added and the mixture stirred for 40 h. Silver chloride (5 g) was added with stirring over 0.5 h, the solid filtered off, washed with hot toluene and the combined filtrates evaporated under reduced pressure to leave a semi-crystalline solid (XX), (36.0 g). NMR CH_3 (acetate). 2.03 ppm, O-H 3.70 ppm (broad) and H-2 5.13 ppm.

2β,3β-Diacetoxy-5α-cholestan-6-one(XXI)

To (XX) (36.0 g) in pyridine (185 ml) at 10°C was added acetic anhydride (165 ml) over a period of 1h. and the mixture allowed to stand over-night. Solvent and reagents were removed *in vacuo* and the residue recrystallized from hexane go give (XXI) (22.3 g) m.p. 190-191°C (lit. 188-190°C). IR ν(C=O) acetate 1737, ν(C=O) 1711 cm^{-1}. NMR 2 x CH_3 (acetate) 2.00, 2.08 ppm, H-2 4.70 ppm and H-3 5.30 ppm.

2β,3β-Diacetoxy-7α-bromo-5α-cholestan-6-one(XXII)

To (XXI) (22.3 g)in acetic acid (400 ml), ether (125 ml) and 45% hydrogen bromide in acetic acid (1.75 ml) under nitrogen was added bromine (6.6 g) in acetic acid (100 ml.) over 1.25 h. During the addition the temperature was slowly raised from 40 to 55°C and then rapidly to 70°C and maintained there for 3h. Ether was removed *in vacuo*, the solution cooled and poured into ice-water, the precipitate collected, air dried and then dried *in vacuo* over phosphorus pentoxide at room temperature to give (XXII) (23.5 g) m.p. 119-120°C after recrystallizing twice from hexane (lit[3] 119-120°C). NMR 2 x CH_3 (acetate) 1.95, 2.05 ppm, H-2 5.30 ppm, H-3 4.35 ppm (broad) and H-7 4.19 ppm.

2β,3β-Diacetoxy-5α-cholest-7-en-6-one(XXIII)

To (XXII) (23.5 g) in dimethylformamide (230 ml.) was added lithium carbonate (23.5 g) and the mixture refluxed under nitrogen for 1.5 h., filtered while hot, cooled and poured into ice-water, the precipitate collected, air dried and dried *in vacuo* over phosphorus pentoxide. The residue was recrystallized from hexane-ether to give (XXIII) 5.8 g m.p. 219-222°C (lit[3] 213-215°C). IR ν(C=O) acetate 1745, ν(C=O) 1676 cm^{-1} and ν(C=C) 1620 cm^{-1}. NMR 2 x CH_3(acetate) 1.99 and 2.05 ppm, H-2 5.30 ppm, H-3 4.6-4.9 ppm (broad) and H-7 5.70 ppm. UV λ_{max} 245 (methanol) $\log_{10} \varepsilon_{max}$ = 4.079.

2β,3β-Diacetoxy-14α-hydroxy-5α-cholest-7-en-6-one(XXIV)

To (XXIII) (4.8 g) in dioxane (175 ml) at 80°C under nitrogen was added in one portion selenium dioxide (4.8 g). After 30 min, the solution was filtered while hot, cooled and poured into ice-water, the precipitate filtered off, air dried and then dried *in vacuo* over phosphorus pentoxide

the residue was recrystallized from acetone-hexane to give (XXIV) (3.6 g) m.p. 231-232°C (lit[3] 230-232°C). UV λ_{max} = 242 (methanol) $\log_{10} \varepsilon_{max}$ = 4.049.

2β,3β,14α-Trihydroxy-5β-cholest-7-en-6-one(V)

To (XXIV) 3.6 g in methanol 290 ml. at 50°C under nitrogen was added a solution of potassium carbonate (7.5 g) in methanol (50 ml.) and water (37 ml.). After stirring for 1h, the volume was reduced to approximately 1/3 in vacuo, diluted with water, the product filtered off and air-dried. TLC on silica gel, eluting with 10% ethanol - chloroform, indicated two components, R_f=0.22 (5α-epimer) and R_f=0.14 (5β-epimer). Separation of the epimers was achieved by preparative layer chromatography on ten plates eluting three times with 10% ethanol-chloroform. Elution of both bands with recrystallization to constant melting-point gave the 5α--epimer (1.26 g) from ethyl acetate m.p. 246 - 248°C (lit[3] 245-249°C) and the 5β-epimer (1.60 g) from methanol, m.p. 207 - 209°C (lit[3] 208-210°C). The purity of the epimers was checked by GC of their TMS ethers on a 3 ft. column of 1% OV-101, at 254°C, 60 ml. min^{-1} N_2, 5α-epimer R_f = 5.48 min, and 5β-epimer R_f 3.35 min.

2β,3β-Dihydroxy-5β-cholest-7-en-6-one(IV)

To (XXIII) (1.0 g) in methanol (130 ml) at 50°C under nitrogen was added a solution of potassium carbonate (0.45 g) in water (15 ml.) After 30 min. the volume was reduced in vacuo, diluted with water, the precipitate filtered off and the residue streaked onto four preparative layer plates eluting three times with 5% ethanol-chloroform. Elution and recrystallization to constant melting-point gave the 5α-epimer (0.4 g) m.p. 207-209°C (lit[3] 208-210°C) and the slower moving 5β-epimer (0.25 g) m.p. 201-204°C (lit[3] 203-205°C).

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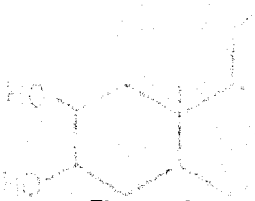
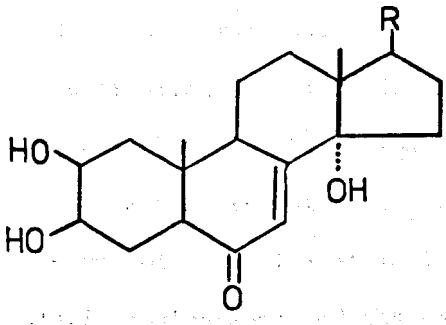
CHAPTER 6



THE ANALYSIS OF ECDYSONES

The ecdysones are a family of polyhydroxy steroids found in both the plant and animal kingdom. Over forty closely related ecdysones in plants and seven in insects have been identified.[1] Their function in the plant kingdom is obscure but in arthropods (insects and crustaceans) they are responsible for regulating the moulting process. The exoskeleton in arthropods restricts their growth, and so the hardened outer cuticle is shed at intervals in the process of ecdysis or moulting. The insect grows rapidly while the new cuticle is soft and elastic, but as soon as this is hardened, usually only a matter of a few hours, the growth is again restricted until the next moult. It is believed that a better understanding of the moulting process could lead to a method of effective control, the "third-generation" of pesticides.[2]

Ecdysones are not readily available for study. Difficulty in extraction from natural sources and lengthy synthetic procedures mean that the hormones can only be obtained in small (mg) quantities. Those available commercially for study are given below



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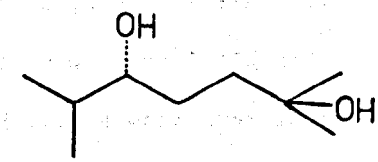
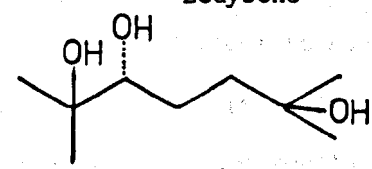
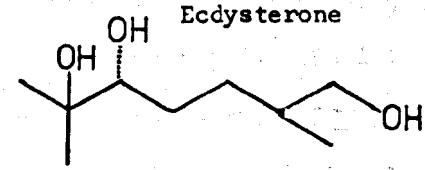


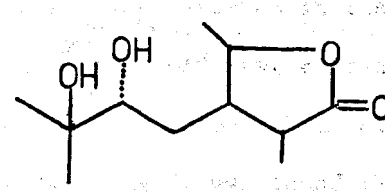
Ecdysone



Ecdysterone



Inokosterone



Cyasterone

A great deal of research has been conducted in devising techniques for the separation and identification of ecdysones at physiological levels in insects. At the onset of this project successful methods for bioassay existed, but these were limited in sensitivity to the 10^{-7} g level and lacked specificity in the analysis of hormone mixtures.[3] A fluorimetric method for ecdysones is known, but its application to crude insect material has not been illustrated and is in doubt.[4] The formation of TMS ethers of ecdysones and their analysis at the sub-microgram level had been indicated[5-7]. The TMS ethers of ecdysones were also known to undergo a non-quantitative exchange reaction with heptafluorobutyrylreagents to give a mixed derivative which could be detected at the picogram level with ECD.[7] The use of silica gel TLC for the separation and identification of

ecdysones on the basis of their R_f values in conjunction with uv visualization or colour forming reactions with vanillin-sulphuric acid was well known[1]. Detection is limited to the microgram level and complete separation of the structurally similar ecdysones is not always achieved. The use of partial acetylation under standard conditions to ascertain the steric environments of the different hydroxyl groups in conjunction with TLC is known.[8] More recently a densitometric procedure for the quantification of ecdysones on TLC plates has been described.[9] Radioimmunoassay techniques could be used to detect ecdysones down to the 0.08 ng. level, but these methods lack specificity and also require an animal colony for the supply of antigens as well as considerable experience in the application of the technique.[10-12] Recent analytical techniques have concentrated on gas chromatographic and high pressure liquid chromatographic methods. In the course of work on the detection of ecdysones it was found that the TMS ethers of ecdysones are sensitive to the electron capture detector, therefore a detailed consideration of electron capture detection and formation of TMS ethers of ecdysones will be given in this chapter.

THE ELECTRON CAPTURE DETECTOR.

The fundamental physical process underlying the operation of all ionization detectors is the conduction of electricity by gases. They owe their success to the fact that at normal temperatures and pressures a gas behaves as a perfect insulator. The introduction of charged molecules or free electrons renders the gas conducting in the direction of an applied electrical field. In the absence of conduction by the gas molecules themselves, the increased conductivity due to the presence of very few charged molecules can be observed. Increasing the strength of the applied electric field, increases the current towards a constant level, the saturation current, corresponding to the collection of all the ions generated.

The length of the saturation current plateau decreases with increasing ion density until at some finite density it vanishes. This upper limit usually determines the maximum current which can be drawn from an ionization detector consistent with a linear response to vapour concentration.[13]

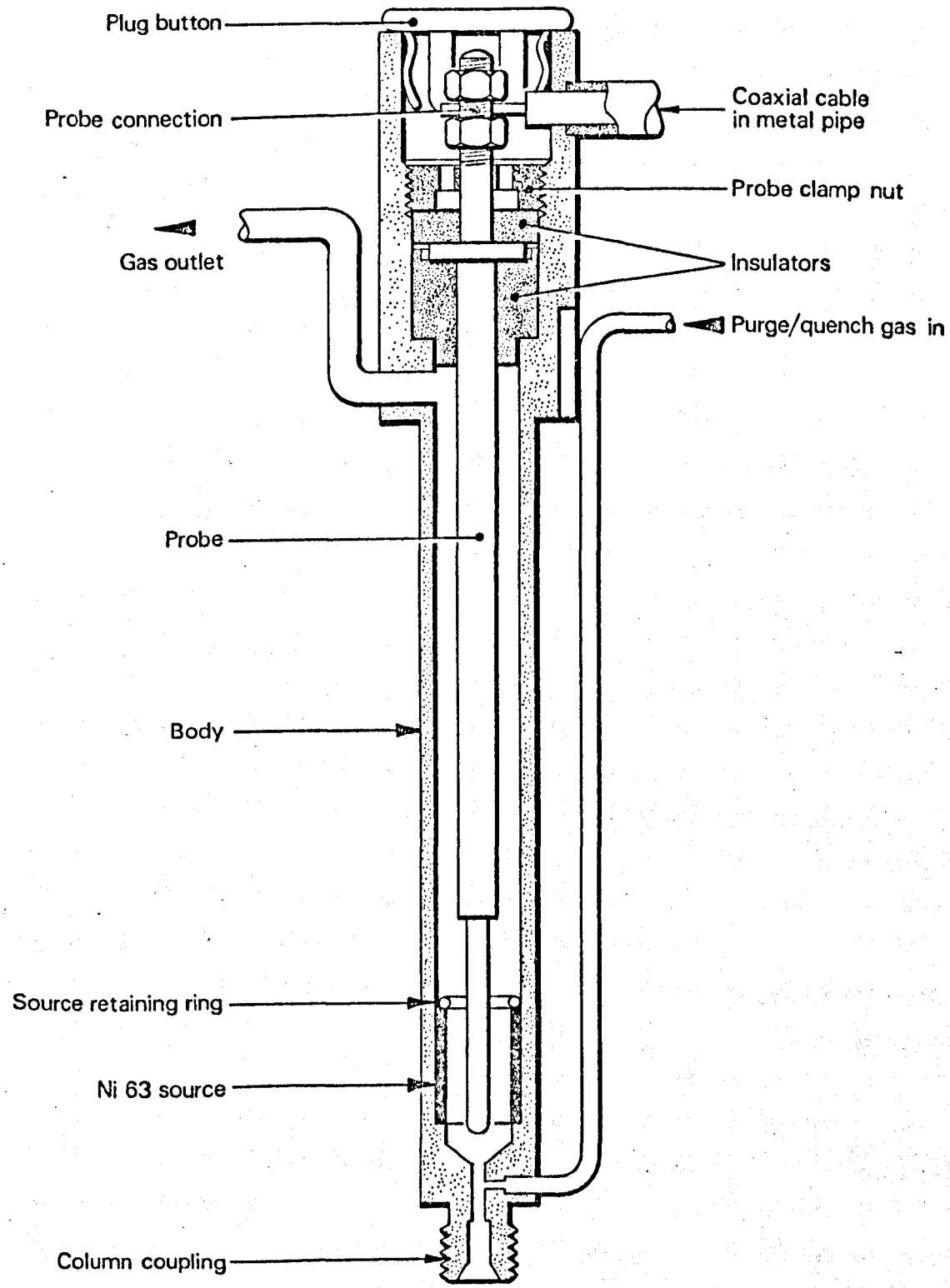
The electron capture detector (ECD) is one of the family of ionization detectors which are used for the estimation of organic vapours in the gaseous effluent from a gas chromatographic column. It differs from the familiar flame ionization detector, FID, in that it uses ionizing radiation (β-particles) to impart a charge to otherwise neutral gas molecules. It is the most sensitive of all gas chromatographic detectors but is limited to those compounds which readily capture thermal electrons.

The ECD has been produced commercially in two basic designs, the concentric tube and parallel plate models.[14-18] A Pye Series 104, model 84, ECD of the concentric tube type was used throughout this work.[19] The detector is shown in a cross section in (Fig. 6.1). A comparison of the various detector designs has been given in three recent reviews.[14-16]

When nitrogen is flowing through the detector a current is produced by secondary electron production resulting from inelastic collisions between β electrons (from Ni^{63} source) and nitrogen molecules. This leads to the production of a plasma within the detector which acts as a reservoir of thermal electrons.[17]

$$\beta^{\ominus} + N_2 \longrightarrow \begin{matrix} N_2^{\oplus} \\ \downarrow \\ N_4^{\oplus} \end{matrix} + e^{\ominus} + e^{*} + \text{energy}$$

The average energy of the primary electrons ($\beta^{\ominus}$) for Ni^{63} are about 67 KeV compared to thermalized plasma electrons 0.01 eV.[19] Under the influence of a potential difference a steady state current is recorded due to


Plug button
Probe connection
Coaxial cable in metal pipe
Probe clamp nut
Gas outlet
Insulators
Purge/quench gas in
Probe
Body
Source retaining ring
Ni 63 source
Column coupling

collection of plasma and primary electrons. The production of primary and thermal electrons is assumed to be constant with respect to time. When an organic vapour enters the detector it captures electrons to form a negative molecular ion or undergoes dissociation with the formation of a neutral radical and a negative ion. The net result is the removal of an electron from the system and the substitution of a negative ion of much greater mass. A rapid decrease in current then occurs due to the fact that the negative ions formed by electron capture readily recombine with the positive ions in the plasma. Recombination of ions occurs 10^5 to 10^8 times faster than the recombination of free electrons and positive ions.[15] Thus the presence of a vapour capable of capturing free electrons is readily observed in terms of this recombination and is quantified as a decrease in the steady state current.

To make measurements of the standing currents in the detector, the application of a potential difference is required. This is either applied continuously (dc) or as a pulse of defined dimensions. In the continuous mode, the dc potential employed for maximum sensitivity depends on the detector configuration, choice of carrier gas, the capturing species as well as detector contamination and is normally determined by trial and error under experimental conditions.[15] It is thus not reproducible between different instruments or the same instrument in different laboratories and must be determined independently for each analysis.[14] In this mode the amount of sample injected should not absorb more than 30% of the standing current to preserve linearity of response and to avoid loss of column resolution by the formation of asymmetric peaks.[16] Anomalous responses with this detection mode have been observed due to the formation of space charge clouds. The positive ion concentration is normally two or three orders of magnitude greater than the free electron concentration as the more mobile free electrons are collected rapidly at the anode compared to

the slow moving positive ions at the cathode. This cloud of positive charge creates a potential in opposition to the detector cell and lowers the concentration of electrons emitted at the cathode.[20] The constant direct potential across the cell also means that all the electrons are not in thermal equilibrium with the carrier gas and thus are less available for capture.

The advantages of a pulsed voltage has been illustrated by Lovelock.[20] During pulsing, the thermal electron concentration in the cell is not constant but varies in a saw-tooth fashion. During the application of a pulse, the electron concentration drops to zero due to the collection of all the electrons at the anode and then builds up after each pulse to a plateau due to $\beta^{\ominus}$ particle radiation of the gas. The magnitude of the electron concentration is dependent on the pulse interval and the detector sensitivity increases to a maximum as the pulse interval increases up to the limit where the time of the pulse becomes equivalent to the time of the natural recombination of electrons and positive ions.[26] When no potential is applied to the cell, the electrons are in thermal equilibrium and the drift of charged ions is negligible, so that space charge cloud effects are eliminated. For strongly electron absorbing compounds the pulse sampling technique may be three or four times more sensitive than the dc method.[20] The amplitude and pulse width should be of sufficient magnitude to afford complete collection of electrons[14]. For the Pye model 84 ECD, these are determined by the manufacturer and set at 47 V positive and 0.75μs respectively.

The response of the ECD is also dependent on the nature of the carrier gas and its flow rate. The noble gases and nitrogen are commonly used. Pure argon is unsuitable because it is readily converted to a metastable form which causes undesirable ionization of the sample molecule.[17] The

presence of methane prevents the formation of metastable argon. The largest response of the ECD must correspond to an optimum flow rate in the column, since the response of the detector depends on concentration and not mass flow rate.[21] Traces of air in the carrier gas have a deleterious effect on sensitivity and linearity as witnessed by an increase in the standing current and the base line drift. Plasma chromatography data, show that the effect is due to a temperature dependent depletion of reactive electrons by formation of $(H_2O)nO_2^{\ominus}$, $(H_2O)nH^{\oplus}$ and $(H_2O)nNO^{\oplus}$ complexes[22,23]. These complexes reduce the response of the detector to organic molecules by reducing the flux of available thermal electrons. The requirement for oxygen-free nitrogen is also well established, as oxygen under operational conditions readily forms negative ions.[19] Column bleed also has a deleterious effect on the operation of the ECD due to capture of thermal electrons, the formation of negative ion complexes which undergo ion-molecule reactions, and by condensation of material onto the radioactive foil reducing $\beta^{\ominus}$ particle emission.[21] The principal advantages of the Ni^{63} source are that being more thermally stable than the tritium source it can be used at higher temperatures to prevent or remove condensed material and the higher energy (67 KeV) of the $\beta^{\ominus}$ particles compared to tritium (18 KeV) means that the electron flux is less affected by small amounts of involatile material.[18] Practical solutions to the problem of overcoming detector contamination have been given by Gosseliu *et al.*[24]. As for contamination, similar arguments have been proposed to demonstrate the poor precision that can be expected if peak areas are measured when the peaks are superimposed on the solvent front or a rising base line.[25]

The temperature of the detector cell is also important. For reliable operation it needs to be controlled to ± 0.3 C.[27] This is usually achieved

by mounting the detector in a separately heated oven block of insulating material. The response of various compounds to the detector is also temperature dependent and variation of response with detector temperature is observed, depending on the mechanism of the electron capture process.[28] From the practical point of view, it is unwise to operate the detector at a temperature less than that of the column oven.

The mechanism of the electron capture process has been extensively studied by Wentworth and co-workers[26-29]. They have developed a steady state model for the detector operated in the pulse mode; embodied in which are the following assumptions,

a) the rate of production of thermal electrons is constant and not affected by the presence of the added capturing species.
b) the reaction zone is localized close to the radioactive foil
c) the reactive plasma is not neutral but rather has an excess of positive species
d) there is an excess of radicals in the plasma over charged species
e) the plasma is homogeneous
f) the reaction zone can be treated as a static system with respect to electron concentration since the flow out of the cell is much slower than the pulse times
g) the primary modes for the loss of electrons in the presence of a carrier gas alone are the recombination of electrons with positive species and the reaction of electrons with radicals
h) the amount of material which undergoes electron capture is small in comparison to the total amount of material present

The electron capture process is either non-dissociative, resulting in the formation of a negative molecular ion, or dissociative, in which bond breaking with the formation of a negative ion and a neutral radical occurs.

$$AB + e^{\ominus} \rightleftharpoons AB^{\ominus} \pm \text{energy} \qquad \text{non-dissociative}$$

$$AB + e^{\ominus} \longrightarrow A + B^{\ominus} \pm \text{energy} \qquad \text{dissociative}$$

In the non-dissociative mechanism the energy of electron attachment is thought to arise from the electron affinity of the molecule, and is subsequently liberated as radiation or translated to other molecules during collisions. In the dissociative capture reaction, the energy balance is maintained by a combination of:

a) the relative kinetic energy of the reactants before and after collision
b) the energy released by the formation of a negative ion from a constituent atom or radical of the compound
c) the energy required or released during the dissociation of the molecule

That both mechanisms of electron capture do exist has been established by measurement of ion mobility spectra of the effluent from an ECD,[22] and in the case of the dissociative mechanism, by identification of the products formed.[30]

The concentration of capturing species and changes in plasma current are related by the expression[27]

$$\frac{I_o - I_b}{I_o} = Ka$$

I_o = maximum of initial standing current before the addition of a capturing species

I_b = current remaining after the introduction of a capturing species

K = capture coefficient

a = concentration of capturing species

The capture coefficient K, can be obtained by integration with respect to

the volume of gas passing through the detector cell during the residence time of a peak if equipment is available for handling the response by analog techniques[29]. Wentworth and Chen have developed an empirical expression for K which can be evaluated directly from the chromatogram.[27]

$$K \equiv A. \frac{F}{S}. \frac{b_o}{b}$$

A = area in cm^2

F = flow rate $cm^3\ min^{-1}$

S = chart speed cm. min^{-1}

b_o = standing current in amps in the presence of pure carrier gas

b = standing current with the column at is operating temperature

On the basis of the kinetic model, if an ideal gas situation is assumed, then a relationship between the capture coefficient K and the absolute temperature T of the detector cell can be derived,[26]

$$\text{Ln}\ KT^{3/2} = \text{Ln}\ Z - \frac{\Delta E}{kT}$$

Z = pre-exponential factor

ΔE = activation energy

k = Boltzmann constant

The Arrhenius plot of Ln $KT^{3/2}$ vs 1/T allows the determination of activation energies, but from the practical point of view, the nature of the slope is more important, as it enables the optimum temperature of the detector for maximum sensitivity to be established. A linear plot with a positive slope is characteristic of a non-dissociative mechanism and linear with a negative slope of a dissociative process. For a non-dissociative process, maximum detector response is obtained at the lowest practical detector temperature. The corollary is also true, which explains the importance of obtaining a plot of this type before optimum analytical conditions can be

established. The molecular basis of the electron capture mechanisms in terms of potential energy diagrams has been described by Wentworth and Chen.[27]

An alternative model has recently been suggested by Lovelock.[31] The assumption made is that the gas and electrons are mixed uniformly within the detector and the reaction proceeds as in a stirred reactor. Unlike the Wentworth model, the formation of an excited negative ion is not important for compounds which capture electrons strongly as under these circumstances the equilibrium is heavily weighted in favour of the formation of negative ions. Moreover, the reactive plasma is not localized. This is more realistic for the high energy Ni^{63} source but may not be true for tritium. The new model leads to a series of differential equations which have been applied successfully to the prediction of response factors for a variety of compounds. Only further experimentation can test the success of the new model.

THE FORMATION OF TRIMETHYLSILYL ETHERS OF ECDYSONES.

The formation of trimethylsilyl, TMS, ethers of ecdysones have been reported using a variety of conditions. Katz and Lensky formed TMS ethers of ecdysone by heating the steroid at 80°C for one minute in a mixture of bis(trimethylsilyl)-acetamide, BSA, and pyridine.[32] A single peak was obtained on GC but no evidence was given for the number of hydroxyl groups reacted. Morgan and Woodbridge formed methoxime-TMS ethers using BSA at room temperature for 70 h.[6] The GC revealed two peaks due to partial separation of the syn-and anti-methoxime isomers. Mass spectrometry showed that under these conditions the C-14 hydroxyl group of ecdysone and the C-14 and C-20 hydroxyl groups of ecdysterone were not silylated.[33] Galbraith et al.[34,35] and Thompson et al.[36] have shown that the 14α-hydroxyl

group in some ecdysone analogues is only incompletely silylated by heating with BSA in dimethylformamide at 80°C for up to 18 h. Ikekawa et al.[7] claimed that all the hydroxyl groups in ecdysterone could be quantitatively silylated with trimethylsilylimidazole, TMSIm, at 100°C for one hour. The C-20 hydroxyl group was found to be the slowest to react and GC-MS was used to confirm the extent of silylation. Similar details are given in a further paper by Miyazaki et al[37]; Lafont et al.[38] have formed TMS ethers using this method. With TMSIm, King et al.[39] suggested that thirty minutes at 96°C was sufficient for complete reaction of ecdysterone, while Borst and O'Connor[11,40] used conditions of 100°C for fifteen minutes. None of these authors provide any evidence for the number of TMS groups introduced, although Borst and O'Connor[11] state that the mass spectra of the TMS ethers of the ecdysones investigated were in agreement with those published by Ikekawa et al.[7]

In an attempt to repeat the work of Ikekawa et al.[7], a sample of ecdysterone was heated at 100°C in TMSIm for 1 h. Analysis of the products by GC (3 ft. 1% OV-101 on Gas Chrom Q, 266°C, 60 ml. min^{-1}) gave two peaks of retention time 5.3 and 4.6 min. Increasing the reaction time gave mainly the peak of retention time 5.3 min. with a trace of a further component of shorter retention time, 4.1 min. This peak of retention time 4.1 min. increased in size on prolonged heating and if the temperature was increased to 140°C, then after 20 h. it was virtually the sole product. Repeating the reaction with 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one, ecdysone, inokosterone and cyasterone, in all cases produced a similar result in that heating at 100°C for 4 h. gave a single product which on heating at 140°C for 20 h. was converted to a peak of shorter retention time. Table 6.1.

TABLE 6.1

RETENTION TIME DATA FOR SOME TMS ETHERS OF ECDYSONES ON A COLUMN OF 3ft. 1% OV-101 ON CQ, 266°C AND 60 ml. min^{-1}.

ECDYSONE TMS ETHER	RETENTION TIME (MIN.)
tetrakis(TMS)ecdysone	3.9
pentakis(TMS)ecdysone	3.1
tetrakis(TMS)ecdysterone	4.6
pentakis(TMS)ecdysterone	5.3
hexakis(TMS)ecdysterone	4.1
pentakis(TMS)inokosterone	6.2
hexakis(TMS)inokosterone	4.5

It was thought that this new product could be an epimer of the fully silylated steroid or involve the formation of a TMS-enol ether. The UV spectra of the product obtained by heating 2β,3β,14α-trihydroxy-5β-cholest--7-en-6-one at 140°C overnight indicated that the conjugated ketone was intact and thus enol-TMS ether formation was not the cause of the product of shorter retention time. Similarly, it was found that the 5α and 5β epimers of 2β,3β,14α-trihydroxycholest-7-en-6-one when heated at 140°C, both produced a peak of shorter retention time Table 6.2. The new product was therefore not the result of a high temperature equilibration of the C-5 proton.

TABLE 6.2

RETENTION TIME DATA FOR SOME TRIHYDROXYCHOLEST-7-EN-6-ONE TMS ETHERS ON A 5 ft. COLUMN OF 1% OV-101 ON CQ, 271°C and 65 ml.min^{-1}.

STEROID	METHOD	RETENTION TIME
2β,3β,14α-trihydroxy-5α-cholest-7-en-6-one	A	8.15
- ditto -	B	4.73
2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one	A	4.63
- ditto -	B	3.48
2β,3α,14α-trihydroxy-5α-cholest-7-en-6-one	A	5.55
- ditto -	B	3.90
2β,3α,14α-trihydroxy-5β-cholest-7-en-6-one	A	4.60
- ditto -	B	3.40

Method A TMSIm room temp. 30 min.

Method B TMSIm, 140°C for 20 h.

Formation of the keto-methoxime derivative and heating at 140°C gave a peak of shorter retention time compared to the room temperature derivative. This result would tend to eliminate epimerization at a carbon centre involving the formation of an enol intermediate, as the methoxime derivative would not be expected to enolize. It was also noted that addition of TMCS to the TMSIm had a marked effect on the rate of formation of the peak of shorter retention time. In spite of the agreement of published results, it was concluded that the most likely explanation was that the product of shorter retention time was indeed the fully silylated derivative while those obtained under milder conditions were incompletely silylated compounds.

It was found that, 2β,3β-dihydroxy-5α-cholestane in TMSIm rapidly gave the completely silylated product at room temperature as witnessed by recording MS, IR, TLC and GC data. When 2β,3β,14α-trihydroxy-5β-cholest--7-en-6-one was heated at 100°C for 1 h., the product by mass spectrometry and infra-red analysis was shown to contain a free hydroxyl group (M^+, m/e 576, ν(OH) = 3460 cm^{-1}). An identical product was obtained when the steroid in TMSIm or BSA was allowed to stand at room temperature for thirty minutes. Morgan and Woodbridge have shown that under these conditions the 14α-hydroxyl group is slow to react in BSA. This was confirmed by the synthesis of 14α-hydroxy-5α-cholest-7-en-6-one and its reaction with TMSIm. Reaction of the 14α-hydroxyl group was slow at 100°C, and at 140°C, twelve hours were required for complete reaction (Fig. 6.2).

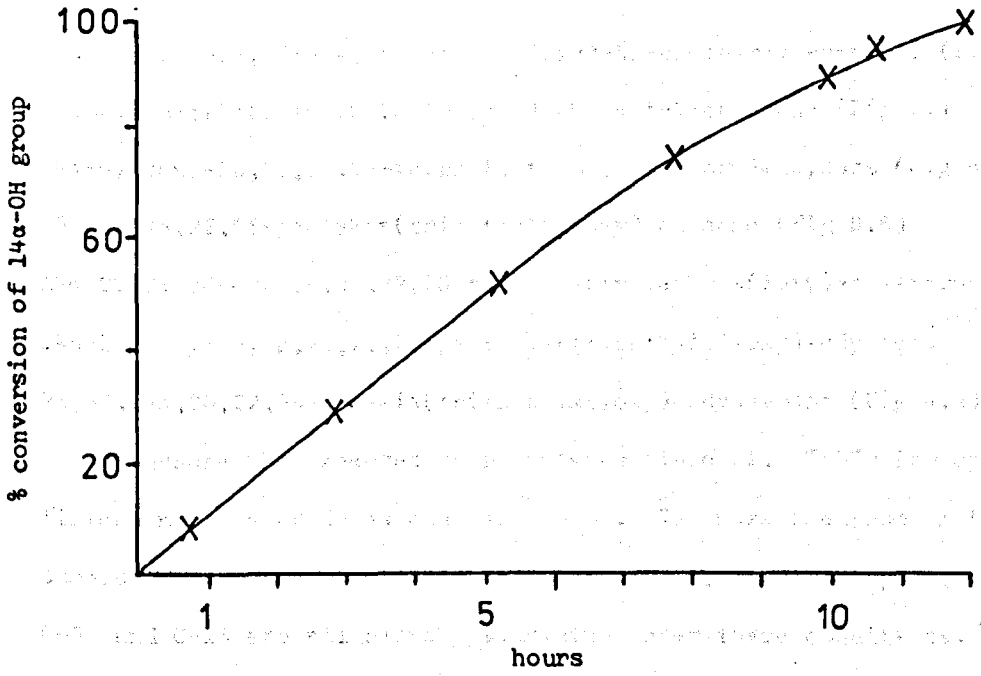


FIG. 6.2

With BSA at room temperature, only starting material was observed while heating at 100°C gave a slow conversion to the TMS ether which was not complete after 48 h. The TMS ether of 14α-hydroxy-5α-cholest-7-en-6-one has been prepared on the gram scale and fully characterized as indicated in Chapter 5 p.146 Maume, Wilson and Horning have shown that the 14β--hydroxyl group in cardiac aglycones (cardenolides) is resistant to silylation and the potent reagent combination of TMSIm:BSA:TMCS at 60°C for three days was required for complete reaction.[41]

The conditions suggested by Ikekawa et al.[7] are unlikely to be severe enough to silylate the 14α-hydroxyl group. Mass spectral data has been obtained to confirm the proposal and for comparison with published spectra.[7,37] Those spectra published to date are probably better interpreted as a mixture of complete and incompletely silylated products. The following mass spectra of the silyl ethers have been recorded via the solid inlet after purification by TLC;

14α-hydroxy-2β,3β-bis(trimethylsiloxy)-5β-cholest-7-en-6-one (Fig 6.3)

2β,3β,14α-tris(trimethylsiloxy)-5β-cholest-7-en-6-one (Fig 6.4)

14α-hydroxy-2β,3β,22,25-tetrakis(trimethylsiloxy)ecdysone (Fig 6.5)

2β,3β,14α,22,25-pentakis(trimethylsiloxy)ecdysone (Fig 6.6)

14α,20-dihydroxy-2β,3β,22,25-tetrakis(trimethylsiloxy)ecdysterone (Fig 6.7)

14α-hydroxy-2β,3β,20,22,25-pentakis(trimethylsiloxy)ecdysterone (Fig 6.8)

2β,3β,14α,20,22,25-hexakis(trimethylsiloxy)ecdysterone (Fig 6.9)

Ecdysone when reacted at room temperature with TMSIm for approximately fifteen minutes produces one peak on GC. The mass spectrum of this compound (Fig. 6.5) indicates that the four hydroxyl groups at C-2, C-3, C-22 and C-25 are all rapidly silylated under these conditions. The 14α-hydroxyl group is only silylated after heating at 140°C for 20 h.

Ecdysterone reacts with TMSIm when heated at 100°C for 1 h. to produce two peaks on GC (Fig. 6.10). The two components were separated by silica

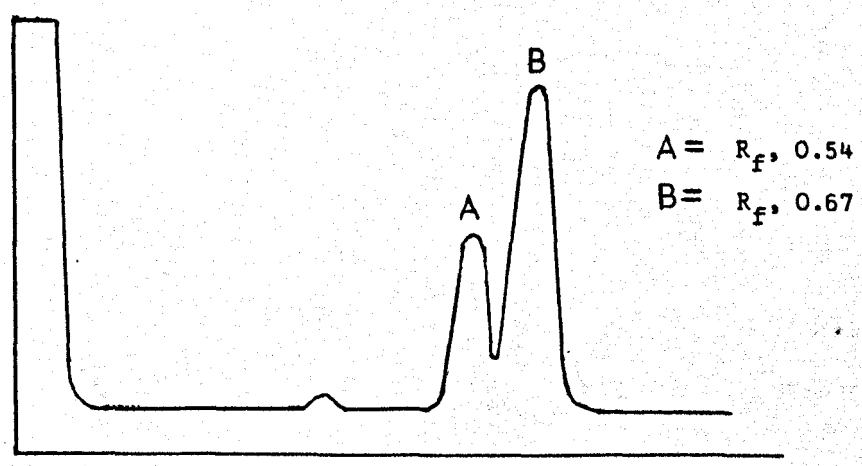
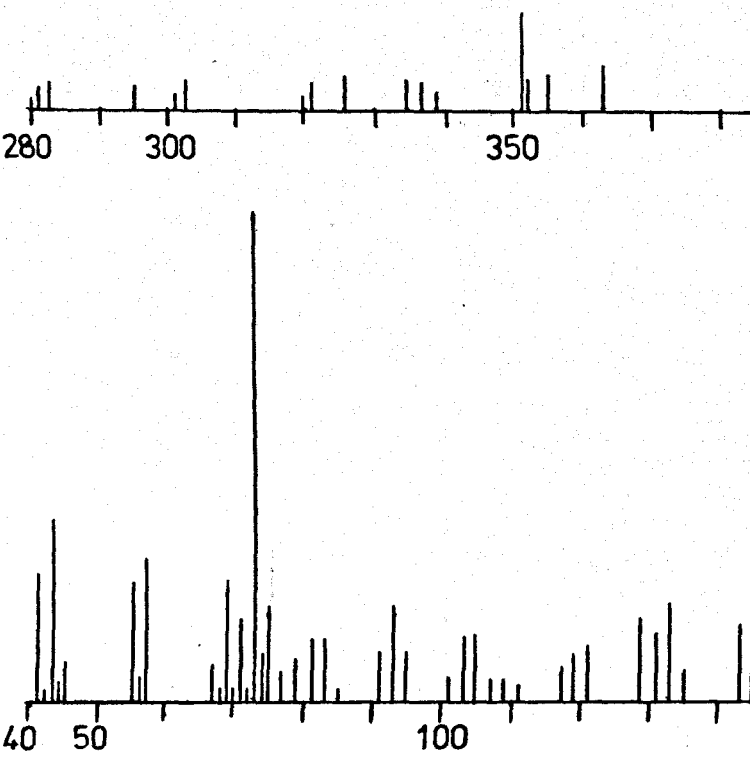


FIG 6.10

gel TLC (toluene-30% ethyl acetate), the products eluted with ether and identified as the tetrakis (TMS) ether, (Fig 6.7), R_f = 0.54 and the pentakis (TMS) ether, (Fig 6.8), R_f = 0.67 on the basis of their mass spectra. The pentakis (TMS) ether is the sole product when ecdysterone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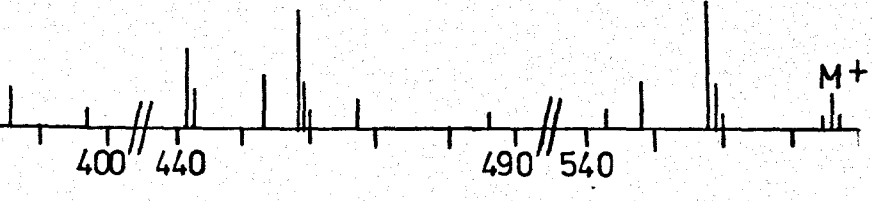
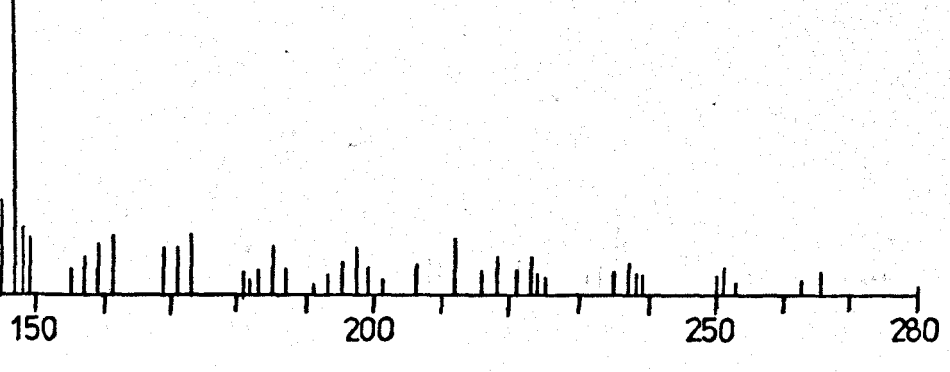
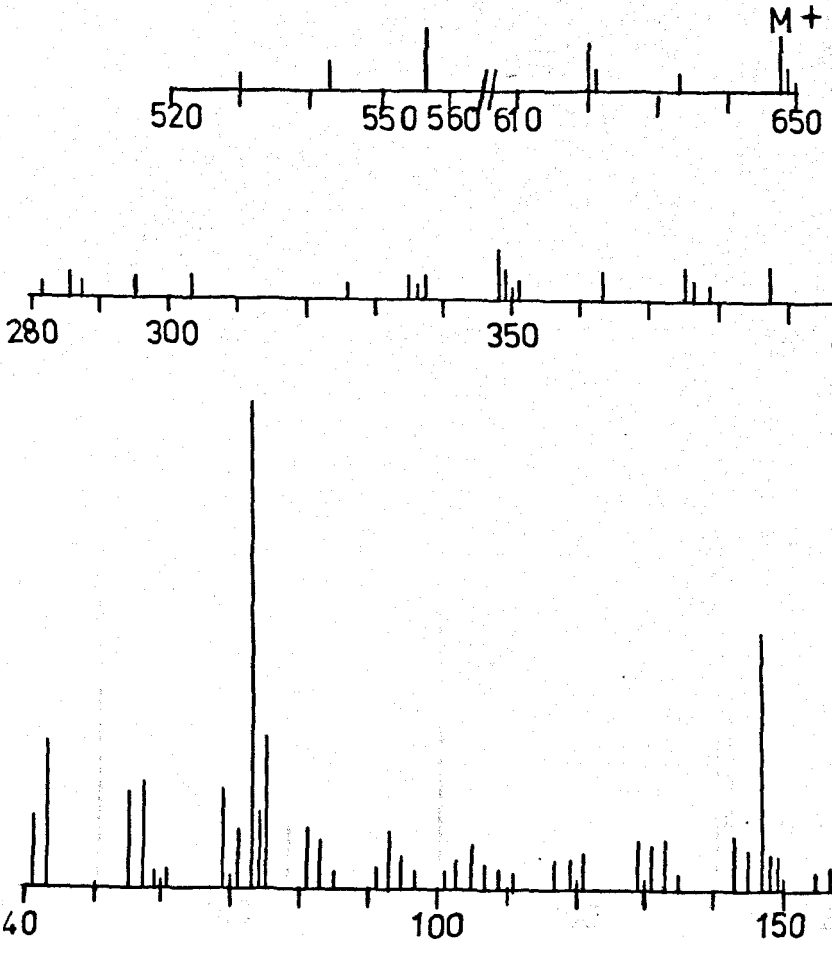


FIG 6.3

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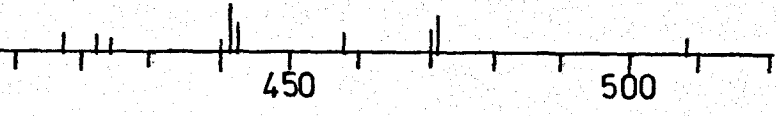
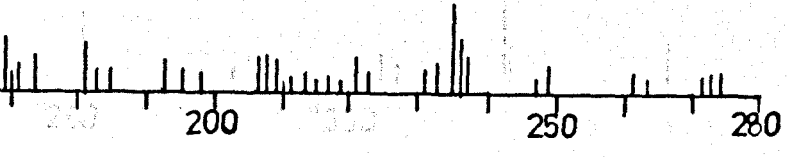


FIG 6.4



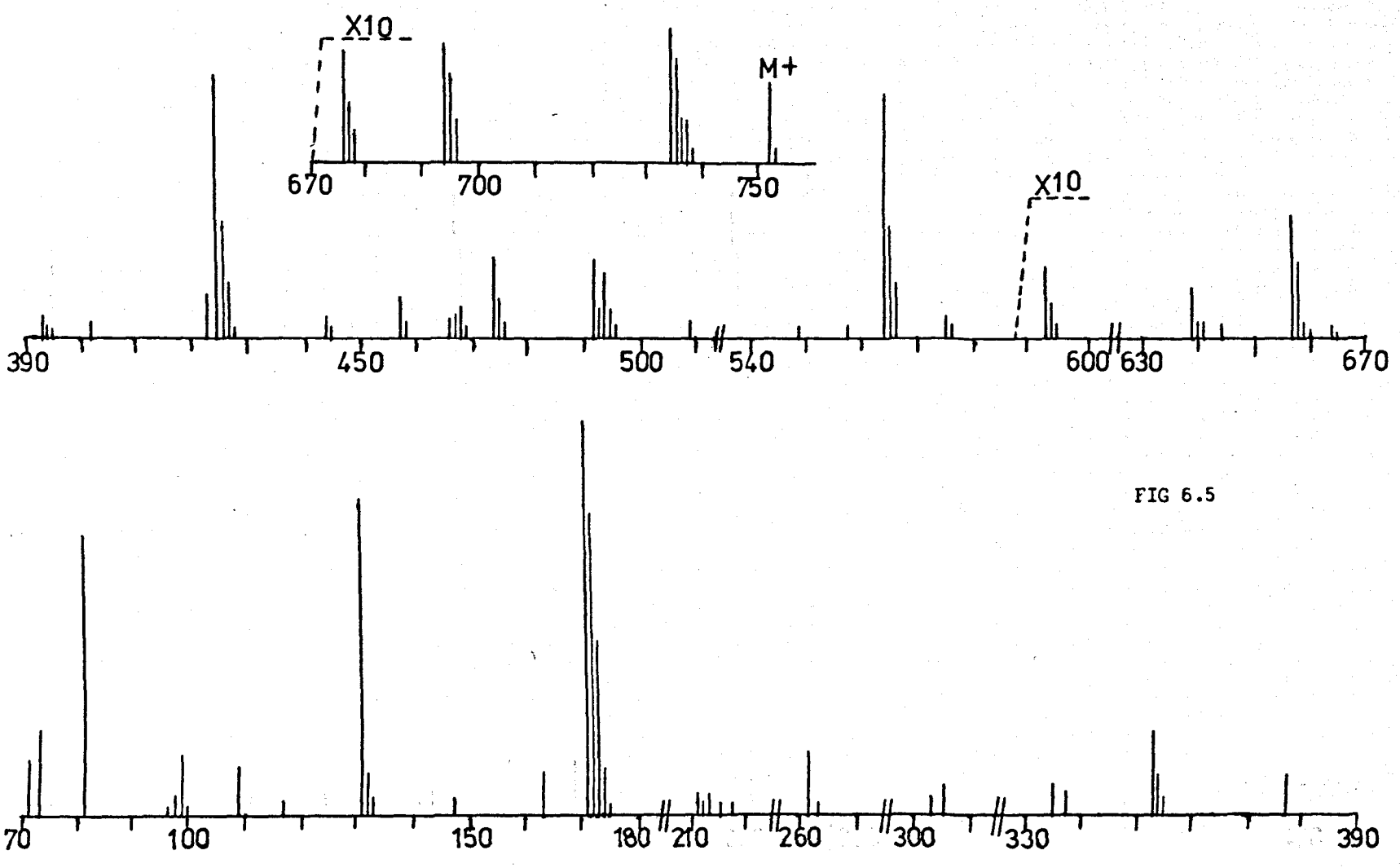
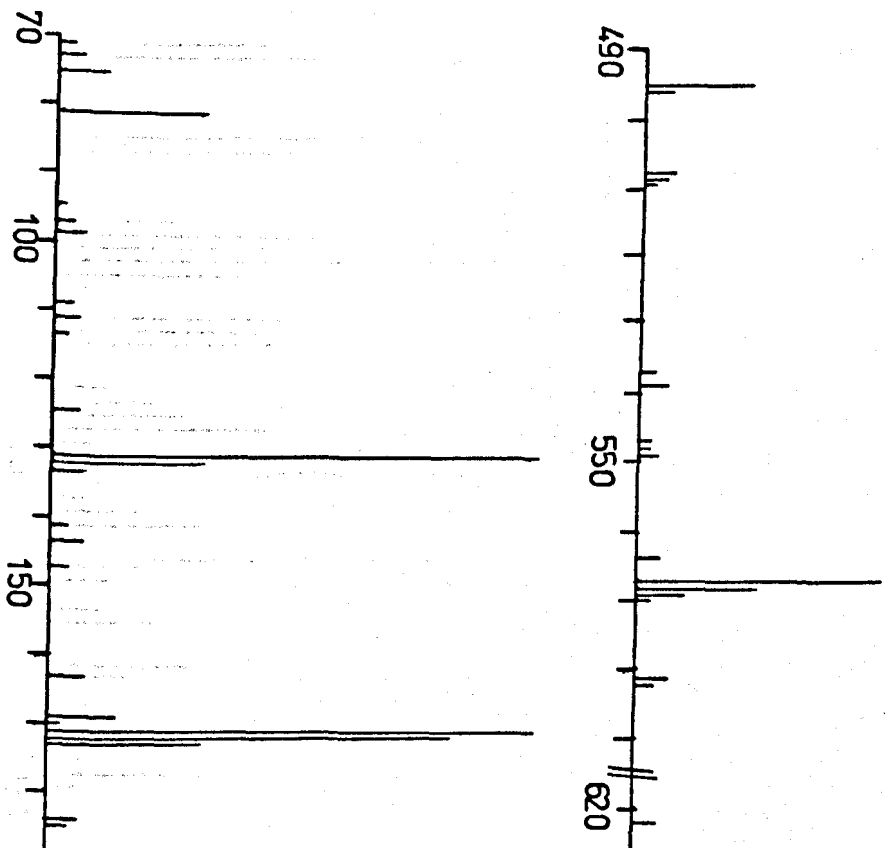

X10
M+
670
700
750
X10
390
450
500
540
600
630
670
70
100
150
180
210
260
300
330
390

FIG 6.5


70
100
150
490
550
620

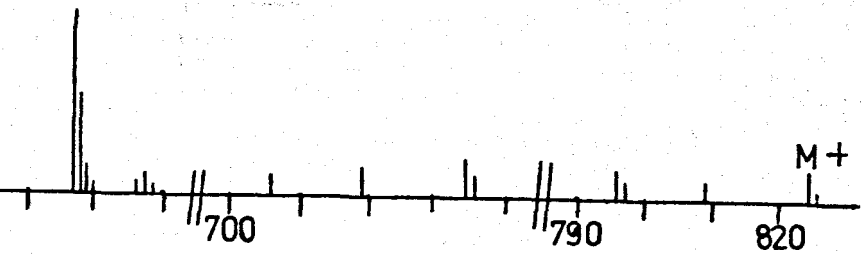
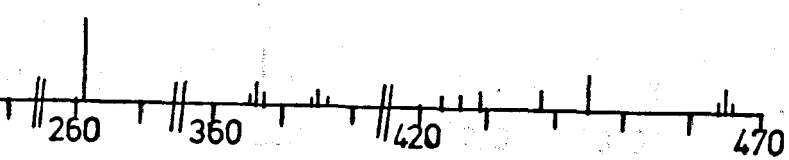
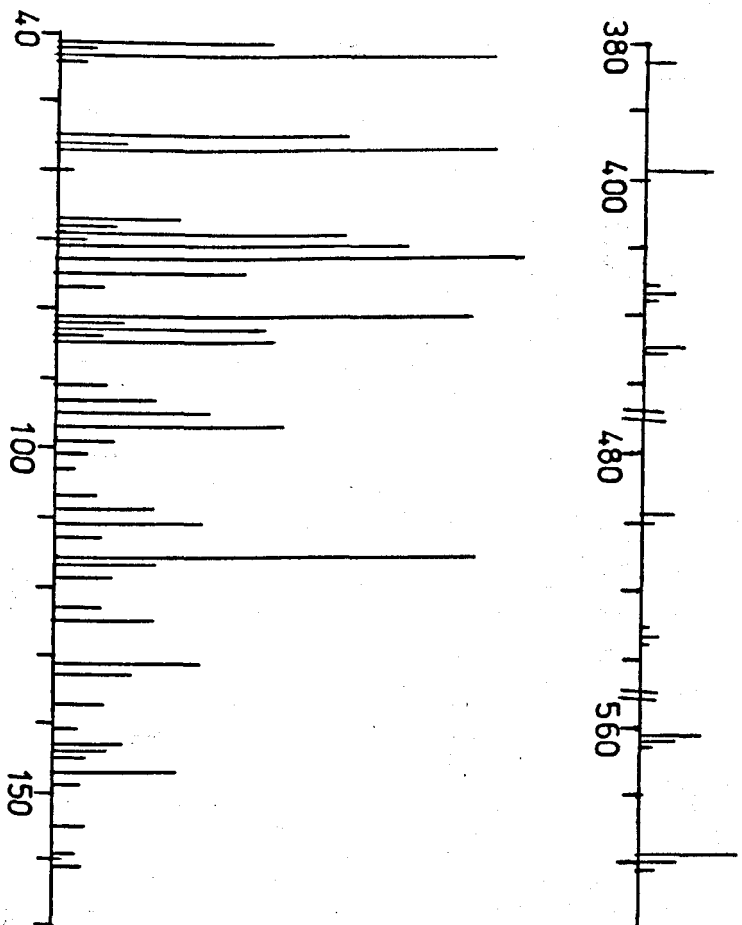


FIG 6.6

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150
380
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480
560

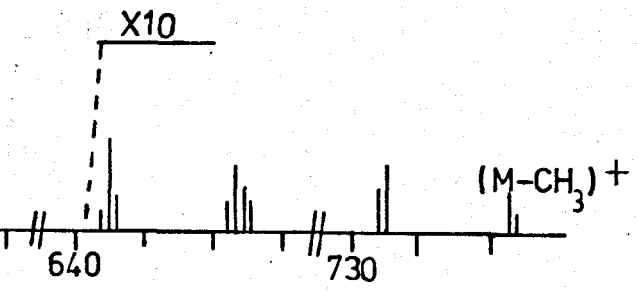
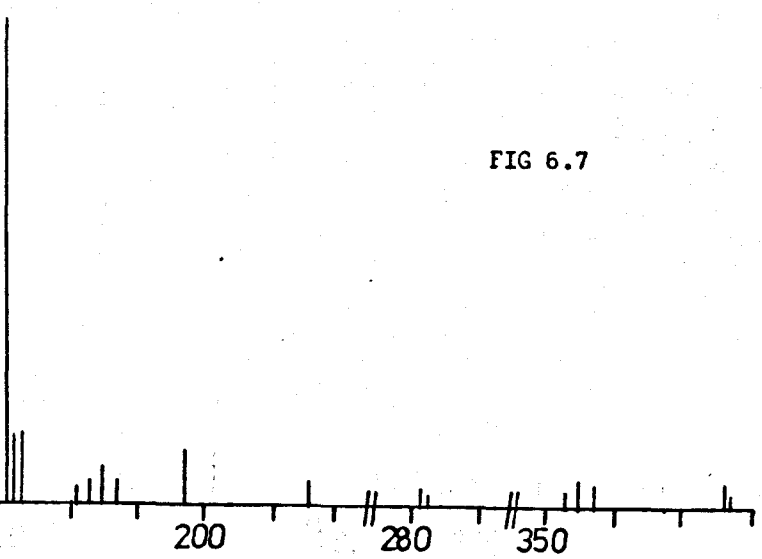
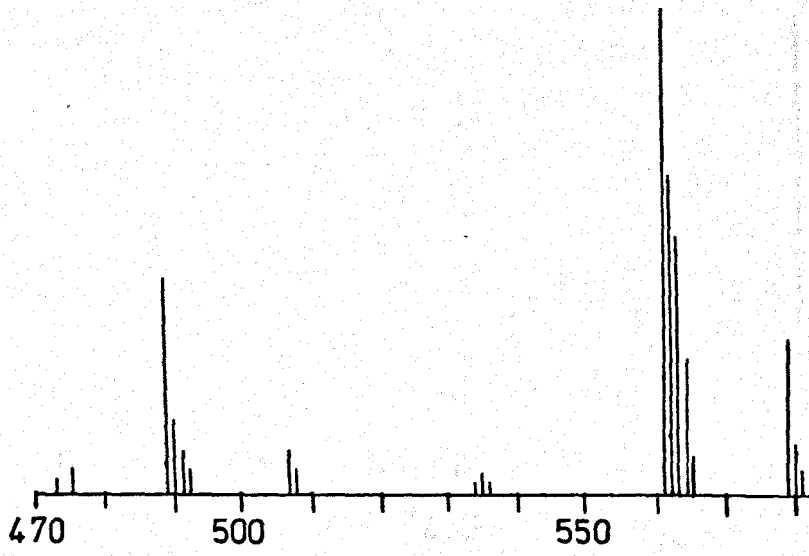


FIG 6.7

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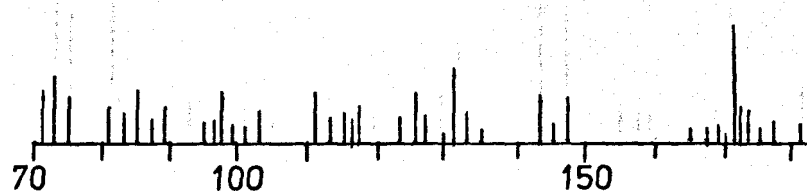
500
550


70
100
150

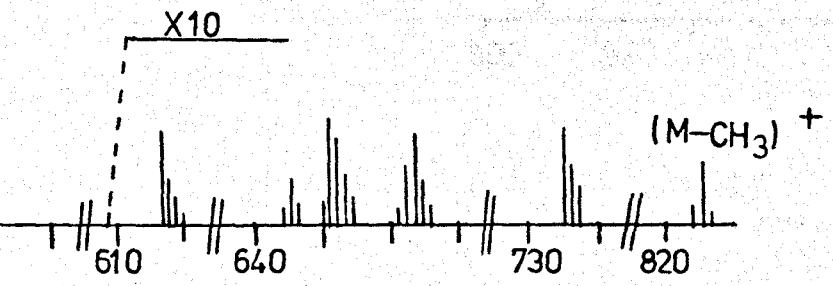
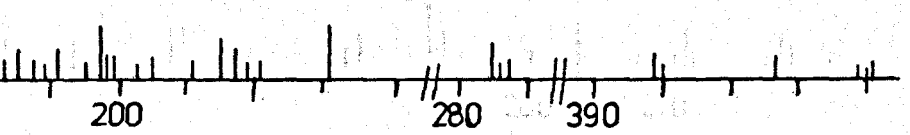


FIG 6.8



350
470
530

70
100
150

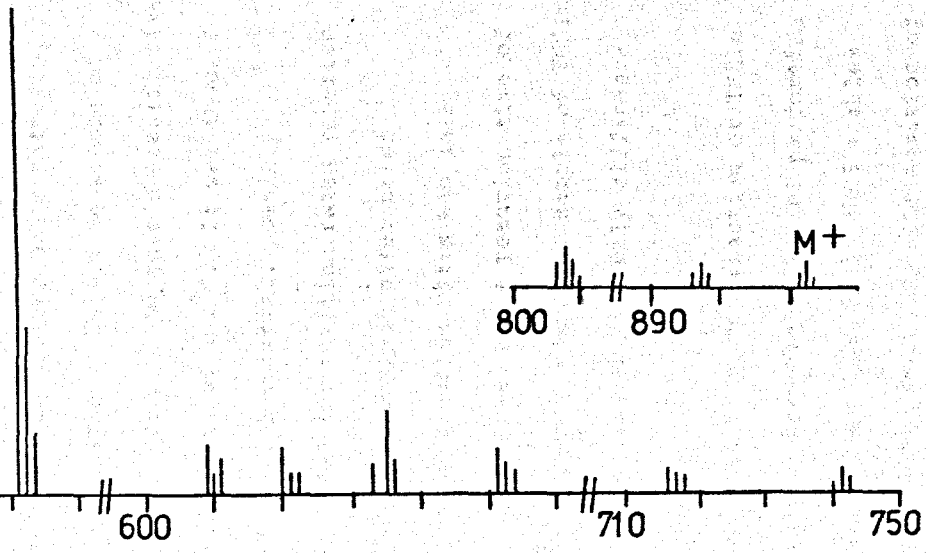
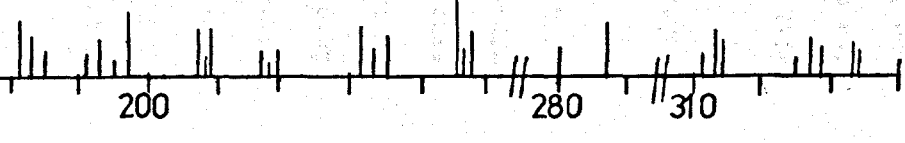


FIG 6.9



is heated in TMSIm at 100°C for 4 h. Access to the 20-hydroxyl group was hindered by the faster reaction of the 22-hydroxyl. The hexakis(TMS) ether was obtained when ecdysterone was heated in TMSIm at 140°C for 20h.

The order in which hydroxyl groups at different positions in the ecdysones react with TMSIm can be stated to be;

C-2, C-3, C-22, C-25 > C-20 >> C-14

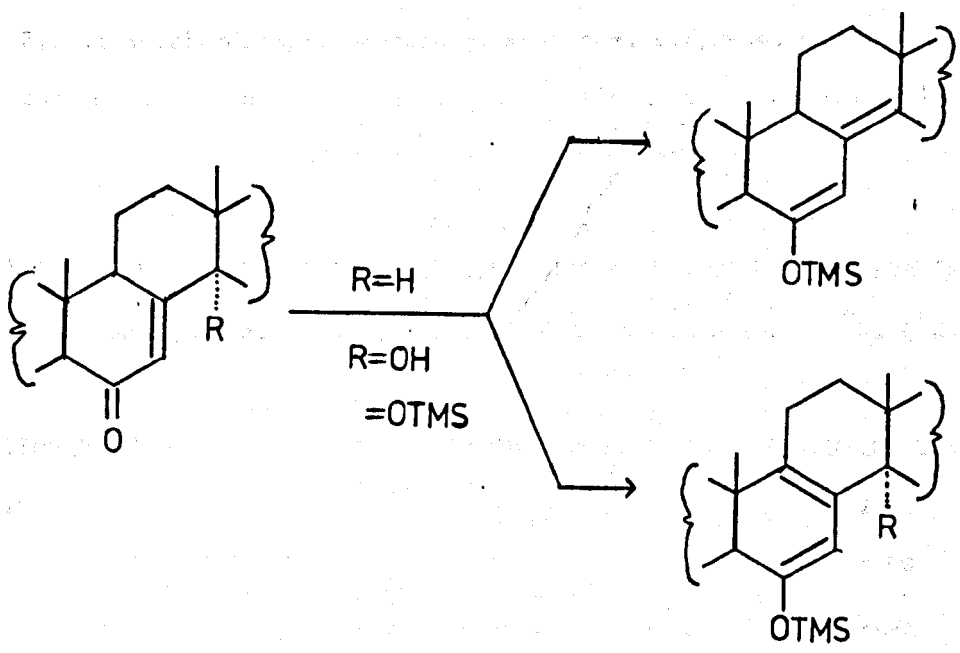
A further problem was encountered in silylating ecdysones, in that during the early part of this work, difficulty was found in reproducing conditions for the quantitative silylation of ecdysones with different commercial samples of TMSIm. The first sample purchased gave a quantitative conversion of the 14α-hydroxyl group with 16h. at 140°c (heated overnight). A second sample from the same supplier required a minimum of 30 h. for complete reaction.

A pure sample of TMSIm prepared in the laboratory under nitrogen, required over forty hours for complete reaction. This same sample of TMSIm after several brief exposures to the atmosphere gave a quantitative conversion of the 14α-hydroxyl group in 12 h. at 140°C. It was thought possible that some impurity in the commercial material, possibly imidazole was catalysing the reaction. All the reaction rates reported in this section refer to the bulk laboratory reagent after brief exposure to the atmosphere. The differing reaction rates was less apparent when crude biological material was used and a reaction time of 20 h. at 140°C was sufficient for all samples. Whichever sample of TMSIm was used, the products were the same in all cases, the only difference being the rate of formation.

A limited study of the effect of catalyst on the reaction of TMSIm with the C-14 hydroxyl group has indicated the possibility of performing the silylation under mild conditions. The addition of 1% TMCS enables the 14α-hydroxyl group to be silylated in 4h. at 100°C. The addition of

further quantities of TMCS leads to the production of by-products, in part due to the formation of TMS-enol ethers. The addition of traces of TMCS is effective for the pure hormone, but with crude biological material its effect was lost, possibly due to deactivation by other material. The reaction was noticeably slower in diglyme or pyridine as solvent. The use of benzylamine, phthalimide and quinol at 100°C resulted in a number of unidentified peaks. The reaction proceeded faster in the presence of pyrrole, piperidine or solid potassium acetate. The addition of potassium acetate to the reaction mixture allowed the 14α-hydroxyl group to be quantitatively silylated in 3h. at room temperature.

The C-14 oxy substituent has a marked effect on the rate of formation and structure of the enol-TMS ether. The use of TMSIm by itself, even at high temperature does not promote the formation of enol ethers. A sample of 5α-cholest-7-en-6-one is easily converted into its enol TMS ether when heated with TMSIm:TMCS (1:1) or at room temperature with TMSIm in the presence of potassium acetate. The UV spectra of the product in hexane, (λ_{max} 255nm, ε_{max} 15,500) supports the hetero-annular diene structure of 6-(trimethylsiloxy)-5α-cholest-6,8(14)-diene, as does the mass spectrum (M^+, m/e 456). In an analogous series of experiments with 2β,3β-dihydroxy--5β-cholest-7-en-6-one similar results were obtained.



In the presence of the C-14 oxy substituent, enol ether formation is considerably reduced. A silylation mixture of TMSIm:TMCS (9:1) at 50°C with 14α-hydroxy-5α-cholest-7-en-6-one gives a non-quantitative conversion to the homo-annular diene 14α-hydroxy-6-(trimethylsiloxy)-5α-cholest--6,8(9)-diene (UV λ_{max} 283 nm, ε_{max} 24,000; mass spectrum M^+, m/e 472). Further heating gives several products, among which is the 14α-TMS ether, the 14α-TMS-enol ether and possibly dehydration products. Likewise, 2β,3β, 6,14α-tetrakis(trimethylsiloxy)-5β-cholest-6,8(9)-diene, (M^+, m/e 720), has been recognized as the principal product in the reaction of BSA:TMCS (5:1) with 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one.

A limitation of the GC technique for the analysis of high molecular weight samples is the shortage of suitable liquid phases of different polarity which can be used at temperatures in excess of 250°C. For the resolution of ecdysone TMS ethers, low loadings of OV-silicone oils are usually preferred. Three such phases were tested under similar conditions, Table 6.3, for the separation of a mixture of fully silylated TMS ethers

of 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one, ecdysone, ecdysterone and inokosterone. The best resolution was obtained on the column of 1% OV-101.

TABLE 6.3

SEPARATION OF TMS ETHERS OF ECDYSONES USING A 3ft. COLUMN, 1% LOADING ON GAS CHROM Q, 60 ml. min^{-1}, TEMPERATURE PROGRAMMED 250-300°C, 6°C. min^{-1}.

STATIONARY PHASE	ECDYSONE TMS ETHER	RETENTION TIME(min)
OV-101	A	2.60
	B	4.50
	C	5.05
	D	5.40
DEXSIL 300 GC	A	3.30
	B	5.30
	C	5.75
	D	5.90
OV-17	A	2.90
	B	4.65
	C	4.90
	D	5.35

A = 2β,3β,14α-tris(trimethylsiloxy)-5β-cholest-7-en-6-one

B = 2β,3β,14α,22,25-pentakis(trimethylsiloxy)-5β-cholest-7-en-6-one (ecdysone pentakis TMS ether)

C = 2β,3β,14α,20,22,25-hexakis(trimethylsiloxy)-5β-cholest-7-en-6-one (ecdysterone hexakis TMS ether)

D = 2β,3β,14α,20,22,26-hexakis(trimethylsiloxy)-5β-cholest-7-en-6-one (inokosterone hexakis TMS ether)

It was the only phase which gave a base line separation of ecdysone and ecdysterone TMS ethers. No phase gave a base line separation of ecdysterone and inokosterone ethers, which differ only in the position of one side chain TMS group. The best resolution of this pair was again achieved on the OV-101 column. Recently, two thermally stable polar phases (Poly-S 179 and PZ-179) of potential use for steroid analysis have been described[42,43]. Their application to ecdysone analysis has not yet been reported.

THE FORMATION OF TRIMETHYLSILOXY-HEPTAFLUOROBUTRYL DERIVATIVES OF ECDYSONE

Ikekawa et al[7] have described a procedure for the formation of a mixed trimethylsiloxy-heptafluorobutryl (TMS-HFB) derivatives of ecdysones. To the TMS ether in TMSIm was added heptafluorobutrylimidazole, HFBIm, with a catalytic amount of heptafluorobutyric acid, HFBA, or heptafluorobutyric anhydride, HFBAn. The conversion was about 90% of theoretical by GC, after heating for 2h at 50°C and the derivative could be detected at the picogram level with an electron capture detector. It was shown that in the case of ecdysterone, one heptafluorobutyrl group was introduced at the C-2 position[37]. Direct reaction of ecdysterone with HFBIm or HFBAn gave a mixture of products.[7,37]

When HFBIm was used without catalyst, no change in the products of the reaction or significant variation in amounts was found, but the reaction time was considerably extended. The addition of a trace of HFBA has a marked effect on reaction rate but not the composition of the final products. As a general observation, the use of HFBAn cannot be recommended as it gives rise to a series of smaller peaks as well as the main products. As reported by Ikekawa *et al.*[7], the presence of TMSIm is essential for a smooth reaction; removal of TMSIm, followed by addition of the HFBIm-HFBA mixture gives complex chromatograms containing several

prominent peaks.

The reported conditions for the formation of TMS ethers of ecdysterone are unlikely to be severe enough to silylate the 14α-hydroxyl group (see previous section).[7] The exchange reaction has therefore been carried out with both the pentakis- and hexakis TMS ethers of ecdysterone. Reaction of the pentakis TMS ether in TMSIm with HFBIm-HFBA (10:1) for 2h. at 50°C gave mainly one peak on GC, B, (Fig 6.11), with a small amount of the unchanged pentakis TMS ether, C, and a further component of shorter retention time, A. Extending the reaction time produced a more complex chromatogram in which the two peaks A and B predominate.

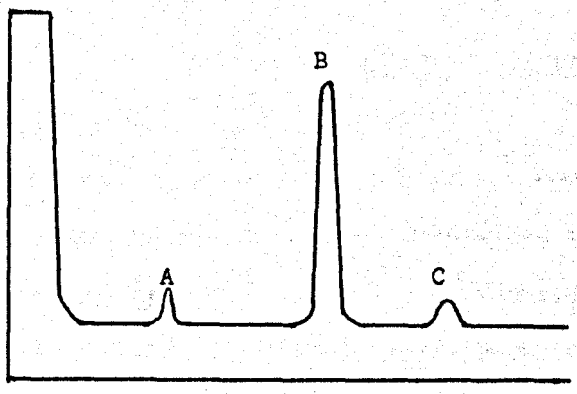


FIG. 6.11

The hexakis TMS ether, under similar conditions gave two peaks on GC with peak D predominating (Fig 6.12). Although neither reaction is quantitative,

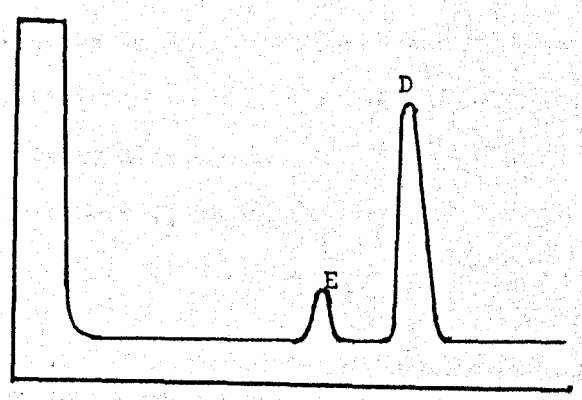


FIG. 6.12

both TMS ethers of ecdysterone give a reasonable yield of the exchange (TMS-HFB) product.

While monitoring the exchange reaction with the electron capture detector, it was found that the TMS ethers of ecdysterone could be detected at the picogram level without forming the mixed TMS-HFB derivative. In the light of this observation, there was little to be gained by forming the mixed derivative, particularly as it introduces a further step in the analytical procedure. The mixed derivatives are useful as an aid to the identity of nanogram amounts of hormone by providing GC retention time data which can be used in conjunction with the retention time data of the TMS ether and compared with known hormones.[44]

STRUCTURAL REQUIREMENTS OF ECDYSONES FOR SENSITIVE DETECTION BY ELECTRON CAPTURE.

Steroids, as a class of compound, do not show strong electron capturing properties and are usually determined at trace levels by GC with electron capture detection after the introduction of an electrophore either as a halogenated acyl ester or halomethyldimethylsilyl ether (see Ch.1). Typical sensitivities of non-halogenated steroids are represented by the first five entries of Table 6.4. To aid comparison the column oven temperature was varied for each compound to produce a peak width at half-height which was the same for each compound on a 3 ft. column of 2% OV-101 on Gas Chrom Q with a flow rate of 85 ml. min^{-1}.

The ketones 5α-cholestan-6-one and 5α-cholest-7-en-6-one, show no special sensitivity to the electron capture detector. The introduction of a substituent at the C-14 position leads to a marked increase in sensitivity (about 1,300 fold in the case of the 14α-TMS ether).

Table 6.4

ECD sensitivity of TMS ethers of ecdysones and their analogues. Measurements with a Pye model 84, Ni^{63} electron capture detector, pulse width 0.75μs, pulse period 50μs, pulse height 47-60V and detector oven temperature of 300°C.

STEROID	LEAST DETECTABLE AMOUNT ng.
3β-(trimethylsiloxy)cholesterol	200
Cholest-4-en-3,6-dione	5
17β(trimethylsiloxy)-17α(methyl)testosterone	5
5α-cholestan-6-one	40
5α-cholest-7-en-6-one	40
5α-acetoxy-cholest-7-en-6-one	0.25
14α-hydroxy-5α-cholest-7-en-6-one	0.06
14α-(trimethylsiloxy)-5α-cholest-7-en-6-one	0.03
2β,3β-bis(trimethylsiloxy)-5α-cholest-7-en-6-one	1
2β,3β-bis(trimethylsiloxy)-5β-cholest-7-en-6-one	1
14α-hydroxy-2β,3β-bis(trimethylsiloxy)-5α-cholest-7-en-6-one	0.06
14α-hydroxy-2β,3β-bis(trimethylsiloxy)-5β-cholest-7-en-6-one	0.06
2β,3β,14α-tris(trimethylsiloxy)-5α-cholest-7-en-6-one	0.005
2β,3β,14α-tris(trimethylsiloxy)-5β-cholest-7-en-6-one	0.005
2β,3β,14α-tris(trimethylsiloxy)-5α-cholest-7-en-6-one	0.005
2β,3α,14α-tris(trimethylsiloxy)-5β-cholest-7-en-6-one	0.005
pentakis(trimethylsiloxy)ecdysone	0.005
tetrakis(trimethylsiloxy)ecdysone	0.06
hexakis(trimethylsiloxy)ecdysterone	0.005
hexakis(trimethylsiloxy)inokosterone	0.005
pentakis(trimethylsiloxy)cyasterone	0.005

The C-14 TMS ether is twelve times more sensitive than the free hydroxyl group. The 2β,3β-bis(trimethylsiloxy)-5α(β)-cholest-7-en-6-one is forty-times more sensitive than the unsaturated ketone, although the two TMS groups are remote from this centre. As both the 5α and 5β epimers are equally sensitive, then the geometry of the A, B ring junction is not important. The 2β,3β,14α-tris(trimethylsiloxy)-5α(β)-cholest-7-en-6-one is some six times more sensitive to detection than the 14α-(trimethylsiloxy)-5α-cholest-7-en-6-one and thus the remote 2β,3β-TMS groups make a small contribution to the electrophore. A 3α-TMS group is as effective as a 3β-TMS substituent. Geometry would seem to play little part in governing the sensitivity of the electrophore, position of the substituent is much more important. Electronic interactions transmitted through carbon bonds probably play an important role. It has been observed previously that conjugated electrophores containing two or more specific groups which are not necessarily electron absorbing in their own right, when linked by specific bridges confer electron absorbing properties on the molecule.[45-47] In these cases a small increase in electron capture sensitivity was noted with substituents remote from the region of the electrophore and was attributed to electronic interactions across the saturated steroid framework.[46,47]

In ecdysones, the electrophore is not simple, involving the 7-en-6-one group and its C-14 substituent with a smaller contribution from groups which are further remote from the unsaturated ketone. There is no evidence to suggest that the presence of weakly electron capturing groups in the side chain exert an influence on the electrophore.

As stated earlier the electron capture process is temperature dependent and a plot of

$$\ln \quad A.\ \frac{F}{S} \cdot \frac{b_o}{b} \cdot T^{3/2} \quad \text{vs} \quad \frac{1}{T}, \quad \text{(Fig 6.13),}$$

for pentakis(trimethylsiloxy)ecdysone, has a positive gradient which according to Wentworth and Chen illustrates that in this case a non--dissociative mechanism is operative.[15,18,27]

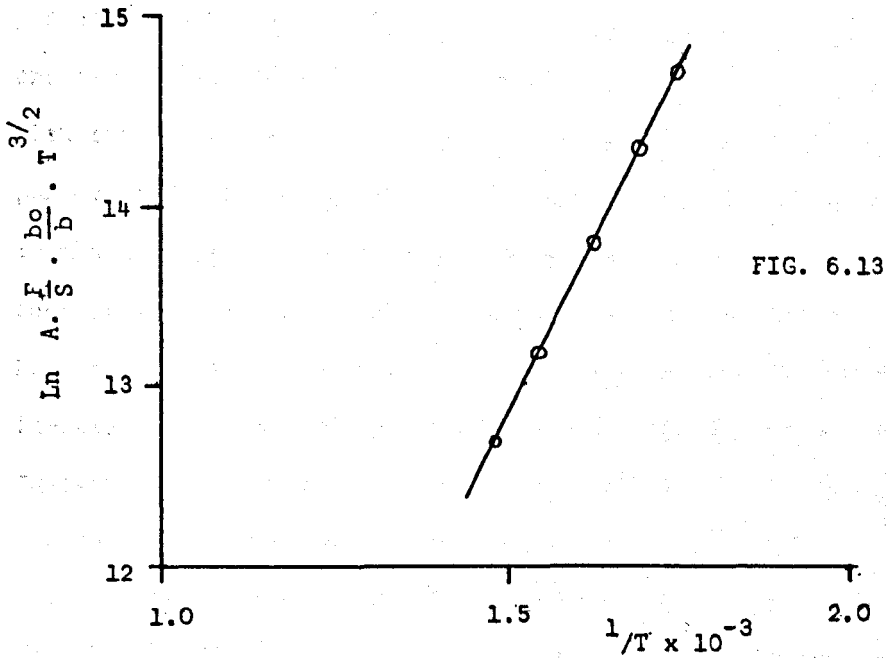


FIG. 6.13

It is evident that the peak area of the injected sample decreases with increase in detector oven temperature, and for maximum sensitivity the lowest practical detector oven temperature should be employed. Moderate retention times for ecdysone TMS ethers requires a column oven temperature of 265°-280° with a slightly higher temperature for the detector so that the best compromise is a detector oven temperature of 300°C.

Using the standard conditions given in Table 6.4 a linear response over the range 5-700 x 10^{-12}g for pentakis(trimethylsiloxy)ecdysone and hexakis(trimethylsiloxy)ecdysterone was obtained. The detector was shown to be linear in the range 60-700 x 10^{-12}g for tetrakis(trimethylsiloxy)-ecdysone.

PRELIMINARY PROCEDURES FOR THE ANALYSIS OF ECDYSONES IN LOCUSTS.

For the analysis of ecdysones in the desert locust (Schistocerca gregaria) by electron capture detection of the TMS ethers, some purification procedure prior to GC was required. This was because the crude material contains electron capturing impurities which co-chromatograph with the TMS-ecdysones masking the response of the detector. For this purpose a TLC procedure was investigated. The extraction of polar material from silica gel is rarely quantitative so TLC of the TMS ethers was considered more appropriate. The formation and subsequent recovery of both the partial and completely silylated ethers of ecdysones was investigated. The TLC data of the TMS-ecdysones used is summarized in Table 6.5.

TABLE 6.5

TMS-ethers of Ecdysones : R_f values on Silica gel.

STEROID	NOS. OF OH GROUPS SILYLATED	SOLVENT SYSTEMS	
		Toluene-Ethyl acetate (9:1)	Toluene-Ethyl acetate (7:3)
2β,3β,14α-trihydroxy-5β-cholest--7-en-6-one	2β,3β	0.39	-
	2β,3β,14α	0.51	-
ecdysone	2β,3β,22,25	-	0.69
	2β,3β,14α,22 and 25	0.58	-
ecdysterone	2β,3β,22,25	-	0.54
	2β,3β,20,22 and 25	0.22	0.67
	2β,3β,14α,20, 22 and 25	0.69	0.75

Kieselgel PF_{254} (E. Merck), purified by extraction with methanol (to remove electron capturing impurities) was spread on 20 x 20 cm glass plates as a slurry in de-ionised, distilled water. The optimum silica layer was 0.6 mm thick, as this enabled the diluted reaction mixture to be streaked directly on the plate without disengagement of the silica from the glass surface. The plates were deactivated by heating at 120°C for at least one hour and allowed to cool to room temperature in a dry box. The smallest amount of ecdysterone TMS ether which could be detected on a fluorescent plate under UV light at λ254 nm was found to be 0.5μg.

The best method for the recovery of ecdysone TMS ethers was found to be elution from packed columns of the silica gel. Partitioning the appropriate area of silica gel between water and benzene gave only moderate recoveries and occasional decomposition was observed. The appropriate area of the TLC plate was packed into a short narrow glass column (10 x 1.1 cm), tapered at one end with a silanized glass wool plug and eluted under gravity. The most efficient eluting solvents were diethyl ether, tetrahydrofuran, methanol, and dichloromethane. Benzene, ethyl acetate and acetone gave consistently lower recoveries. Diethyl ether was selected because it gave high recoveries, contained very few electron capturing impurities and decomposition of small amounts of the TMS ethers was not observed to occur. It does not dissolve silica gel from the column, has sufficient volatility to be evaporated easily and is cheap and readily available. The use of 8 ml. of diethyl ether, typically gave recoveries in the range 75-90% for 0.005-10μg of the pentakis(trimethylsiloxy)-ecdysterone ether. For the hexakis(trimethylsiloxy)ecdysterone ether, 6 ml. of diethyl ether gave recoveries of 85-100% in the same sample range. A similar set of results were obtained with the bis and tris(trimethyl-siloxy) ethers of 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one.

To extract the ecdysones from locust material, the whole insects were ground in methanol using a liquidizer and the solid residue removed by filtration through a glass sinter (porosity 4). The methanol extract was evaporated to a residue in vacuo at 50°C, a fraction of the residue silylated and analysed by GC-ECD after TLC. The analysis was not successful as the ecdysone region of the chromatogram was completely masked by electron capturing impurities (Fig. 6.14a). The methanolic residue was partitioned between light petroleum (b.p. 40-60°C) and aqueous methanol (4:1, water: methanol), the aqueous phase reduced to a residue in vacuo at 50°C, silylated and analysed by GC-ECD after TLC. The detector was again masked in the ecdysone region (Fig 6.14b). The aqueous methanolic residue was partitioned between butanol and water (K ecdysterone = 5.3, ecdysone = 10)[1], the butanol phase reduced in vacuo at 50°C, a fraction of the residue silylated and analysed by GC-ECD after TLC. A clear indication of the presence of ecdysones was obtained, but the broad solvent front was too large for adequate quantitative analysis (Fig. 6.14c). A further partition between ethyl acetate and water (K ecdysterone = 0.06, ecdysone = 0.32)[1], evaporation of the aqueous phase in vacuo at 50°C, followed by silylation and GC-ECD analysis after TLC gave a steady base line and the ecdysones were well removed from the solvent front, which enabled them to be easily quantified (Fig. 6.14d).

As an alternative to partition procedures, the residue from the butanol extract was purified on a silica gel TLC plate, developing in chloroform - - 96% ethanol (2:1); R_f (ecdysterone) = 0.41. The area R_f (0.2 - 0.6) was scraped off and eluted with methanol. The residue was silylated, partitioned between toluene and water, the organic phase dried with molecular sieves, and injected into the GC. Good separation of ecdysones from electron capturing impurities was obtained, but the overall recovery

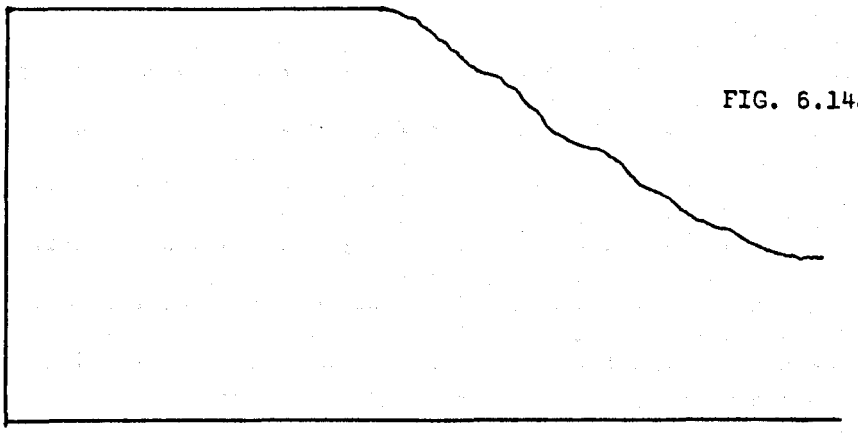


FIG. 6.14a

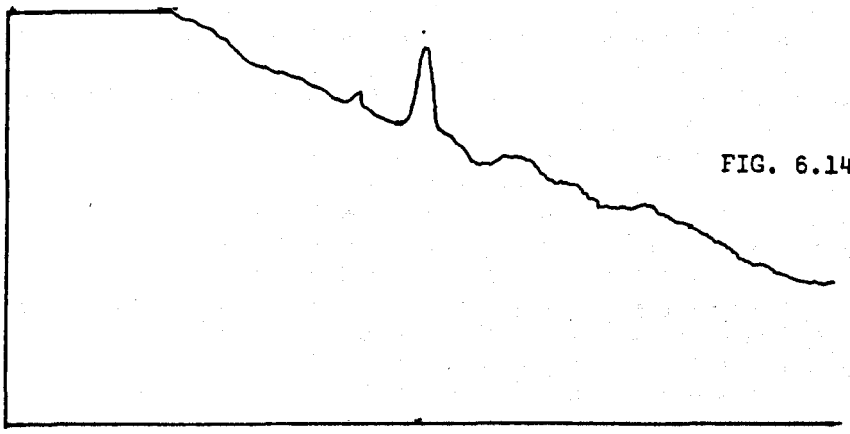


FIG. 6.14b

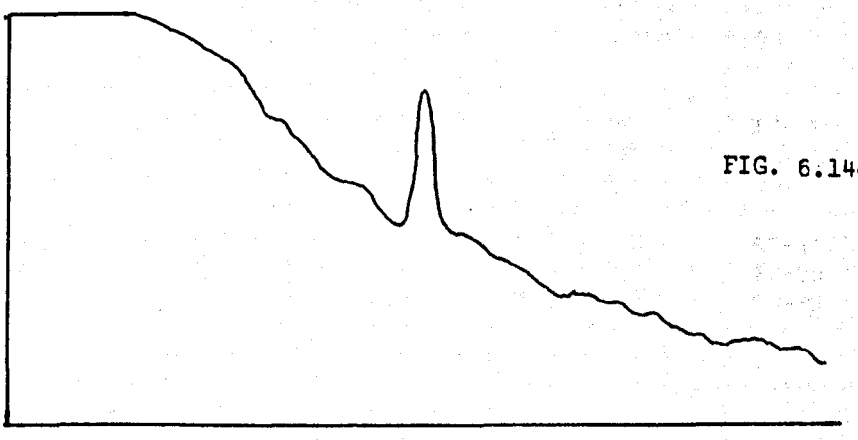


FIG. 6.14c

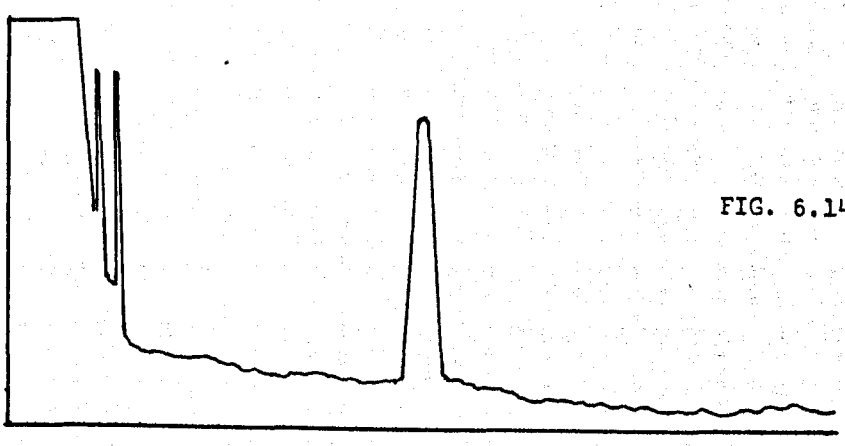


FIG. 6.14d

of added ecdysterone was poor (40-60%) and offered no advantages over the partition procedure in terms of simplicity, recovery and time per analysis. The partition of ecdysone TMS ethers in TMSIm with water and toluene, benzene or hexane does not result in their hydrolysis, and provides a convenient method for the removal of the silylating reagent prior to GC.

The overall recovery of ecdysterone, as ascertained by the addition of known quantities to the methanolic extracts obtained from adult locusts, which contain no hormone was found to be greater than 70% in all samples investigated. Table 6.6

TABLE 6.6

RECOVERY OF ECDYSTERONE FROM "SPIKED" ADULT LOCUSTS

TMS ETHER FORMED	AMOUNT OF ADDED ECDYSTERONE(μg)	NUMBER OF SAMPLES ANALYSED	RANGE OF RECOVERIES (%)
pentakis(trimethylsiloxy)--ecdysterone	10-1	5	80-90
	1-0.1	7	75-85
	0.01-0.001	10	70-85
hexakis(trimethylsiloxy)--ecdysterone	10-1	4	90-100
	0.1-0.01	6	80-90
	0.001-0.0001	10	70-85

The procedure adopted for the recovery experiments was direct comparison of the peak area on GC-ECD of the spiked sample with a standard prepared directly and purified of exceeding trimethylsilyl reagents by partition between toluene and water. The recoveries of the pentakis(trimethylsiloxy) ether was marginally lower than the completely silylated form. In both cases, most of the hormone that was lost occurred at the TLC and elution

stage; very little ecdysterone was lost in the partitioning stages.

The standard extraction procedure, outlined in the experimental section, has been designed for the analysis of ecdysones in crude biological material. For the analysis of culture medium, haemolymph or isolated glands, it might prove possible to eliminate some of the steps in the sequence. For those cases in which maximum sensitivity is required, the fully silylated derivative is approximately ten-times more sensitive than the partially silylated derivative. When maximum sensitivity is not required the partially silylated derivative is more convenient in use, being more rapidly formed.

DETERMINATION OF ECDYSONES AT DAILY INTERVALS IN THE 4th AND 5th INSTAR OF THE DESERT LOCUST.

The hormone titre at daily intervals in the 4th (Fig 6.15) and 5th instar (Fig 6.16) of the desert locust (Schistocerca gregaria) were determined GC-ECD using the extraction procedure given in the experimental section.

The plots of the daily titre of ecdysones in both instar indicate that the maximum titre of ecdysterone occurs more than 24 h. before ecdysis. Ecdysone is detectable throughout the instar in amounts always less than that of ecdysterone, reaching its maximum titre on the day before that of ecdysterone. On the day of maximum ecdysterone titre, the amount of ecdysone is very small. This is in agreement with the view that ecdysone is a prohormone for ecdysterone (see introduction). A further electron capturing peak of longer retention time was observed whenever the titre of ecdysterone was high. This unidentified peak is potentially a third hormone having some function in the moulting process or the first inactivation product. Its retention time suggests some modification to the ecdysterone skeleton, perhaps the presence of a further hydroxyl group.

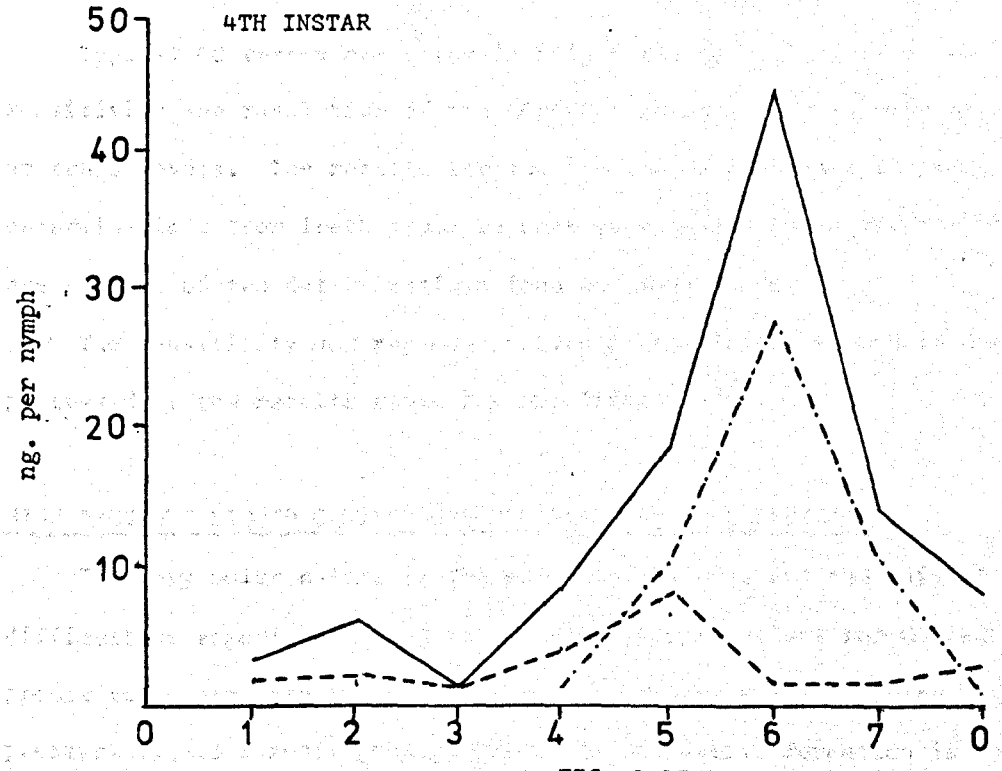


FIG. 6.15

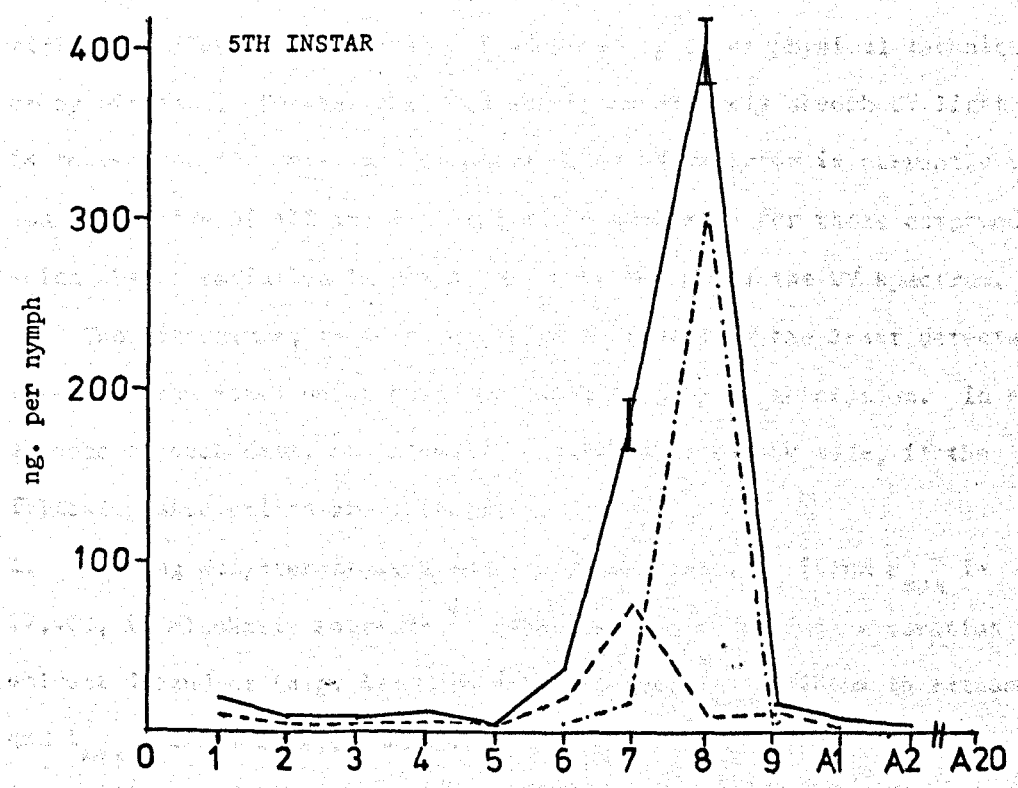


FIG. 6.16

ecdysterone
ecdysone
unknown

Typical GC traces are given in (Fig 6.17) and illustrates the sensitivity and resolution of the GC-ECD technique for ecdysone analysis at trace levels. The results for the 5th instar are the average of five determinations from fresh stock in each case, while those for the 4th are average of two determinations from the same stock.

The sensitivity and reproducibility of the GC-ECD method is clearly portrayed by the results given for the fifth instar.

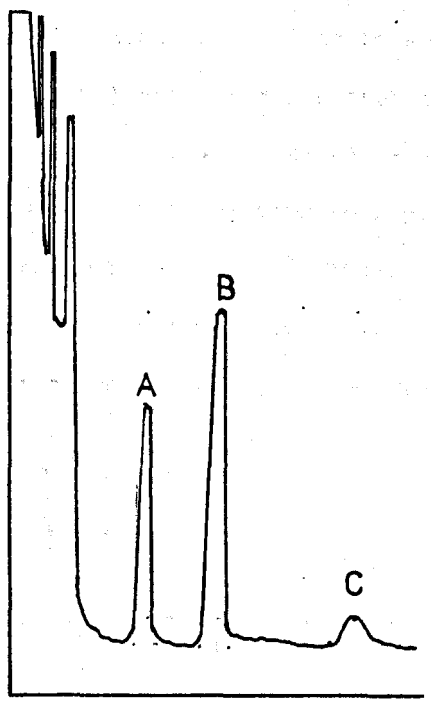
HIGH PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF ECDYSONES.

The very polar nature of the ecdysone molecule and the initial difficulties experienced in forming suitable derivatives for GC led several groups to investigate the possibility of ecdysone analysis using high pressure liquid chromatography, HPLC. As derivative formation is not required for analysis, ecdysones might be determined directly and the effluent collected for subsequent analysis by other physical techniques or by bioassay. Furthermore, the ecdysones strongly absorb UV light which is convenient for detection purposes. The UV detector is currently the most sensitive of all the HPLC detectors available for those compounds which absorb radiation in the appropriate region of the UV spectrum.

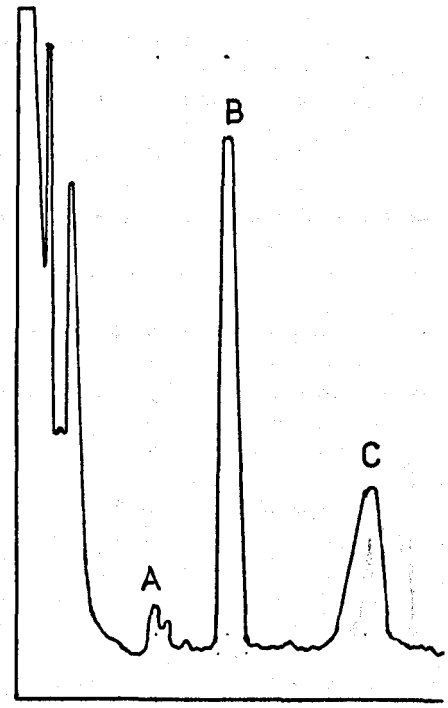
The literature, to date, contains no report of the least detectable amount of ecdysones which could be identified by UV absorption. In the absence of such data, an estimate of this value can be made, if the following assumptions are allowed;

1. Using ecdysterone as a model ecdysone, at λ_{max} 242nm ε_{max} is 12,400, in alcoholic solvents.[48] The position of maximum absorption is solvent dependent (e.g. 5α-cholest-7-en-6-one, λ_{max} = 244nm in methanol and λ_{max} = 235nm in cyclohexane.

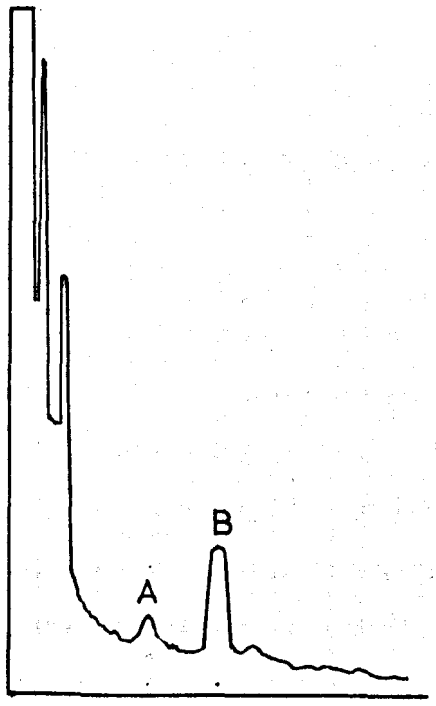
2. Fixed wavelength detectors using low pressure mercury discharge lamps as a source of energy emit radiation of λ254nm. The absorption of



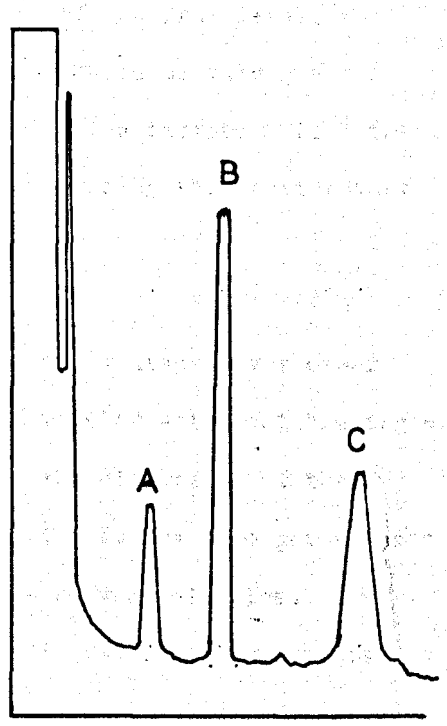
5TH INSTAR DAY 7



5TH INSTAR DAY 8



5TH INSTAR DAY 3



4TH INSTAR DAY 5

A ecdysone
B ecdysterone
C unknown

FIG. 6.17

ecdysterone in methanol at 254nm is approximately 9,300.

3. The path length of the detector cell is 1 cm. and total volume 10µl.

4. The maximum sensitivity range of the detector is 0.01 absorption units full scale. The minimum absorption to give a peak height of 0.5 cm on the recorder is 2.5×10^{-4} absorption units (FSD = 20 cm).

5. Using the relationship OD = ε C l, where OD = optical density, ε = absorption coefficient, C = concentration, ($M\ l^{-1}$), l = path length of cell:

$$C = \frac{2.5 \times 10^{-4}}{9,300 \times 1}$$

$$W = \frac{2.5 \times 10^{-4} \times 480}{9,300 \times 10^{3}} \approx 13ng\ cm^{-3}$$

where W = grams per cm^3 and 480 is the molecular weight of ecdysterone. Thus ecdysones should be detectable at the tens of nanogram level, the least detectable amount, being dependent on peak shape as this governs the volume of effluent in which it elutes. All those factors which affect column efficiency will also be important in determining the least amount of steroid detected.

Reversed-phase chromatography

Reversed-phase chromatography has important advantages over other forms of adsorption chromatography for the analysis of water soluble organic compounds. The separation process is based on the attraction of the hydrophobic stationary phase.[49] Hydrophobic solvents are stronger eluents because they are more capable of displacing the sorbed molecules. For ecdysones, the reversed-phase technique is particularly attractive, as ecdysones are more soluble in water-alcohol mixtures than in chlorinated hydrocarbons, the very polar impurities found in crude extracts (e.g. sugars and glucosides) are eluted from the column before the less polar

ecdysones and the column material can be made ready for re-use by solvent recycle. Hori described the separation of ecdysones by reversed-phase chromatography using Amberlite XAD-2 with a water-alcohol eluting gradient.[50] The Amberlite resin is not available in a particle size suitable for HPLC and had to be ground and sieved prior to use. Since the resin is compressible, maximum pressures were limited, which set an upper limit on flow rates. Although the separation of structurally similar ecdysones was often quite good, the retention times were inconveniently long (ecdysterone 420 min, ecdysone 540 min). The technique has been used for the separation of ecdysones in plant[50,51] and insect material[52,53]. Separations on the preparative scale have been described for ecdysones in crude plant extracts.[55] The authors caution that for this application, the very polar ecdysones may not be sufficiently retained by the resin (low column capacity factor) to give an adequate separation from impurities. For this purpose more polar Amberlite resins might be used to advantage.[49] The separation of ecdysones on the semi-rigid porous polymer, Poragel PN, has been described.[54,55] Poragel PN is a copolymer of divinylbenzene and ethylene glycol dimethacrylate with heavy cross-linking (40%) compared to Amberlite XAD-2 which is a lightly cross-linked polystyrene polymer. The ester groups of the Poragel confer a degree of polarity to the polymer which probably explains the greater retention volume of ecdysterone on Poragel compared to Amberlite XAD-2. The separation of milligram quantities of ecdysones was achieved but analytical separations (sub-microgram level) were poor, due in part to the inherently slow mass-transfer and limited capacity factor of the polymer, particularly at high flow rates.[54,55]

The reversed-phase separation of ecdysones in crude plant extracts using polyamide has been described.[56] Pellicular polyamide phases suitable for HPLC are available commercially (Pellidon, Reeve Angel) but their

application to the analysis of ecdysones has not been reported. King et al[57] have described the analysis of ecdysones in culture medium using Vydac reversed-phase (C_{18} hydrocarbon permanently bonded to a pellicular support) and eluting with a mobile phase of 25% methanol in water. The capacity of the C_{18} hydrocarbon phase is very much dependent on the polarity of the mobile phase. Uisng Corasil C_{18}, and a mobile phase of 35% water in methanol 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one had a retention time of 12.0 min (1.0 ml. min^{-1}, 1 m. x 2.1 mm). Under these conditions ecdysterone is poorly retained and elutes with the injection solvent. The change in capacity factor for the model steroid with solvent composition can be illustrated by the following data;

0.5 ml min^{-1}, 29% water in methanol R_t = 5.75m

0.5 ml min^{-1}, 25% water in methanol R_t = 1.40m

In an attempt to increase the capacity factor by using a more polar reversed-phase column (ETH Permaphase), ecdysterone was not retained when water-alcohol mixtures were used as the mobile phase.

Liquid-Liquid partition chromatography

Liquid partition chromatography of ecdysones is difficult due to a lack of permanently bonded polar stationary phases. Those separations which have been achieved have used polar liquid phases mechanically held on a solid support. To prevent stripping of the stationary phase the mobile phase has to be pre-saturated. Gradient elution is not practical and high flow rates usually sheer the stationary phase from the support. The most successful analyses have been achieved with 1% β,β'-oxydipropionitrile (BOP), on Zipax using hexane mixed with tetrahydrofuran as the mobile phase.[58,59] A method for increasing the percentage loading of BOP on Zipax has been reported.[60] Permanently bonded liquid phases such as Corasil C_{18} and ETH Permaphase show no retention of ecdysones using hexane mixed with chlorinated hydrocarbons as the mobile phase.

Liquid-solid absorption chromatography

From the early days of ecdysone analysis, the separation of the hormones by silica gel chromatography has been important. The extension of this work to HPLC using highly efficient small particle silica columns was not difficult to predict. Chino et al.[61] separated ecdysone from ecdysterone on ZORBAX SIL (25 cm x 2.1mm, 0.3 ml. min^{-1}, dichloromethane methanol 9:1). Peak shapes were reasonably sharp and the separation quite good (R_t for ecdysone = 23 min and ecdysterone = 30 min.). Nigg et al.[62] have described the separation of ecdysones on Corasil II (a high capacity pellicular silica) using mixtures of chloroform-ethanol as the mobile phase.

With Porasil A, an irregular shaped porous silica (37-75μ) and tetrahydrofuran or tetrahydrofuran mixed with methanol or water, ecdysterone is rapidly eluted. With 40% tetrahydrofuran in dichloromethane, 2β,3β,14α--trihydroxy-5β-cholest-7-en-6-one is eluted in 2.25 min (1.5 ml min^{-1}, 500mm x 2.1mm) but the retention time of ecdysterone is very long. The optimum conditions was found to be a mobile phase composition of tetrahydrofuran-methanol-dichloromethane (20:1:29). The addition of a small amount of methanol as a polar phase modifier reduced peak tailing substantially. With a flow rate of 1ml. min^{-1}, the retention time of ecdysterone was 11.0 min. and for 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one was 2.6 min., (Fig 6.18).

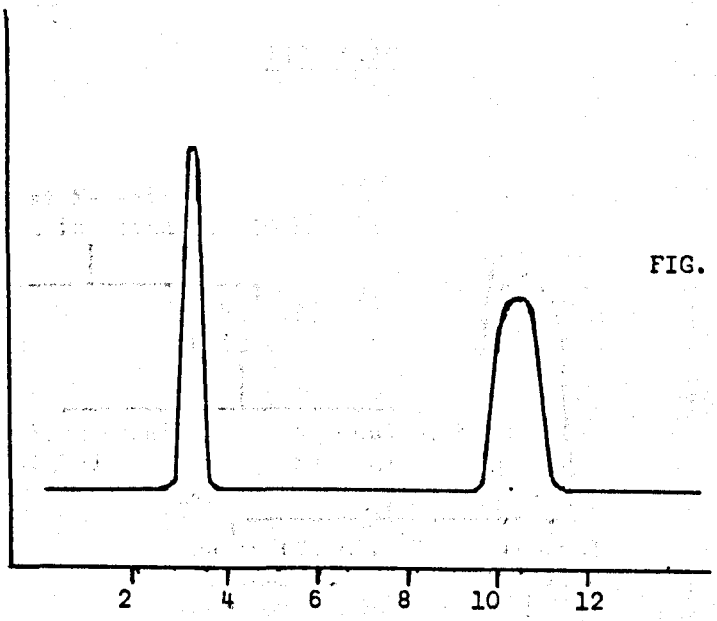


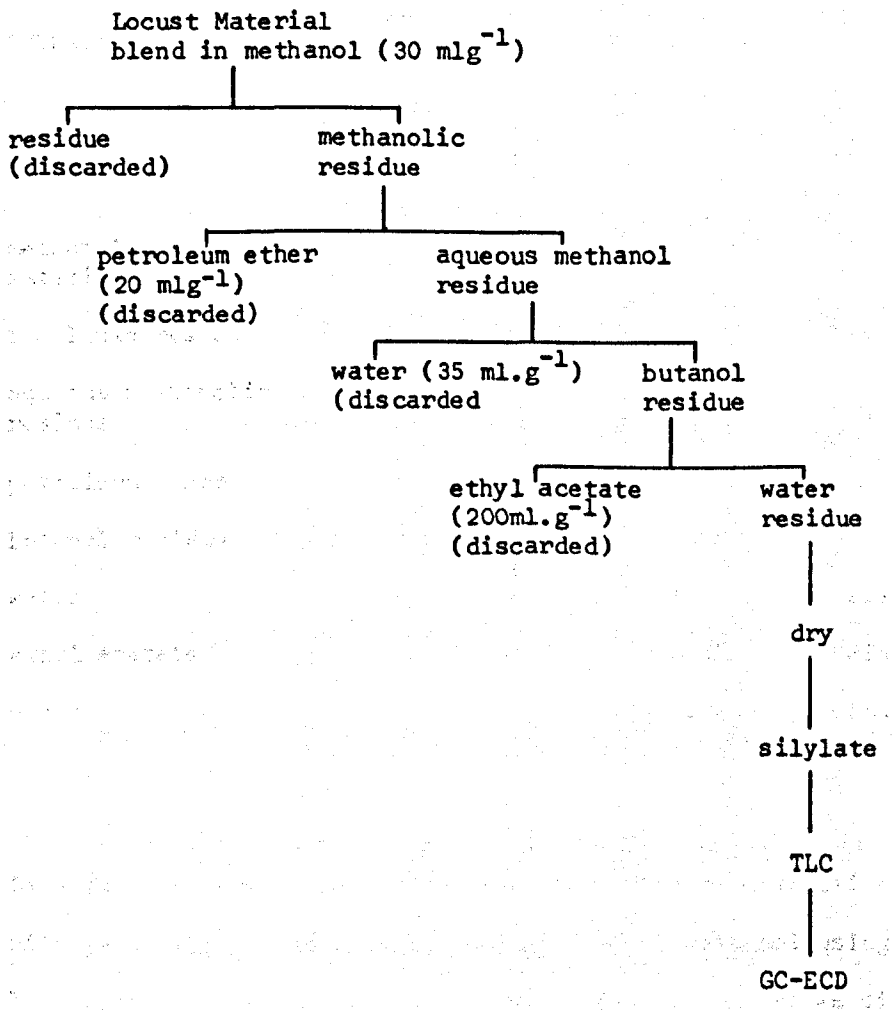
FIG. 6.18

The still relatively broad peak for ecdysterone is probably a consequence of the effect of diffusion in the stagnant mobile phase in the pores of the support making an important contribution to the mass transfer term. To overcome this problem the use of more efficient silica gel adsorbents is required. Using Porasil A, the peak shapes of 2β,3β,14α-trihydroxy--5β-cholest-7-en-6-one (R_t = 5.1 min) and ecdysterone (R_t = 12.5 min) with a mobile phase of dichloromethane-methanol (9:1, 1.6 ml. min^{-1})[61] are broad indicating poor efficiency.

EXPERIMENTAL.

The standard extraction procedure used for the analysis of ecdysones in desert locust material (Schistocerca gregaria) is given diagramatically in (Fig. 6.19).

FIG. 6.19



Typical volumes of partitioning solvents and weights of the extracted residues are given in Table (6.7) for ten adult locusts.

TABLE 6.7

WEIGHT OF EXTRACTABLE RESIDUES OBTAINED BY THE USE OF THE STANDARD EXTRACTION PROCEDURE FOR THE ANALYSIS OF TEN LOCUSTS.

PHASE	VOLUME (ml)	WEIGHT (g)	FATE
methanol extractable material	250	2.0 g	
insoluble residue	-	4.9 g	discarded
aqueous methanolic residue	40	0.8 g	
petroleum ether	40	1.2 g	discarded
butanol residue	30	0.15 g	
water	30	0.65 g	discarded
ethyl acetate	25	0.10 g	discarded
water	25	0.05 g	analysed

Live locusts were anaesthetized and frozen before extraction in liquid nitrogen. The frozen insects were blended in methanol using a liquidizer. The solid residue was removed by vacuum filtration (glass sinter porosity 4), the residue washed with methanol and the methanolic residue obtained by removal of solvent *in vacuo* at 50°C. The residue was distributed between equal volumes of light petroleum (b.p. 40-60°C) and aqueous methanol (20% methanol in water). The hydrocarbon phase was back-washed with a small volume of aqueous methanol and the combined phases evaporated *in vacuo* at 50°C. The solid residue was partitioned between equal volumes of water -saturated butanol and water. The aqueous phase was back extracted with a small amount of butanol, the combined butanol phases washed with a small

volume of water and the butanol phase evaporated to a residue in vacuo at 50°C. In a similar manner the butanol residue was partitioned between ethyl acetate saturated with water and water. The aqueous residue was dissolved in a small amount of "Spectrosol" methanol and transferred to a 1 ml. reacti-vial. The solvent was removed with a stream of nitrogen and the residue dried at 56°C and 0.01mm. for at least thirty minutes in a drying pistol. The residue was then silylated by the addition of 100µl of pyridine and 35µl of TMSIm and heated at 100°C for 6 h. Pyridine was removed with a stream of nitrogen, the silylating medium diluted with 200µl of toluene for chromatography.

Silica gel for TLC plates was extracted with methanol, dried and 20 x 20 cm plates prepared in the usual way and activated by heating at 120°C, for at least one hour and allowed to cool in a dry box. The material for analysis was streaked along one edge of a plate, and the plate developed with ethylacetate-toluene (3:7), the plate dried with a hair dryer and the area R_f 0.5-0.9 scraped off and packed into a narrow glass tube (10 x 1.1cm) containing a glass wool plug. The ecdysone TMS ethers were extracted by elution under gravity with 10 ml. of sodium-dried diethyl ether in a 10ml. conical centrifuge tube. The ether was removed with a stream of nitrogen at 35°C and the residue dissolved in purified toluene for injection. The GC separation was carried out on a 3ft. column of 2% OV-101 on Gas Chrom Q, nitrogen flow rate 85ml. min^{-1} and column oven temperature 270-280°C. The ECD was operated with a detector oven temperature of 300°C and pulse space 50µs. The quantity of ecdysones present was estimated by comparison with a calibration graph prepared with known quantities of pure ecdysterone and ecdysone.

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