A Thesis

entitled

"APPLICATIONS OF GAS CHROMATOGRAPHY - MASS SPECTROMETRY IN STEROID CHEMISTRY"

Submitted in part fulfilment of the requirements for admittance to the degree of

Doctor of Philosophy

in

The University of Glasgow

by

T.A. Baillie, B.Sc.

University of Glasgow 1973.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. C.J.W. Brooks for his guidance and encouragement at all times, and to Professors R.A. Raphael, F.R.S., and G.W. Kirby, for the opportunity to carry out this research.

Thanks are also due to my many colleagues for useful discussions, and in particular to Dr. B.S. Middleditch who was associated with me in the work described in Section 3 of this thesis.

The work was carried out during the tenure of an S.R.C. Research Studentship, which is gratefully acknowledged.

Finally, I would like to thank Miss J.H. Bennie who typed the manuscript, and Dr. J.D. Gilbert for photographing many of the diagrams which appear in this thesis.

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Certain sections of the work described in this thesis have been published:

T.A. Baillie, C.J.W. Brooks and B.S. Middleditch.
Comparison of corticosteroid derivatives by gas chromatography - mass spectrometry.
Analyt. Chem., 44, 30 (1972).

T.A. Baillie, C.J.W. Brooks and E.C. Horning.
O-Butyloximes and O-pentyloximes as derivatives for the study of ketosteroids by gas chromatography.
Analyt. Letters, 5, 351 (1972).

T A. Baillie, C.J.W. Brooks, E.M. Chambaz, R.C. Glass and C. Madani.
Comparison of isopentyloxime and benzyloxime trimethylsilyl ethers in the characterisation of urinary steroids of newborn infants.
Proceedings of the International Symposium on Mass Spectrometry in Biochemistry and Medicine, Milan, 7th -9th May, 1973; in press.

CONVENTIONS AND NOMENCLATURE

In the text that follows, numbers appearing as a superscript, followed by a single bracket thus [54)] denote references, while those written in line with the text, between paired brackets thus (48) indicate drawings of chemical formulae, and may be referred to in the text as 'structure (48)'. In drawings of structure, stereochemistry is not implied unless specifically indicated; a thickened or dotted bond denotes a substituent located respectively above or below the plane of the paper.

Wherever possible, trivial names of steroids have been used in the text, the systematic names of which are given in the list below. In Tables 19-21 and in Appendix I, the following abbreviations of steroid types have been employed:-

A = Androstane

A' = Androstene

P = Pregnane

P' = Pregnene

P" = Pregnadiene

Trivial Name	Systematic Name
Estetrol	3,15α,16α,17β-Tetrahydroxy-estra-1,3,5(10)-triene.
Estradiol	3,17β-Dihydroxy-estra-1,3,5(10)-triene.
Estriol	3,16α,17β-Dihydroxy-estra-1,3,5(10)-triene.
Estrone	3-Hydroxy-estra-1,3,5(10)-trien-17-one.
Androstenedione	4-Androstene-3,17-dione.
Androsterone	3α-Hydroxy-5α-androstan-17-one.
Dehydroepiandrosterone (DHA)	3β-Hydroxy-5-androsten-17-one.
Dianabol	17α-Methyl-17β-hydroxy-1,4-androstadien-3-one.
Etiocholanolone	3α-Hydroxy-5β-androstan-17-one.
Etiocholenic acid	17β-Carboxy-4-androsten-3-one.

16α-Hydroxydehydroepiandrosterone/

Trivial Name	Systematic Name
16α-Hydroxydehydroepiandrosterone (16α-OH-DHA)	3β,16α-Dihydroxy-5-androsten-17-one.
16β-Hydroxydehydroepiandrosterone (16β-OH-DHA)	3β,16β-Dihydroxy-5-androsten-17-one.
16-Ketoandrostenediol (16-Keto-AD)	3β,17β-Dihydroxy-5-androsten-16-one.
11-Ketoandrosterone	3α-Hydroxy-5α-androstane-11, 17-dione.
11-Ketoetiocholanolone	3α-Hydroxy-5β-androstane-11,17-dione.
5α-Dihydrotestosterone	17β-Hydroxy-5α-androstan-3-one.
Testosterone	17β-Hydroxy-4-androsten-3-one.
Aldosterone	11β,21-Dihydroxy-4-pregnene-3,20-dion-18-al.
Betamethasone	9α-Fluoro-16β-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione.
Cortexolone (Reichstein's S)	17α,21-Dihydroxy-4-pregnene-3,20-dione.
Corticosterone (Kendall's B)	11β,21-Dihydroxy-4-pregnene-3,20-dione.
Cortisol (Kendall's F)	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione.
Cortisone (Kendall's E)	17α,21-Dihydroxy-4-pregnene-3,11, 20-trione.
Cortol	5β-Pregnane-3α,11β,17α,20α,21-pentol.
β-Cortol	5β-Pregnane-3α,11β,17α,20β,21-pentol.
Cortolone	3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one.
β-Cortolone	3α,17α,20β,21-Tetrahydroxy-5β-pregnan-11-one.
11-Dehydrocorticosterone (Kendall's A)	21-Hydroxy-4-pregnene-3, 11,20-trione.
6,7-Dehydrodexamethasone	9α-Fluoro-16α-methyl-11β,17α, 21-trihydroxy-1,4,6-pregnatriene-3,20-dione.
11-Deoxycorticosterone (DOC)	21-Hydroxy-4-pregnene-3,20-dione.
Dexamethasone	9α-Fluoro-16α-methyl-11β,17α, 21-trihydroxy-1,4-pregnadiene-3, 20-dione.

Dihydrocortexolone (DHS)	17α,21-Dihydroxy-5β-pregnane-3,20-dione.
1,2-Dihydro-6,7-dehydrodexamethasone	9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-4,6-pregnadiene-3,20-dione.
Dihydrocortisol (DHF)	11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione.
allo-Dihydrocortisol (allo-DHF)	11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione.
Dihydrocortisone (DHE)	17α,21-Dihydroxy-5β-pregnane-3,11,20-trione.
20α-Dihydroprednisolone	11β,17α,20α,21-Tetrahydroxy-1,4-pregnadien-3-one.
9α-Fluorocortisol	9α-Fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione.
6α-Fluoroprednisolone	6α-Fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione.
9α-Fluoroprednisolone	9α-Fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione.
6β-Hydroxycortisol	6β,11β,17α,21-Tetrahydroxy-4-pregnene-3,20-dione.
16α-Hydroxypregnenolone	3β,16α-Dihydroxy-5-pregnen-20-one.
17α-Hydroxypregnenolone	3β,17α-Dihydroxy-5-pregnen-20-one.
21-Hydroxypregnenolone	3β,21-Dihydroxy-5-pregnen-20-one.
16α-Hydroxyprogesterone	16α-Hydroxy-4-pregnene-3,20-dione.
17α-Hydroxyprogesterone	17α-Hydroxy-4-pregnene-3,20-dione.
18-Hydroxyprogesterone	18-Hydroxy-4-pregnene-3,20-dione.
16-Ketopregnenolone	3β-Hydroxy-5-pregnene-16,20-dione.
Prednisolone	11β,17α,21-Trihydroxy-1,4-pregnadiene-3,20-dione.
Prednisone	17α, 21-Dihydroxy-1,4-pregnadiene-3,11,20-trione.
Pregnanediol	5β-Pregnane-3α,20α-diol.
Pregnanetriol	5β-Pregnane-3α,17α,20α-triol.
Pregnanolone	3α-Hydroxy-5β-pregnan-20-one.
Pregnenolone	3β-Hydroxy-5-pregnen-20-one.
Progesterone	4-Pregnene-3,20-dione.
Tetrahydrocorticosterone (THB)	3α,11β,21-Trihydroxy-5β-pregnan-20-one.
allo-Tetrahydrocortiscosterone (allo-THB)	3α,11β,21-Trihydroxy-5α-pregnan-20-one.

Tetrahydrodehydrocorti costerone (THA)	3α,21-Dihydroxy-5β-pregnane-11,20-dione.
allo -Tetrahydrodehydrocorticosterone (allo-THA)	3α,21-Dihydroxy-5α-pregnane-11,20-dione.
Tetrahydrocortisol (THF)	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one.
allo-Tetrahydrocortisol (allo-THF)	3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one.
Tetrahydrocortisone (THE)	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione.

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SOURCES OF REFERENCE COMPOUNDS AND REAGENTS

Dimethyldiacetoxysilane - gift from Dr. J.C. Orr, Huntington Laboratory, Massachusetts General Hospital, Boston, U.S.A.

Sodium bismuthate - gift from Dr. J.K. Norymberski, Unit for Endocrine Chemistry, Department of Zoology, the University, Sheffield.

The following reagents were donated by Dr. D.J. Outred (Beecham Research Laboratories):

sec-Butoxyamine hydrochloride (1-Aminoxy-1-methylpropane hydrochloride)

Isobutoxyamine hydrochloride (1-Aminoxy-2-methylpropane hydrochloride)

Isopentoxyamine hydrochloride (1-Aminoxy-3-methylbutane hydrochloride)

n-Pentoxyamine hydrochloride (1-Aminoxypentane hydrochloride)

Other alkoxyamines were commercial samples.

5α-Androstan-17-one, 5α-pregnan-20-one and 16-dehydropregnenolone were gifts from Dr. G.F. Woods (Organon Laboratories, Newhouse, Scotland).

3β,11β-Dihydroxy-5-androsten-17-one and 3β,11β,16α-trihydroxy-5-androsten-17-one were supplied by Dr. R.W. Kelly (MRC Cinical Endocrinology Unit, Edinburgh, Scotland).

The following steroids were donated by Professor W. Klyne and Dr. D.N. Kirk (MRC Steroid Reference Collection, Westfield College, London):-

5-Androstene- 3β,16β,17β-triol

3β,16β-Dihydroxy-5-androsten-17-one

5-Pregnene - 3β,17α,20β-triol

5-Pregnene - 3β,20α,21-triol

5-Pregnene - 3β,20β,21-triol

5-Pregnene - 3β,17α,20α,21-tetrol

5β-Pregnane - 3β,16β,20α-triol

3β,11β-Dihydroxy-5α-pregnan-20-one

3β,16α-Dihydroxy-5α-pregnan-20-one

3β,16α-Dihydroxy-5β-pregnan-20-one

3α,17α-Dihydroxy-5β-pregnan-20-one

3β,17α-Dihydroxy-5α-pregnan-20-one

3β,17α,21-Trihydroxy-5-pregnen-20-one

16α-Hydroxyprogesterone

18-Hydroxyprogesterone

Professor E.C. Horning (Institute for Lipid Research, Baylor College of Medicine, Houston, Texas, U.S.A.) kindly supplied the following compounds:-

5-Androstene-3β,11β,17β-triol
5-Androstene-3β,16α,17α-triol
5-Androstene-3β,16α,17β-triol
3β,16α-Dihydroxy-5-androsten-17-one
5-Pregnene-3β,16α,20α-triol
5-Pregnene-3β,17α,20α-triol
3β,16α-Dihydroxy-5-pregnen-20-one
3β,21-Dihydroxy-5-pregnen-20-one
3β,11β,17α,21-Tetrahydroxy-5-pregnen-20-one
3β,16α,17α,21-Tetrahydroxy-5-pregnen-20-one
5β-Pregnane-3α,6α,20β-triol
5β-Pregnane-3β,20β,21-triol
5β-Pregnane-3α,6α-diol-20-one
allo-THA
allo-THB

Other steroids used in this work were commeccial samples, obtained from the following suppliers:-
The Upjohn Co., Kalamazoo, Michigan, U.S.A.
Sigma Chemical Co. St. Louis, Missouri, U.S.A.
Koch-Light Laboratories Ltd. Colnbrook, Bucks, England.
Pfizer Ltd., Sandwich, Kent, England.
Eastman Organic Chemicals, Rochester, N.Y. U.S.A.
B.D.H. Laboratory Chemicals Division, Poole, England.
Ikapharm, Ramat-Can, Israel.
Schwartz-Mann Inc., New York, N.Y., U.S.A.
Fluka AG, Buchs, Switzerland.

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CONTENTS

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SUMMARY

Steroids have, for many years, been recognised as an important class of compounds, because of their occurrence in almost all living systems and widespread use in pharmaceutical preparations. Many steroids, e.g. the corticosteroids, possess hormonal activity, and their analysis in biological fluids is consequently of considerable interest.

In the work described in this thesis, gas—liquid chromatography (GLC) and combined gas chromatography-mass spectrometry (GC-MS) were applied to the analysis of steroids of biological significance, with particular interest centring on the corticosteroid family. As a result of the thermal lability of many of these compounds, particularly those possessing the "dihydroxyacetone" side-chain, modification of the basic structure is a prerequisite in any gas-phase analytical procedure. Of the two approaches currently employed in this area, namely oxidative cleavage of the corticosteroid side-chain, and conversion of functional groups to suitable derivatives, the latter technique was studied in some detail. The possible value of the 17α,21-anhydro (oxetanone) derivative in stabilising steroidal dihydroxyacetones for gas chromatography was investigated for fourteen representative examples of this type. Although these cyclic derivatives were found to possess desirable GC-MS properties, their application to analytical separations was hindered by the lack of a suitable preparative method. A number of other derivatives which have been described previously for the gas-phase separation of corticosteroids were also examined; these included trimethylsilyl (TMS) ethers, O-methyloxime trimethylsilyl (MO-TMS) ethers, acetonides, dimethylsiliconides and cyclic boronate esters. These derivatives were compared with respect to their ease of preparation, stability, retention characteristics and mass spectra. The technique of/

of "single ion monitoring" (SIM) was applied to the quantitative detection of five representative derivatives of Reichstein's Substance S. The sensitivity with which single, characteristic fragment ions in their mass spectra could be measured varied considerably between derivatives, the limit of detection in the most favourable case (the 17α,21-anhydro derivative) being 400 pg. However, the derivative of choice for general use was found to be the MO-TMS ether, on account of its convenience of preparation, range of detectable concentration and applicability to all six types of corticosteroid side-chain.

In view of the above findings on the suitability of oxime-TMS ethers for the characterisation of steroids by GC-MS, several O-alkylhydroxylamines were evaluated as reagents for the conversion of reactive ketosteroids to their O-alkyloxime derivatives. O-Butyloximes and O-pentyloximes proved to be useful in this respect by virtue of their structurally informative mass spectra and the convenient increments in retention time accompanying their formation. In particular, O-isopentyloxime trimethylsilyl (iPO-TMS) ethers were of value in affording gas-phase "group" separations of keto- from hydroxysteroids. This type of derivative, together with the O-benzyloxime trimethylsilyl (BO-TMS) ether, was applied to the GC-MS determination of urinary steroid profiles from newborn infants: samples from both normal and pathological cases were examined. An advantage of the iPO-TMS ethers in profile analysis was that derivatives of steroidal diketones were eluted at normal operating temperatures, whereas di-BO derivatives remain undetected due to their excessively long retention times. In the "glucuronide" fraction from a normal infant, three such compounds were detected and partially characterised.

The results presented in this thesis confirm the scope and potential of GC-MS in steroid chemistry. In particular, the technique affords a highly powerful means by which complex mixtures,/

mixtures, such as steroid hormone metabolites in urine, may be examined. The sensitivity and selectivity of GC-MS in this area may be enhanced by single or multiple ion detection methods, and by the use of high-efficiency GLC solumns. The current development of relatively low-cost GC-MS systems could well provide the clinical chemist with an invaluable tool for both routine analysis, and metabolic research purposes.

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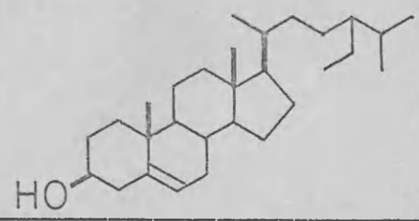
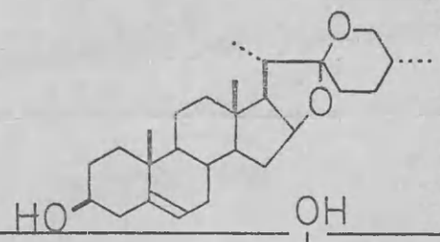
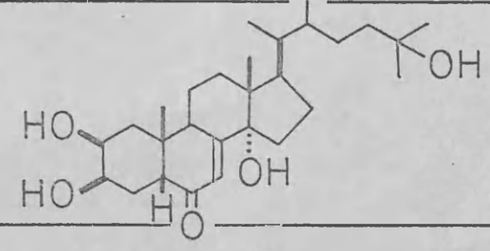
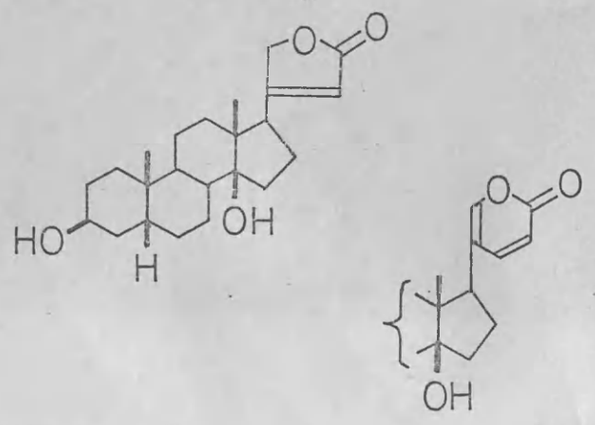
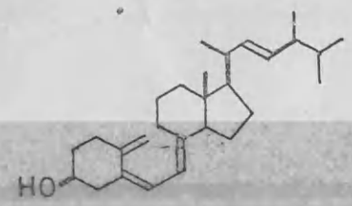
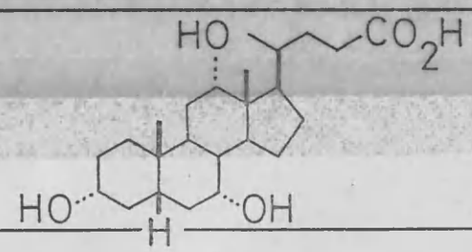
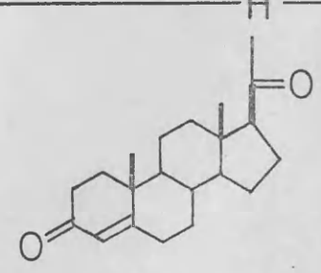
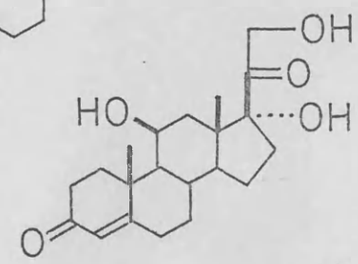
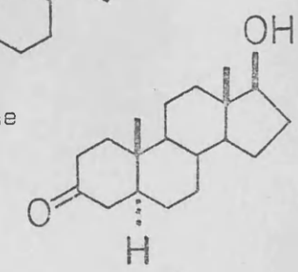
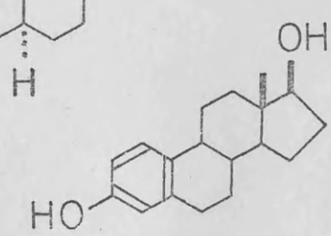
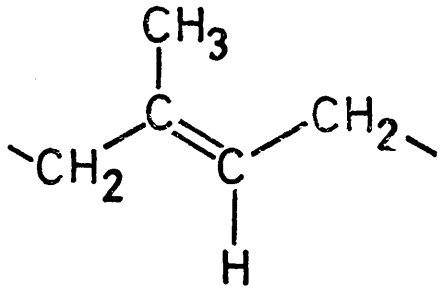
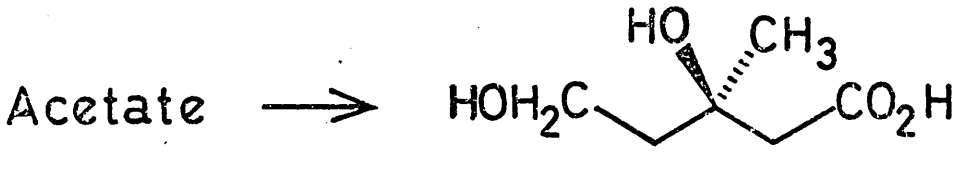
Group	No. of C atoms	Representative Structure	Occurrence and properties
Sterols	27-29	 Sitosterol	Sterols are structurally the most similar to cholesterol itself, and are abundantly distributed in the plant an animal kingdoms.
Steroid sapogenins	27	 Diosgenin	Characterised by spiroketal moiety and occur in plants as glycosides. Diosgenin is an important starting material for the commercial preparatio of steroid hormones.
Insect-Moulting Hormones	27-29	 Ecdysone	Occur in both animals and plants, and control moulting in insects. Their common feature is the Δ^7-6-keto function and 14 α-hydroxyl group.
Cardiac genins: (a) Cardenolides	23	 Digitoxigenin	They occur in certain plant, animal and insect families as glycosides, and exert a specific and powerful action on the cardiac muscles on injection in man or animals. They are characteris by their 14 β-hydroxyl group and unsaturated lactone ring.
(b) Bufadienolides	24	Bufalin	
Vitamin D series *	27-29	 Vitamin D_2	Compounds in this series are produced by U.V.-irradiation of $\Delta^{5,7}$-unsaturated sterols, and exhibit antirachitic activity.
Bile acids	24	 Cholic acid	When conjugated with amino acids, thes compounds play an important role in lipid metabolism. In man, approx. $\frac{1}{2}$ of the body cholesterol is catabolised to bile acids and eventually removed via the faeces.
Steroid hormones: (a) Progesterone	21	 Progesterone	See text
(b) Corticosteroids	21	 Cortisol	
(c) Androgens	19	 5 α-Dihydrotestosterone	
(d) Estrogens	18	 Estradiol	

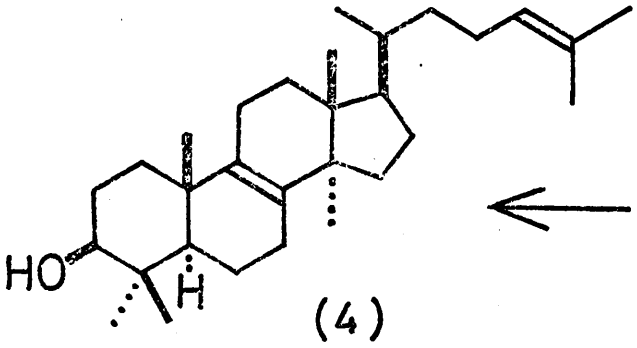
Table 1: The major steroid classes, grouped according to structural characteristics.
* These are seco-steroids, and not true steroids.



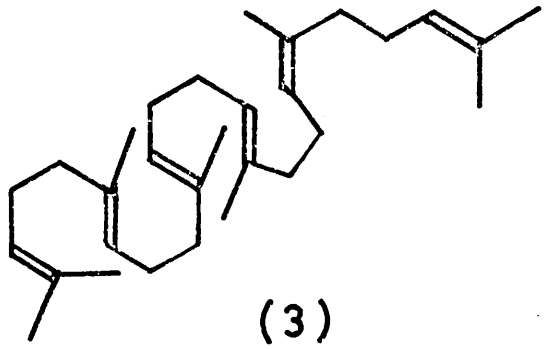
(1)



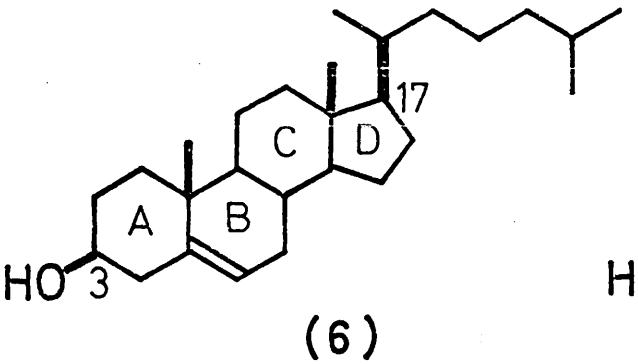
(2)



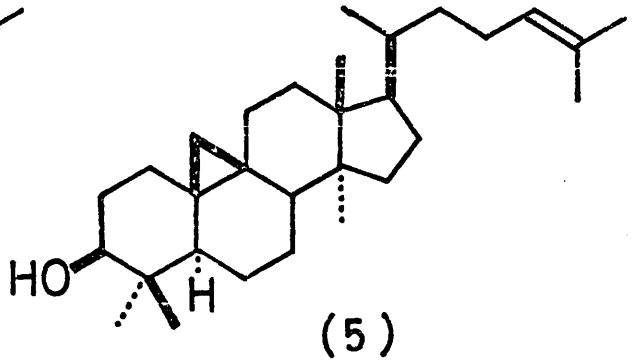
(4)



(3)



(6)



(5)

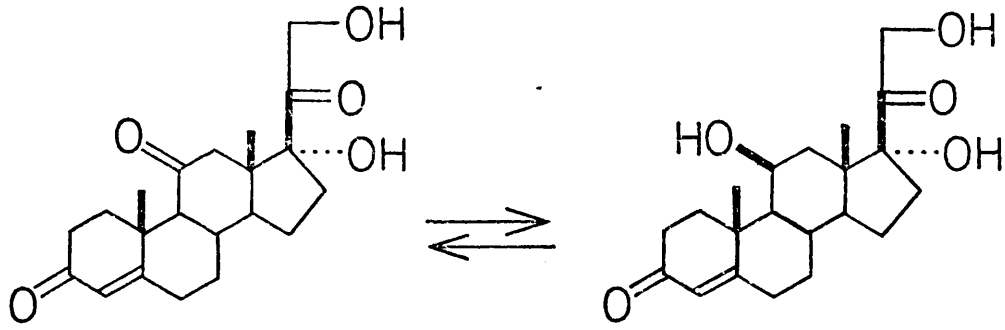
Fig.1: The biosynthesis of cholesterol (6).

1.1 GENERAL INTRODUCTION

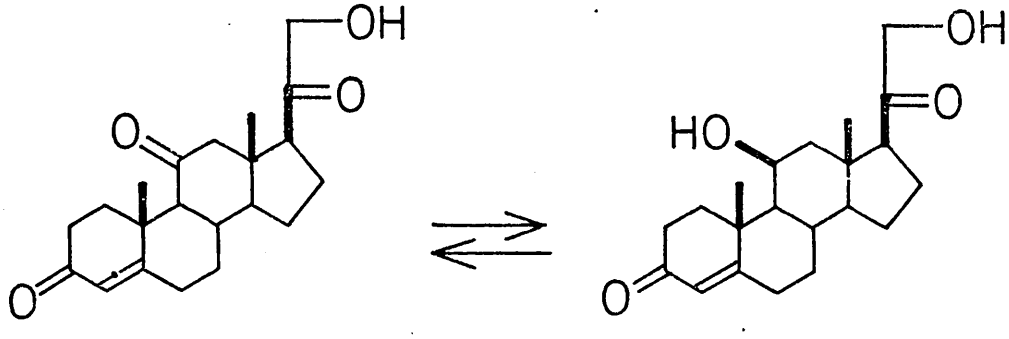
1.1.1 STEROIDS: THEIR OCCURRENCE AND SIGNIFICANCE

Steroids belong to a class of organic compounds known as terpenoids or polyisoprenoids, a vast family of substances which have one common link: they are (or are presumed to be) biogenetically derived from the same parent "building block", the five-carbon "isoprene unit" (1).[1-3] The biosynthesis of cholesterol (6), a key intermediate in the formation of almost all naturally occurring steroids, has been studied in great detail.[4,5] The pathway (fig.1) has been shown to proceed in four phases: synthesis of (-) mevalonic acid (2) from thiol esters (mainly acetyl-Coenzyme A), formation of squalene (3) - essentially a polyisoprene chain - *via* pyrophosphate derivatives, and then stereospecific cyclisation. These three steps result, in animals, in the formation of lanosterol (4) (while the closely related triterpenoid cycloartenol (5) is produced in plants). Finally, loss of three carbon atoms (though oxidative eliminations) from the tetracyclic skeleton, and formation of the Δ^5-stenol system produces cholesterol.

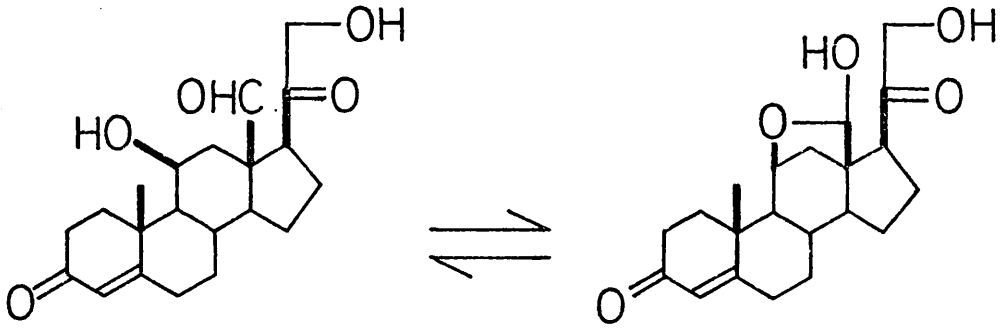
Cholesterol is present in practically all living organisms, and further elaboration of its structure (or of the structure of its immediate precursors) by, *e.g.* oxidation and side-chain cleavage, affords a host of compounds, which may be grouped according to their basic structure (Table 1).[6,7] One such group, the steroid hormones, is of particular importance, and a brief discussion of the main types is relevant to the work presented later in this thesis.



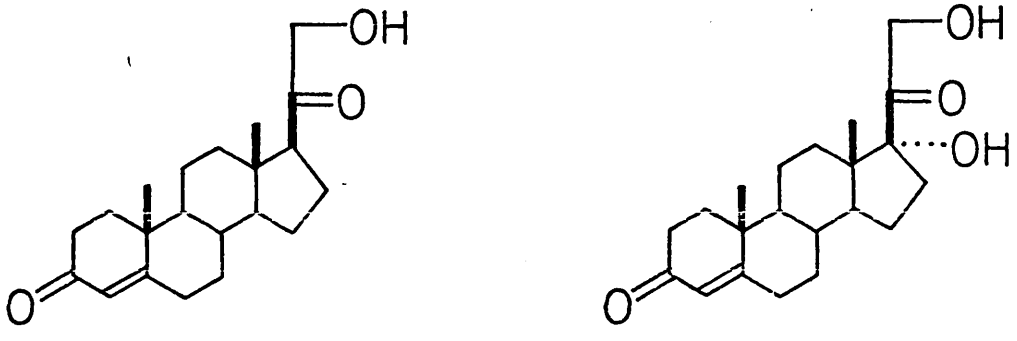
Cortisone (E) Cortisol (F)



11-Dehydrocorticosterone (A) Corticosterone (B)



Aldosterone



11-Deoxycorticosterone (DOC) Cortexolone (S)

Fig.2: The adrenocortical hormones.

1.1.2 THE STEROID HORMONES

This group comprises progesterone, the corticosteroids, androgens and estrogens (Table 1).

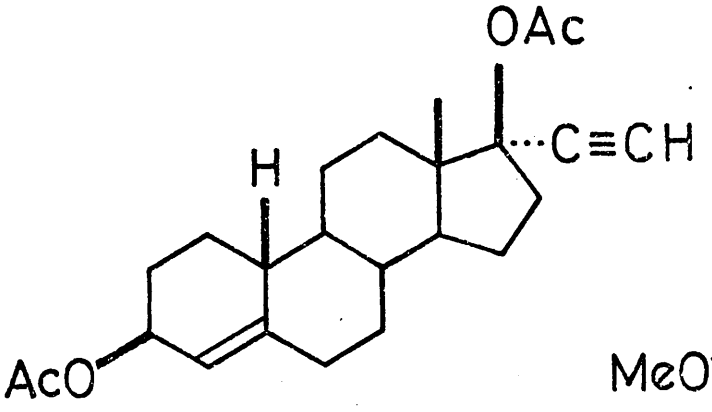
Progesterone is a key intermediate in the biosynthesis of steroids with 21 or fewer carbon atoms. It is closely associated with the reproductive process in mammals, creating a favourable medium for the implantation of the fertilized ovum and the maintenance of pregnancy.

Fig. 2 shows the seven adrenocortical hormones which are produced by the human adrenal gland. Of these, cortisol, corticosterone and aldosterone are the three principal corticosteroids secreted into the bloodstream. These compounds have profound effects on many metabolic processes in animals and possess two main types of activity: mineralocorticoid activity, where the electrolyte balance is controlled through sodium retention, and glucocorticoid activity, which deals with protein catabolism. While aldosterone is the most potent mineralocorticoid, cortisol is the prominent glucocorticoid hormone.

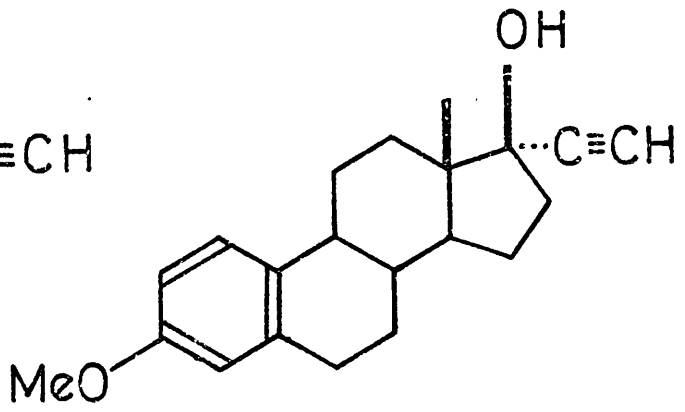
The androgens are C_{19} steroids and result from the complete removal of the side-chain attached to C-17 in cholesterol. They stimulate the development of the male reproductive organs and secondary sex characteristics, and also possess anabolic activity.

Finally, the estrogens are the corresponding female sex hormones. Their biosynthesis from C_{19} precursors involves oxidative elimination of the C-19 methyl group, followed by aromatisation of ring A. During pregnancy, estrogens act with progesterone to maintain gestation.

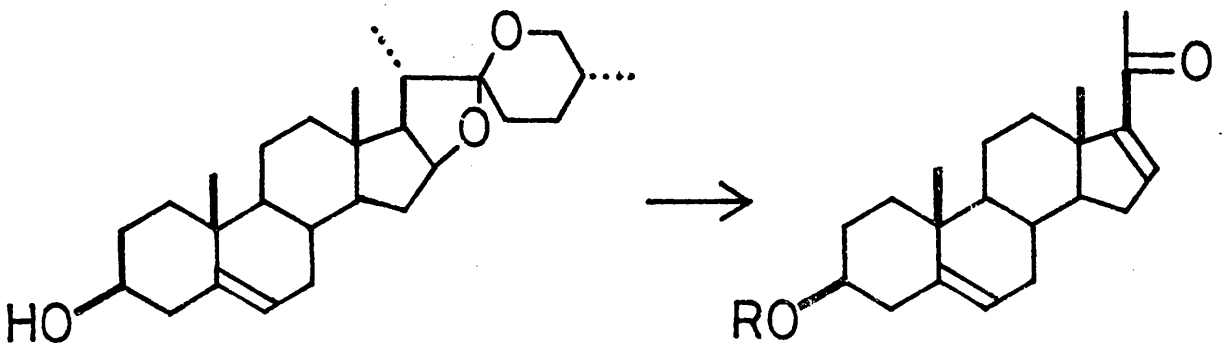
In addition to the naturally occurring hormones, a/



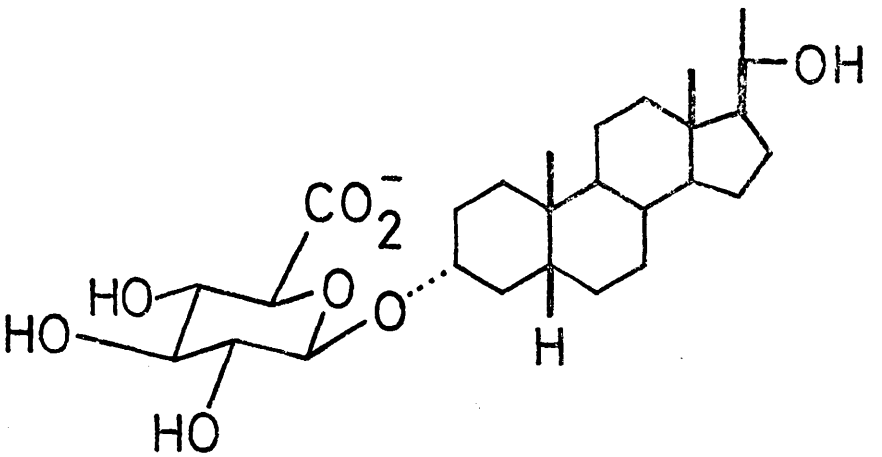
(7)



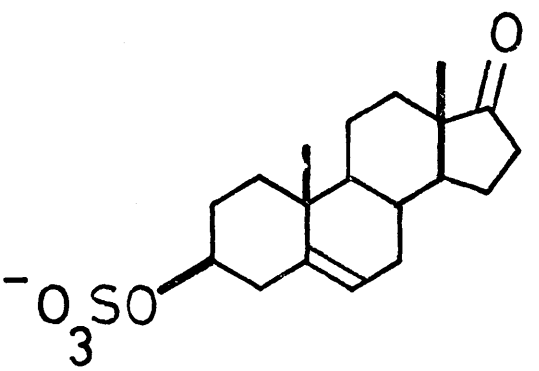
(8)



(9) (10)



Pregnanediol 3-glucuronide



Dehydroepiandrosterone sulphate

Fig.3: Steroid Conjugates.

a large number of synthetic analogues have been produced by the pharmaceutical industry for therapeutic use. The demonstration, in 1949, of the anti-arthritic effects of cortisone acted as a powerful stimulus to drug research. However, attempts to separate the beneficial anti-inflammatory properties of corticosteroids from their glucocorticoid activity have been largely unsuccessful. In view of the undesirable side-effects associated with corticosteroid therapy, the present trend in pharmaceutical research is towards non-steroidal anti-inflammatory drugs. However, analogues of progesterone, e.g. ethynodiol diacetate (7) have found widespread use as ovulation inhibitors. When combined with a small amount of an estrogen, such as the synthetic compound mestranol (8), highly effective oral contraceptive preparations result. Diosgenin (9), from Mexican barbasco root (Discorea tubers), is an important starting material for the commercial preparation of synthetic hormones. Degradation of this sapogenin affords 16-dehydropregnenolone acetate (10: R=Ac) from which a variety of C_{21} and C_{19} steroid hormones can be prepared.[8)]

1.1.3 METABOLISM OF STEROID HORMONES

The metabolism of corticosteroids in man occurs primarily in the liver and takes place in two stages. Firstly, the hormone is inactivated by reduction of the α,β - unsaturated carbonyl group to yield predominantly the 3α-hydroxy-5β-pregnane derivative. (Reduction at C-20 may also occur). Secondly, the 3α-hydroxyl group is conjugated, through the action of a glucuronosyl transferase and uridine diphosphoglucuronic acid (UDP-glucuronic acid).[9)] This yields the water-soluble glucuronide (fig. 3) which is finally excreted, via the kidneys, into the urine.

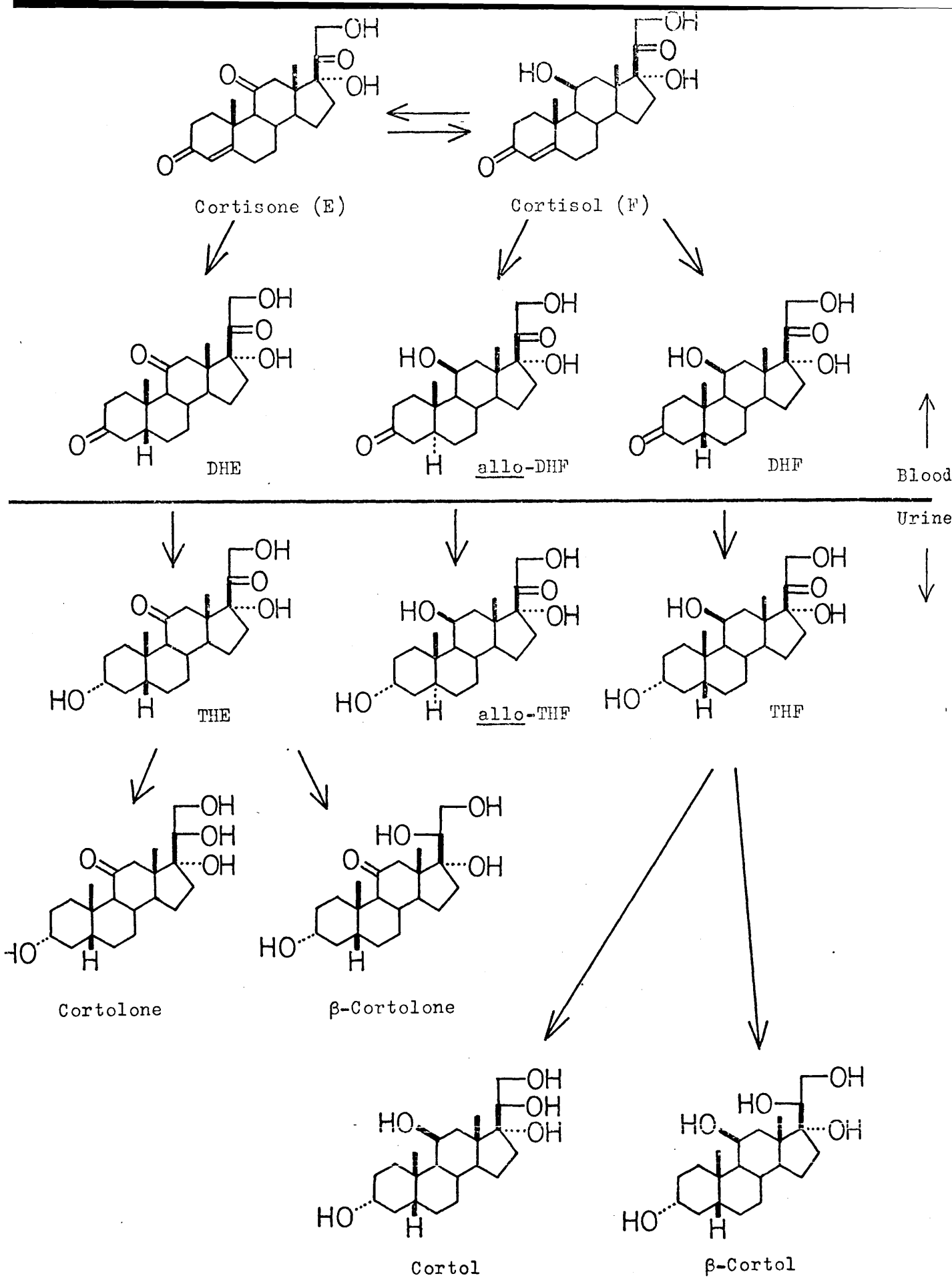


Fig.4: The metabolism of cortisol.[7)]

A minor fraction is also conjugated with sulphuric acid, transferred from 3-phosphoadenosine 5-phosphosulphate (PAPS) by a sulphokinase enzyme [9], while an even smaller amount of the parent hormone is excreted either unchanged, or as its 6β-hydroxylated derivative. The major metabolites of cortisol (F), readily interconvertible in the body with cortisone (E), are shown in fig.4. Analogous metabolites are obtained from corticosterone (B) and dehydrocorticosterone (A), while the principal aldosterone metabolite is the 3α, 5β-tetrahydro compound.

An alternative pathway for the metabolism of 17-hydroxylated corticosteroids involves degradation to the corresponding 17-ketosteroid; this provides a route to the androgens, which are themselves metabolised by reduction and subsequent conjugation. Androgens with a 3β-hydroxyl group, such as dehydroepiandrosterone (DHA) are generally excreted as sulphate conjugates (fig.3).

The estrogens may be combined with sulphuric or glucuronic acid, or both. The sulphate group is invariably attached to C-3, but the glucuronide moiety may be conjugated with any of the free hydroxyl groups of estrogens.

1.1.4 THE ANALYSIS OF STEROIDS IN URINE

In order to obtain an indication of the rate of steroid production from a given endocrine gland, e.g. the adrenal gland, numerous methods have been devised for the estimation of hormone metabolites in urine. This approach, however, assumes the existence of a direct relationship between the level of active hormone in blood and that of its inert metabolite in urine, [10] and it is essential for the validity of urinary assays that some fairly constant relationship does exist between the two. Furthermore/

Furthermore a certain urinary metabolite may originate from more than one source; a given hormone may be secreted by various glands, while different hormones may ultimately give rise to the same urinary metabolite. Consequently, results based on urinary steroid levels should be treated with some caution when assessing endocrine performance.

R. Borth[11] has suggested four criteria by which the validity of a given analytical method may be judged. These are:

Precision - the measure of agreement between replicate estimations.

Accuracy - the extent of agreement between the measured amount and the actual amount of compound in the sample.

Sensitivity - the least amount of the substance which can be measured.

Specificity - the extent to which the procedure measures only the substance in question.

The use of modern analytical methods has greatly improved specificity in steroid assays, although accuracy can still be a problem due to uncertainties in the techniques used for sample preparation.

Normally, procedures for the analysis of steroids in urine entail three stages. Firstly, steroid conjugates are cleaved; sulphate esters may be solvolysed, while glucuronides require a hydrolysis step - this may be achieved either by treatment with hot mineral acid, or, preferably, by incubation with a suitable enzyme preparation. An advance in this area came with the introduction of Amberlite XAD-2, a neutral, cross-linked polystyrene resin.[12] On passing a urine sample through a column of this material, free steroids and their conjugates are selectively retained, while other components e.g. urea and uric acid, are eluted. The steroid fraction is recovered by washing the column with an alcohol, such as/

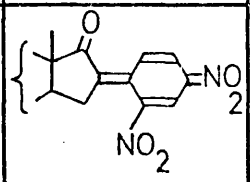
Name of Reaction	Substrate	Reagent	Product and max.	Ref.
Kober	estrogens	hydroquinone in aq. H_2SO_4	oxidised form 515-520nm	16, 17
Zimmermann	17-ketosteroids	m-dinitro-benzene in alkali	 520nm	18, 19
Tetrazolium	20,21-ketols	blue tetrazolium	a formazan 525 nm	20, 21
Porter-Silber	17 α,21-diol-20-ones	phenylhydrazine in aq. H_2SO_4	phenylhydra-zone 410 nm	22, 23

Table 2: Principal colour reactions in steroid analysis.[24)]

as methanol, and the conjugates are hydrolysed as before. This procedure thus eliminates many non-steroidal impurities at an early stage. Quantitative aspects of steroid losses on XAD-2, either by non-adsorption or by failure to elute, have been studied by J.A. Luyten[13]. Using ^{14}C-estriol and ^{14}C-DHA, recoveries of 99% and 88%, respectively, were obtained from the column.

Secondly, a fractionation or purification step is often desirable to reduce the complexity of the mixture and to eliminate, as far as possible, material which may interfere with the subsequent analysis. Various chromatographic techniques have found widespread application in this connection, e.g. column chromatography on alumina or silicic acid, paper chromatography and thin-layer chromatography.[14, 15] A combination of some of these techniques is frequently necessary to obtain extracts of sufficient purity, depending on the analysis procedure chosen.

Thirdly, the individual steroids, or groups of structurally-related steroids of interest, are determined using an appropriate method. Most of the classical methods for steroid determination (many of which are still in routine use) centre on the colour reaction produced when some specific reagent reacts with a group of steroids, each of which possesses a particular structural feature. The intensity of the colour produced is directly proportional to the amount of substrate present, and may be measured by colorimetric methods. The four most important colour reactions in steroid analysis are summarised in Table 2.

Numerous variations on these basic procedures, aimed at increasing the sensitivity and specificity of the original method, have been published. For example, the Zimmermann reaction may be extended to determine the so-called "17-ketogenic" steroids,/

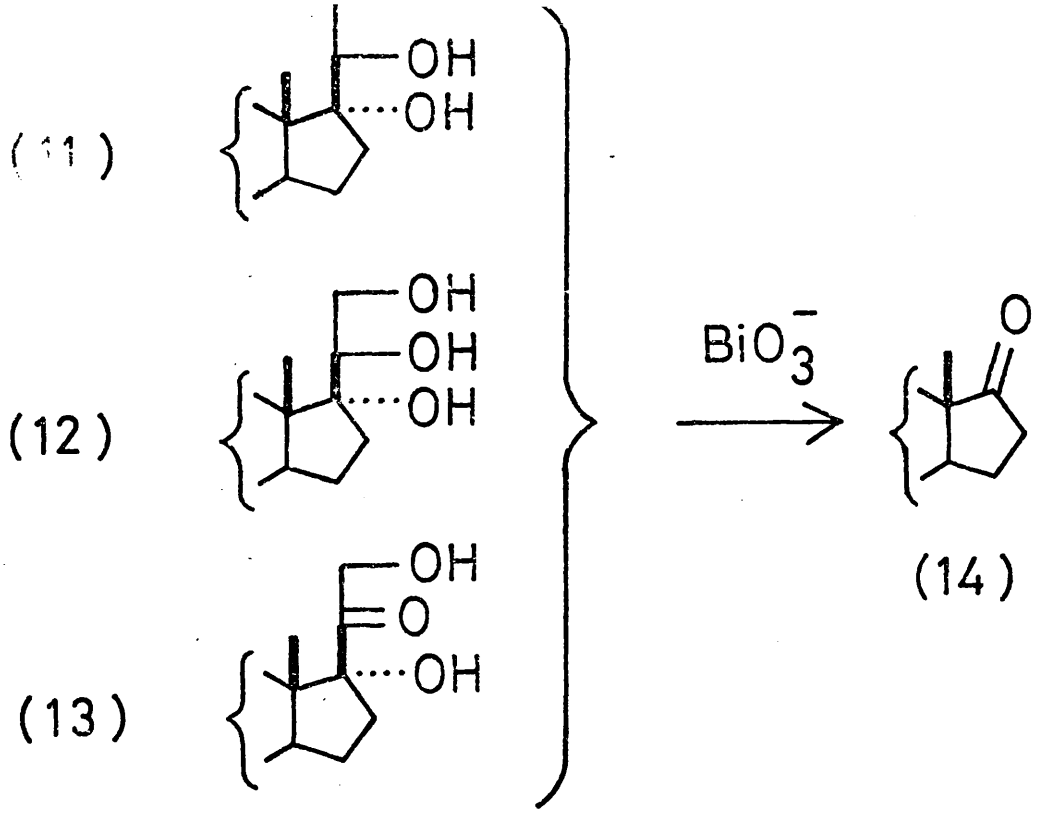


Fig.5: Side-chain cleavage with bismuthate.

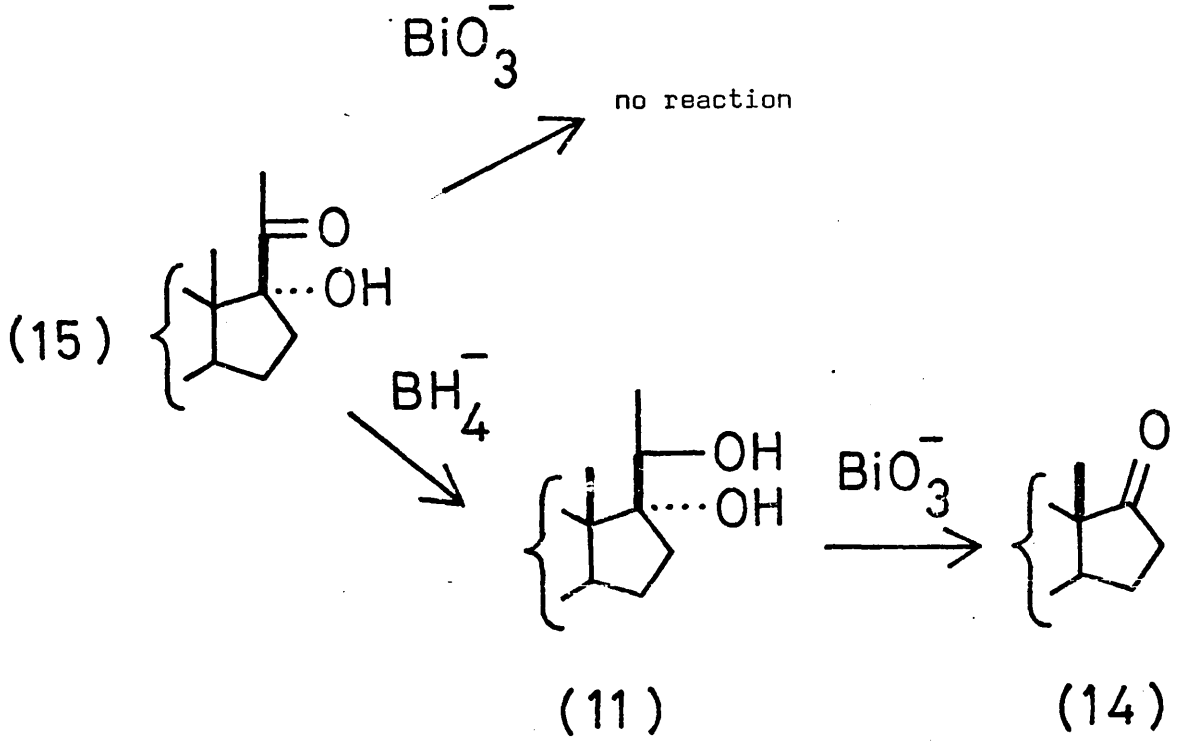


Fig.6: Reduction - oxidation sequence for 20, 17-ketols.

steroids, which possess 17α,20-diol (11), 17α,20,21-triol (12), or 17α,21-diol-20-one (13) side-chains (fig. 5). These compounds undergo oxidation with sodium bismuthate, which results in side-chain cleavage and formation of the corresponding 17-ketones (14).[25,26] Thus, an analysis of the 17-ketosteroid content of aliquots of an extract prior to, and after bismuthate oxidation affords an estimate of these types of adrenal steroids.[27,28] A further elaboration of this approach is described by Appleby et al.[29], where a sodium borohydride reduction precedes the bismuthate cleavage. In this determination, 20,17-ketols (15) are also included since they are reduced to the 17α,20-diols and therefore also undergo subsequent oxidation to the 17-ketones (fig.6). Furthermore, the 17-ketosteroids present in the original extract are reduced to 17-hydroxysteroids, so that in the final Zimmermann reaction one obtains a measure only of 17-hydroxylated corticosteroids. Alternatively, 20,17-ketols may be selectively determined by a three-step bismuthate/borohydride/bismuthate sequence (fig.6.).[30] The 17-ketosteroids so produced originate only from this type of side-chain. Reviews of these selective degradations of various corticosteroid side-chains are given by J.K. Norymberski [31] and in the textbook by A.I. Scott.[24]

Ultra-violet spectrometry may be used to quantify steroids possessing a suitable chromophore, the Δ^4-3-one being the most common system, while methods based on fluorescence have been applied to the determination of e.g. cortisol [32] and the estrogens.[33] However, all analytical procedures which depend on the measurement of light absorption (or emission) suffer from the disadvantage that trace impurities frequently interfere with the determination. Consequently, meticulous purification of extracts is often necessary prior to the final estimation. Nevertheless, these classical /

classical methods are considered to yield data sufficiently accurate for diagnostic purposes in many cases.

Techniques based on radioisotopic dilution procedures have proved valuable in the estimation of certain steroids which are normally present in biological fluids at very low concentrations, e.g. aldosterone.[34] A higher degree of sensitivity, and also selectivity, may be achieved by this method. In recent years, however, considerable interest has focused on assay procedures which utilise the interaction between steroids and specific binding proteins. These methods are known as saturation analysis techniques; the term competitive protein binding (CPB) is used where the protein is a natural binding protein, and radioimmunoassay where the protein is an antibody. Minute amounts (10-100pg) of some hormones may be estimated in this way, and although the procedures were initially developed for plasma samples, they have been shown to be equally applicable to urinary extracts. A feature which often complicates such analyses is the presence of interfering binding proteins, or interfering steroids, in the sample under study. Rigorous pre-purification of the extract is therefore necessary, and chromatography on Sephadex LH-20 has been found useful in this respect.[35,36] Thus, CPB and radioimmunoassay offer highly sensitive and selective methods for the determination of those steroids for which a suitable binding protein exists. General details of these methods are given in reviews by Diczfalusy [37] and Midgley and Niswender.[38]

1.1.5 GAS-LIQUID CHROMATOGRAPHY (GLC) AND COMBINED GAS CHROMATOGRAPHY - MASS SPECTROMETRY (GC-MS) IN STEROID ANALYSIS

Although GLC was introduced in 1952,[39] it was not /

not until several years later that the first application in the steroid field was reported.[40] Significant early developments in the technique were those of columns containing 1-3% of thermostable polysiloxane stationary phases,[41] and the flame ionisation detector [42] for quantitative work with small (μg or sub-μg) samples. The introduction of derivatives, particularly trimethylsilyl ethers,[43] was found to overcome many of the problems associated with the low volatility of steroids, and their susceptibility to decomposition on GLC columns. Accurately determined retention data were shown to be of great value for characterisation and structural elucidation purposes, and have been widely applied in the steroid field. These advances, together with improved methods of column preparation,[44] led to a rapid increase in the use of GLC in steroid analysis.

Greater sensitivity has subsequently been achieved by the use of the electron capture detector (ECD)[45] in conjunction with suitable aromatic and halogen-containing derivatives, e.g. chloroacetates, [46,47] chloromethyl dimethylsilyl ethers [48] and heptafluorobutyrates.[49] By this method, detection limits of the order of 5pg [50] may be attained. However, where such high sensitivity is desired, a more extensive sample pre-purification is necessary, together with a modified GLC injection system incorporating a by-pass valve to eliminate the solvent front.

The next major advance in the gas-phase analysis of steroids came with the development of the mass spectrometer as a GLC detector. In some aspects, the effluent from a GLC column is suitable for study by a mass spectrometer i.e. the sample is eluted in a gaseous state, separated from other components of the mixture, and present in amounts (normally 1-5μg) which are adequate to/

to yield interpretable mass spectra. However, the high gas flow and relatively low sample concentration in the column effluent are undesirable features. Some form of interface between the two instruments was clearly necessary, particularly as the operating pressure in a mass spectrometer ion source is in the region of 10^{-9} atmospheres. Further criteria for the mass spectrometer as a GLC detector were that the scan must be fast enough (1-5 sec) to be completed before the composition or concentration of the effluent changed significantly. Also the accessible mass range for steroid work had to be at least 1-750 atomic mass units (a.m.u.). These problems were effectively overcome by the development of the "molecule separator" and quick-scan system of R. Ryhage,[51] which resulted in the creation of the first practical GC-MS system for steroids. Thus, the combined technique involves the separation of a mixture and the study, by mass spectrometry, of its components. By the use of suitable internal standards, quantification and positive identification of individual compounds may be achieved. (The role of derivative formation in structural studies will be discussed in section 3). The potential of the method, especially for the analysis of complex mixtures such as one obtains in biological fluids, was quickly appreciated.[52,53] The mixed O-methyloxime-trimethylsilyl ether (MO-TMS) derivatives, introduced by Gardiner and Horning,[54] stabilised the thermally-labile "dihydroxyacetone" side-chain and hence permitted the direct analysis of corticosteroid metabolites in urine. By employing a temperature-programmed GLC separation, a urinary steroid "profile" could be obtained, affording a multi-component analysis of androgen, estrogen and corticosteroid (as well as drug) metabolites.[55-57] This facility to determine simultaneously/

simultaneously individual hormone metabolites is highly desirable for diagnostic purposes. Thus, while variations in the urinary levels of certain structurally-related compounds may indicate a certain pathological condition, group determinations based on colour reactions would fail to detect any alteration in the normal metabolic process where the total values lie within the normal range.

The sensitivity and specificity of a GC-MS combination may be greatly enhanced by the techniques of single ion monitoring (SIM)[58-60] and multiple ion detection (MID).[61-65] When used in the former mode, the mass spectrometer is focused to detect ions of a single m/e ratio, which are chosen to be sufficiently characteristic of the compounds concerned. Detection limits in the order of 15-20pg have been obtained by this method in suitable cases.[66,67] In multiple ion detection, two or more ions (within a moderate range of m/e values) may be measured by alternate focusing; when employing this mode of operation, one of the ions monitored is frequently the corresponding ion of a deuterated analogue introduced as an internal standard.[68]

In conclusion, it may be said that the combined technique of GC-MS is one of the most powerful analytical tools available to the chemist. Currently its capabilities are being expanded by the use of high-efficiency capillary GLC columns,[13, 69, 70] quadrupole-type mass spectrometers,[71] chemical ionisation sources,[72] and data processing by computers.[67, 73, 74] Some applications of the technique in steroid chemistry will be presented in the following sections.

1.2 GENERAL EXPERIMENTAL METHODS

1.2.1 CHROMATOGRAPHIC AND SPECTROSCOPIC PROCEDURES *

Thin-layer chromatography (TLC) was carried out on glass plates (20x20cm or 5x20cm), coated with "Kieselgel-G" (E. Merck AG, Darmstadt, Germany), using 0.25mm layers for analytical purposes and 1.00mm layers for preparative use. The following reagents were used for the detection of bands:-

5% (w/v) Ceric sulphate in 10% sulphuric acid - after spraying the developed chromatograms with this reagent, the plates were heated in an oven at 130° for 1-2 min. This gave rise to coloured bands, the colours frequently being diagnostic for the compounds present. Prolonged heating of the chromatograms, however, caused the bands to turn black or brown.

2,4-Dinitrophenylhydrazine - this was used for the detection of compounds containing free aldehyde or keto groups, which stained yellow or orange without heating. The reagent was prepared by suspending 1g of powdered 2,4-dinitrophenylhydrazine in 30ml of stirred methanol, and cautiously adding 2ml of concentrated sulphuric acid.

Iodine-vapour - brief exposure of the chromatogram to iodine vapour was sufficient to locate the bands. This method was used routinely in preparative TLC, being non-destructive in nature.

An alternative procedure for preparative work was to use silica gel with an added fluorescent material - "Kieselgel HF_{254}". In this case, bands were located by their quenching of the fluorescence of the modified layer when viewed under an ultraviolet lamp. This method is highly sensitive for compounds containing a chromophore, and was found particularly suitable for the detection of Δ^4-3-keto steroids.

* These details are generally applicable throughout this thesis.

Table 3: Instruments and Columns for GLC

Type of Instrument	Length and Shape of Columns	Packings used
Pye Series 104	5' spiral	1% OV-1 1% Dexsil- 300GC
Perkin-Elmer F-11	6' spiral	1% OV-1 1% OV-17
Carlo Erba Model GV	12' W-shaped	1% OV-1 1% Dexsil-300GC.

Gas-liquid chromatography (GLC) was performed on one of the three dual-column instruments listed in Table 3, each of which was fitted with flame ionisation detectors. Columns were made up from Pyrex glass tubing, 3mm i.d., and were 5ft, 6ft, or 12ft in length. Packings were prepared by the methods of Horning *et al.*,[44] using the following stationary phases:-

1. 1% OV-1 and 1% SE-30 (Methyl siloxane polymers)
2. 1% OV-17 (phenyl/methyl siloxane polymer (50% phenyl))
3. 1% Dexsil -300 GC (poly - *m* - carboranylene siloxane)

The OV-1, SE-30 and OV-17 phases were obtained from Applied Science Laboratories Inc., State College, Pennsylvania, U.S.A., while the Dexsil - 300GC was supplied by Analabs Inc., North Haven, Connecticut, U.S.A. In all cases, the solid support used was Gas Chrom Q, 100-120 mesh (Applied Science Laboratories Inc.).

Samples for GLC were prepared, mainly in ethyl acetate solution, at a concentration of 1-3 mg/ml. Aliquots ranging from 0.2 - 5μl in volume were injected by means of a 10μl Hamilton syringe. Nitrogen was used as the carrier gas, with a flow rate of 40 ml/min, and the injection port heaters were maintained at temperatures between 20^{o} and 50^{o} higher than that of the analyser oven.

n - Alkanes, co-injected with the samples, were used to standardise the retention data of the steroids studied: the values are expressed as Kováts retention indices.[75]

Mass spectra were recorded, at electron energies 22.5 eV and 70 eV, using an LKB 9000 gas chromatograph - mass spectrometer (LKB-Produkter AB, Bromma, Sweden). This instrument is equipped with a two-stage jet separator of the Becker-Ryhage type which enables removal of 99% to 99.5% of the carrier gas (helium) and retention of 50% to 75% of the sample eluted from the gas chromatographic column.

The separator and ion source were maintained at 250-270°, and a carrier gas flow rate of 25-35 ml/min was used. The trap current was 60μA, accelerating voltage 3.5kV, electron multiplier voltage 2.1 KV; exit slit, 0.2mm; entrance slit, 0.12mm. Spectra were obtained using a recording oscillograph, and with a scan time of approximately 5 sec. The gas chromatographic columns used in this instrument were glass spiral columns, 6ft or 12ft in length, with i.d. 0.3mm.

Single ion monitoring was carried out, at 22.5eV, with a Rikadenki dual pen potentiometric recorder. An output from the galvanometer amplifier of the mass spectrometer was connected via a 2.5cps filter (in the accelerating voltage alternator unit) to one pen set on the 10V fsd range and offset to the centre of the chart paper. The total ion current (T.I.C.) was simultaneously monitored with the second pen, set on the 10mV fsd range. The T.I.C. attenuation and electron multiplier voltage were selected to give recordings of suitable amplitude on the chart paper. Much of the electrical "noise" originating in the electron multiplier was filtered via a 25,000 μF capacitor.

For samples unsuited to GLC, mass spectra were recorded on an AEI MS-12 mass spectrometer, at electron energy 70eV.

High resolution mass spectrometry was carried out on an AEI MS902 instrument, with direct probe sampling, at electron energy 70eV.

Infra-red spectra were obtained using a Unicam SP1000 or Perkin-Elmer Model 257 spectrophotometer.

N.M.R. spectra were recorded at 100 MHz with the Varian Model HA-100 spectrometer, and at 60 MHz with the Varian Model T-60 spectrometer.

Melting points were determined on a Kofler micro hot stage.

Thanks are due for the services of Mr. J.M.L. Cameron and Miss F. Cowan for microanalyses, Mrs. F. Lawrie (Infra-red laboratory), Mrs. J. Borthwick and Dr. R.C. Glass (GC-MS), and Mr. A. Heitzmann and Mr. J. Gall (NMR). The line diagrams which appear in this thesis were drawn with the aid of a computer program devised by Dr. J.A. Wilson.

GENERAL CHEMICAL TECHNIQUES

1.2.2 PREPARATION OF MANGANESE DIOXIDE [76]

A solution of potassium permanganate (15g) in water (200ml) was added dropwise to a stirred solution of manganous sulphate (21g) in water (30ml), held at 90°. The addition took place over a period of 1¼h, during the last 30min of which aliquots (approx. 1 ml) were withdrawn from the reaction mixture, centrifuged and the supernatants examined. As soon as a faint pink coloration was observed, addition of permanganate was stopped, and the reaction mixture stirred for a further 15 min at 90° before being cooled to room temperature.

The chocolate-brown precipitate of manganese dioxide was collected by filtration, washed with hot water (500ml), then methanol (100ml) and finally ether (100 ml). The product was then dried overnight at 130°, and finely powdered prior to use. Total yield of manganese dioxide was 17.5g.

1.2.3 PREPARATION OF 17α, 20α - AND 17α, 20β - DIHYDROXY - 4 - PREGNEN -3- ONE [77]

(a) Lithium Aluminium Hydride Reduction of 17-Hydroxyprogesterone

Lithium aluminium hydride (174mg; 4.59 m mole) was placed in a 25 ml, three-necked, pear-shaped flask, equipped with dropping funnel and water condenser, and dry, redistilled tetrahydrofuran (THF; 5.5 ml) added. A solution of 17α -hydroxyprogesterone/

Table 4: TLC Data on Compounds Involved in Synthesis of 17α,20ξ-Dihydroxy-4-pregnen-3-ones.

Reaction	Rf EtOAc/$CHCl_3$ (1:1 v/v)	Colour with $Ce(SO_4)_2$/ heat	Reaction with U.V. light	Reaction with 2,4-D.N.P.	Structure
-	0.54	brown	+	+	17α-Hydroxyprogesterone
LAH reduction	0.34	purple	-	-	4-Pregnene-3ξ 17α,20ξ-triols
	0.26	purple	-	-	
	0.19	purple	-	-	Saturated pregnanetriols.*
	0.13	purple	-	-	
MnO_2 oxidation	0.34	reddish-brown	+	+	17α,20β-Dihydroxy-4-pregnen-3-one
	0.27	reddish-brown	+	+	17α,20α-Dihydroxy-4-pregnen-3-one
	0.18	purple	-	-	Saturated pregnanetriols.*
	0.13	purple	-	-	

* Postulated structures - formed by over-reduction of the enone group.

hydroxyprogesterone (97 mg; 0.294 m mole) in THF (3.0 ml) was then added over a period of 5 min, when slight effervescence took place. The contents of the flask were heated under reflux for $1\frac{1}{2}$ h, after which no starting material could be detected by analytical TLC (mobile phase: ethyl acetate/chloroform (1:1 v/v)). Ethyl acetate was added to destroy excess reagent, followed by saturated sodium sulphate solution (5 ml) and finally solid sodium sulphate (1g). The precipitated salts were filtered off and washed with THF. Evaporation of the filtrate afforded the product (93 mg) as a colourless solid. Examination of this material by analytical TLC (Table 4) revealed two major spots (R_f 0.34 and 0.26), while GLC of the TMS ethers (prepared by overnight reaction with BSA) gave one broad peak on both OV-1 and OV-17 columns ($I^{230^o}_{OV-1}$ = 2965; $I^{230^o}_{OV-1}$ = 3095) and several minor peaks with lower retention indices. This mixture of reduction products was not separated at this stage, but was used directly for the following oxidation step.

(b) Manganese Dioxide Oxidation of the Reaction Product from (a).

The product from step (a) above (71mg) was taken up in dry THF (3.8ml) and freshly prepared manganese dioxide (375mg) added. The mixture was then stirred at room temperature for 4h. The manganese dioxide was removed by filtration through celite, washed with a little THF, and the combined filtrates were evaporated yielding a pale yellow oil (60mg). This material again gave two major spots on TLC (Table 4), although these could now be detected by both 2,4-dinitrophenylhydrazine reagent and ultraviolet light (λ=254nm), confirming the formation of a Δ^4 -3-one system. GLC of the TMS ethers (prepared as in (a) above) gave a partly resolved doublet on OV-1 ($I^{230^o}_{OV-1}$ = 2965 and 2985) and a single, broad peak on OV-17 ($I^{230^o}_{OV-17}$ = 3315). Several early (minor) peaks were also observed.

(c) Isolation of 17α, 20α - and 17α, 20β -Dihydroxy -4-pregnen -3-one/

The crude oxidation product from step (b) (50mg) was applied to two preparative TLC plates (20x20cm; 0.5mm Kieselgel HF_{254}), and the chromatograms developed using ethyl acetate/chloroform (1:1 v/v) as mobile phase, with a double elution. The bands corresponding to 17α, 20α-dihydroxy-4-pregnen-3-one and the 20β epimer were located with ultraviolet light (λ = 254nm) and extracted with ethyl acetate. Evaporation of the solvent afforded 22mg of the less polar (20β) isomer, and 11mg of the more polar (20α) isomer.

17α, 20β-Dihydroxy-4-pregnen-3-one, recrystallised from acetone, gave broad crystals (19mg) m.p. 203-206° (lit. 204-205.5°). Infra-red: ν_{max} (KBr) 3478 cm^{-1} (broad, s), 1647 cm^{-1} (vs) and 1614 cm^{-1}(s). GC-MS of the methylboronate ester ($I^{225°}_{OV-17}$ = 3145) gave $M^{+\cdot}$ at m/e 356.

17α,20α-Dihydroxy-4-pregnen-3-one, recrystallised from methanol, gave fine needles (5mg) m.p. 190-200° (lit. 208-210°). Infra-red: ν_{max}(KBr) 3427cm^{-1} (broad, s), 1658 cm^{-1} (vs) and 1610cm^{-1} (s). GC-MS of the methylboronate ester ($I^{225°}_{OV-17}$ =3170) gave $M^{+\cdot}$at m/e 356.

1.2.4 HYDROLYSIS OF DIHYDROXYACETONE 21-ACETATES

In order to provide substrates for oxetanone formation (section 2.2), hydrolysis of the commercially available 21-acetates was necessary in the cases of prednisolone and 9α-fluorocortisol.

(a) Hydrolysis of Prednisolone Acetate (11β, 17α,21-trihydroxy-1,4 - pregnadiene - 3,20-dione 21-acetate)[78]

Two solutions, one containing prednisolone acetate (201 mg, 0.502 m mole) in methanol (20 ml), and the other potassium bicarbonate (200mg) in distilled water (2.0ml), were bubbled (5min) with nitrogen to remove dissolved oxygen. The solutions were then mixed in a 100ml two-necked conical flask, equipped with magnetic stirrer and nitrogen inlet. (At this point, some precipitation occurred, although this material re-dissolved as the reaction

progressed.) The reaction mixture was stirred, at room temperature and under a nitrogen atmosphere, for a period of 4h. (During this period, large aqueous globules formed in the reaction mixture, in agreement with the reported heterogeneous character of this procedure). Analytical TLC of the product (mobile phase: ethyl acetate/light petroleum, b.p. 60-80°, (4:1 v/v) indicated complete hydrolysis of the acetate.

3.1 ml of a solution containing glacial acetic acid (3ml) in ice-water (60ml) was then added, and the resulting mixture concentrated in vacuo, without heating, and left overnight in the refrigerator (5°). This produced a crop of fine, yellowish needles, which were collected by filtration, washed with a little ice-water and dried overnight in a vacuum desiccator (yield=114mg). A second crop of crystals, amounting to 24mg, was obtained by further cooling of the filtrate. Total yield of prednisolone: 138 mg (0.384 m mole, yield: 77%); Infra-red: νmax (Nujol) 3400 cm^{-1} (m, broad), 1708 cm^{-1}(m), 1658 cm^{-1}(s), 1612 cm^{-1}(m), and 1590 cm^{-1}(m).

(b) Hydrolysis of 9α-Fluorocortisol Acetate (9α-fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione 21-acetate).

This was carried out in a similar fashion to that described above for prednisolone acetate. In this case, 198mg of the acetate afforded 116mg of 9α-fluorocortisol as colourless crystals in 65% overall yield. Infra-red: ν_{max} (Nujol) 3450 cm^{-1}(m, broad), 1720 cm^{-1}(m) and 1655 cm^{-1}(s).

1.2.5 SOLVENTS AND REAGENTS [79]

For large scale preparative work, solvents of analytical grade were used, while "Nanograde" solvents (Mallinckrodt Chemical Works, St. Louis, Missouri) were employed for the handling of small (less than 2mg) samples, and urinary extracts.

Acetone (BDH AnalaR) was dried by distilling from anhydrous/

anhydrous potassium carbonate.

Acetonitrile was dried by shaking with Linde type 5A molecular sieve and distilling from calcium hydride (b.p. 82°).

Benzene (BDH AnalaR, 300ml) was purified by shaking with conc. sulphuric acid (50ml), followed by water (100 ml). It was then shaken with 2N sodium hydroxide (100ml) and finally water (100ml). Drying was accomplished by distilling from calcium hydride.

Dimethylformamide was dried by prolonged storage over Linde type 4A molecular sieve, and redistillation.

Dimethyl sulphoxide was dried by prolonged standing over either Linde type 5A molecular sieve or calcium hydride, followed by distillation under reduced pressure (b.p. 40° at 0.6mm Hg).

1,4-Dioxan (BDH AnalaR) was purified by distilling from sodium borohydride.

Hexane (BDH Spectroscopic grade) was redistilled from sodium hydride.

Methylene Chloride was purified by shaking with calcium chloride, redistilling and storing over Linde type 5A molecular sieve.

Pyridine (b.p. 114°) was redistilled from potassium hydroxide pellets and stored over calcium hydride.

Tetrahydrofuran (b.p. 67°) was redistilled from lithuim aluminium hydride.

Reagents

Calcium Chloride was made anhydrous by heating at 85°/0.5mm Hg for 17h.

Methanesulphonyl Chloride was distilled from phosphorus pentoxide (b.p. 160-161°).

Silylating Reagents were redistilled and stored under anhydrous conditions. HMDS had b.p. 124°, BSA 148° and TSIM 214°.

_p_-Toluenesulphonic Acid was recrystallised from benezene, and dried by heating at 100° for 4h under water pump vacuum.

Triethylamine was redistilled from calcium hydride (b.p. 91°).

Triphenylphosphine was dried overnight in a drying pistol, with phosphorus pentoxide as the dehydrating agent. Conditions were 60° at 0.25mm Hg.

SECTION 2

OXETANONES AS DERIVATIVES FOR THE GAS CHROMATOGRAPHY OF STEROIDS WITH THE DIHYDROXYACETONE SIDE-CHAIN.

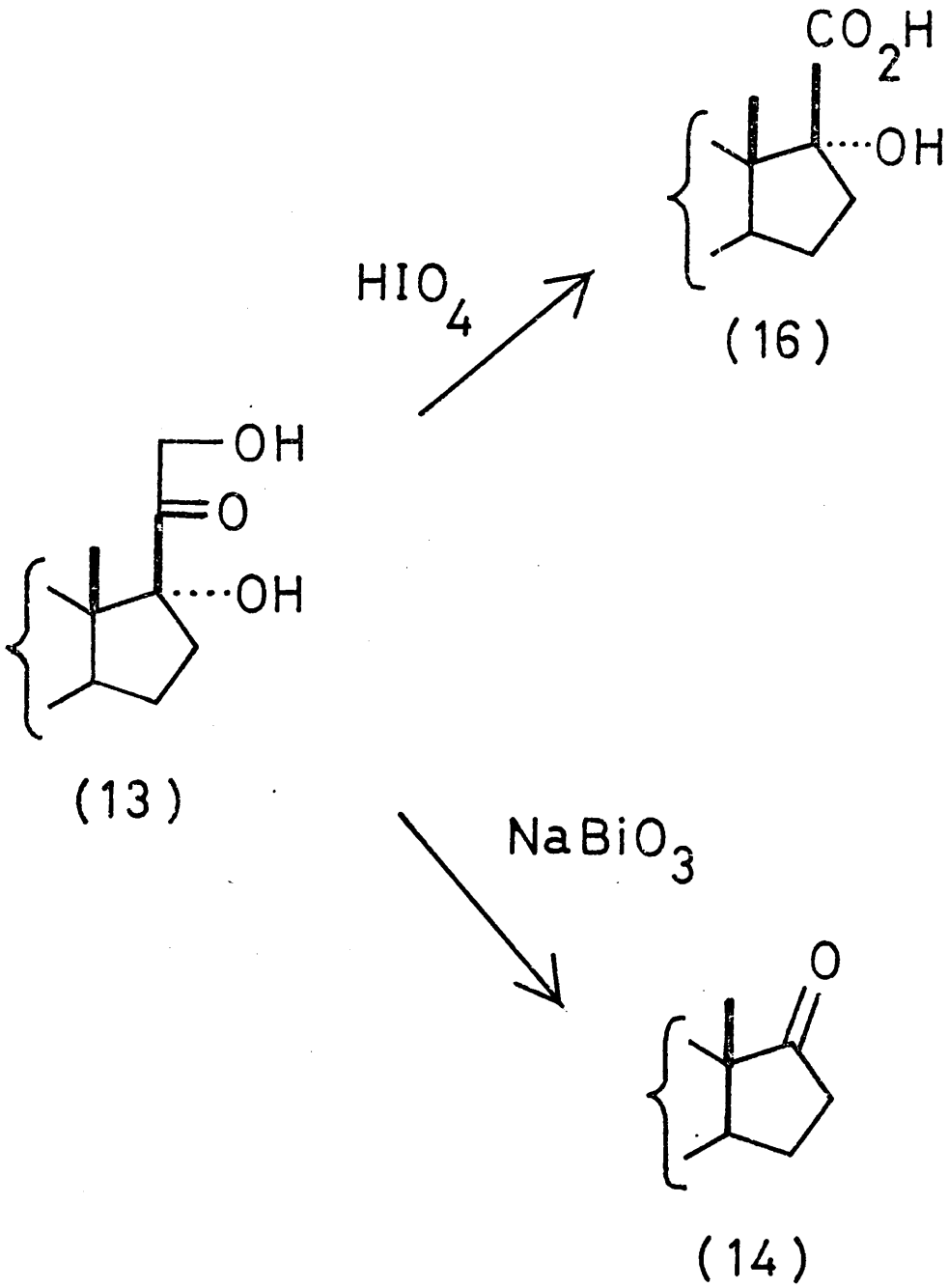
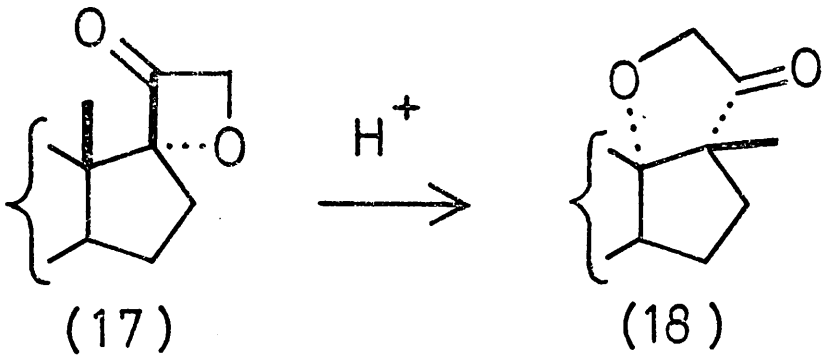


Fig. 7: Oxidation of the dihydroxyacetone side-chain.

2.1 INTRODUCTION

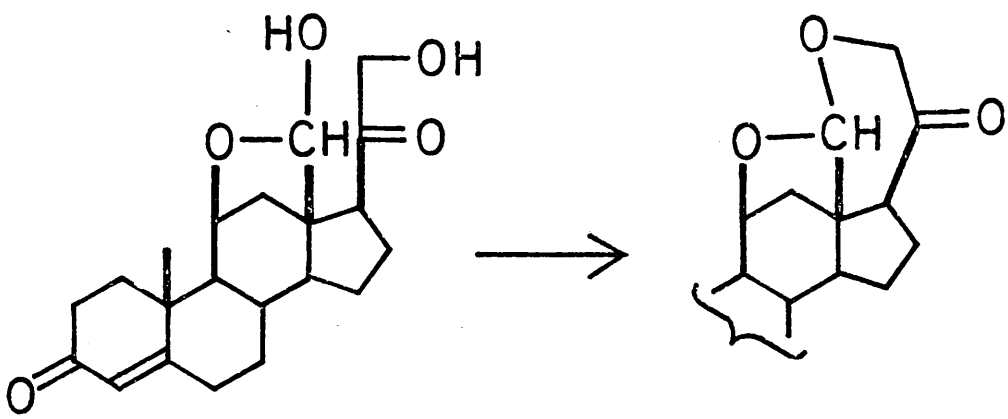
Among the types of adrenocortical hormones normally encountered in biological fluids, those possessing the 17α,21-diol-20-one (dihydroxyacetone) side-chain (partial formula 13) constitute an important group. Many synthetic corticoids exhibiting anti-inflammatory activity also incorporate this structure.[80] An investigation into the GLC properties of such compounds revealed that decomposition to the corresponding 17-ketosteroid occurred in the flash heater zone of the gas chromatograph.[81] An application of this finding was made by Luetscher and Gould, who determined urinary tetrahydrocortisone (THE), allo-tetrahydrocortisone (allo-THE) and tetrahydrocortisol (THF) as their thermal cleavage products, and reported good agreement with the values obtained from colorimetric estimations.[82] However, quantitative studies of this thermal dissociation indicated that the yield of 17-ketone was in the order of 20 to 30%, although the exact value depended on both the nature of the parent corticosteroid and, to some extent, on the amount injected.[83-85] It therefore appeared that modification of the dihydroxyacetone structure in some way was desirable prior to the quantitative estimation of such steroids by GLC. Two approaches have been developed for this purpose: oxidative cleavage of the side-chain (a reproducible process known to proceed in high yield) and stabilisation of the parent molecule through derivative formation.

The dihydroxyacetone structure may be oxidised (fig. 7), either by periodic acid to yield an etiocholenic acid derivative (partial formula 16), which may be chromatographed following methylation,[86] or by sodium bismuthate which completely removes the side-chain affording the thermally stable 17-ketosteroid (14).[26] Both of these procedures have been successfully applied to the analysis of complex mixtures of cortiscosteroids, when the respective oxidation products are estimated by GLC.[87,93]



(17) (18)

Fig. 8: Acid-catalysed rearrangement of 17α,21-oxido steroids.



(19) (20)

Fig. 9: Stabilisation of aldosterone through acetal formation.

Numerous derivatives have been used, or proposed, to stabilise steroidal dihydroxyacetones for GLC. The most widely used of these are probably the O -methyloxime trimethylsilyl ethers (MO-TMS) introduced by Gardiner and Horning in 1966.[54] However, cyclic boronate esters and dimethylsiliconides,[96,97] in which the oxygen functions at C-17α and C-21 are incorporated into a six-membered ring, have proved useful in many instances. A survey of existing corticosteroid derivatives is presented in section 3.

Currently derivative formation is preferred to the side-chain degradation of corticosteroids, since in the former case the identity of the parent steroid is conserved to a greater extent. However, when the derivatives are to be analysed by a GC-MS system, their molecular weights must be taken into account. Conversion of a 17α, 21-diol-20-one to its 20-MO 17α, 21-diTMS derivative increases the molecular weight of the steroid by 173 a.m.u., while still further mass increments result from the derivatisation of functional groups elsewhere in the steroid nucleus. Derivatives of high molecular weight (greater than 700 a.m.u.) are undesirable for study by common low-resolution GC-MS instruments. Furthermore, derivatisation of corticosteroids, as opposed to side-chain cleavage, increases the GLC retention time, although this is usually less severe a problem. Hence, a derivative which stabilises the corticosteroid side-chain, and which has a low molecular weight and short retention time, would clearly be a welcome addition to the existing range. The object of this study was to evaluate 17α, 21-oxido steroids (partial structure 17) as derivatives which might fulfil these requirements.

The change in molecular weight accompanying oxetanone/

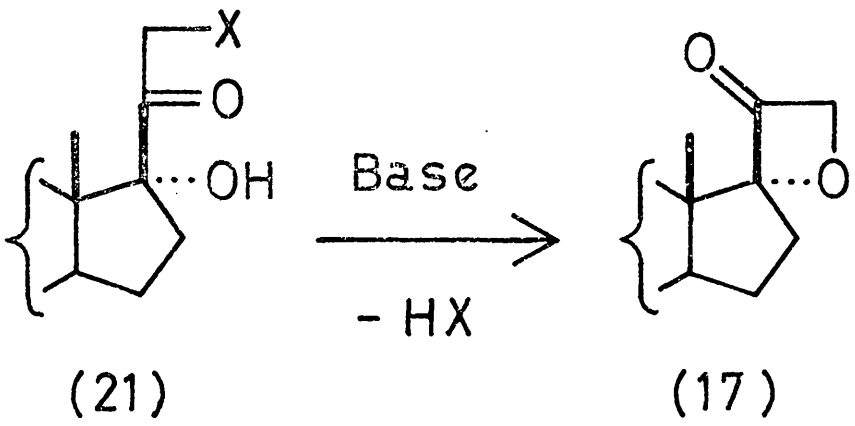


Fig. 10: Synthesis of 17α,21-oxido steroids.

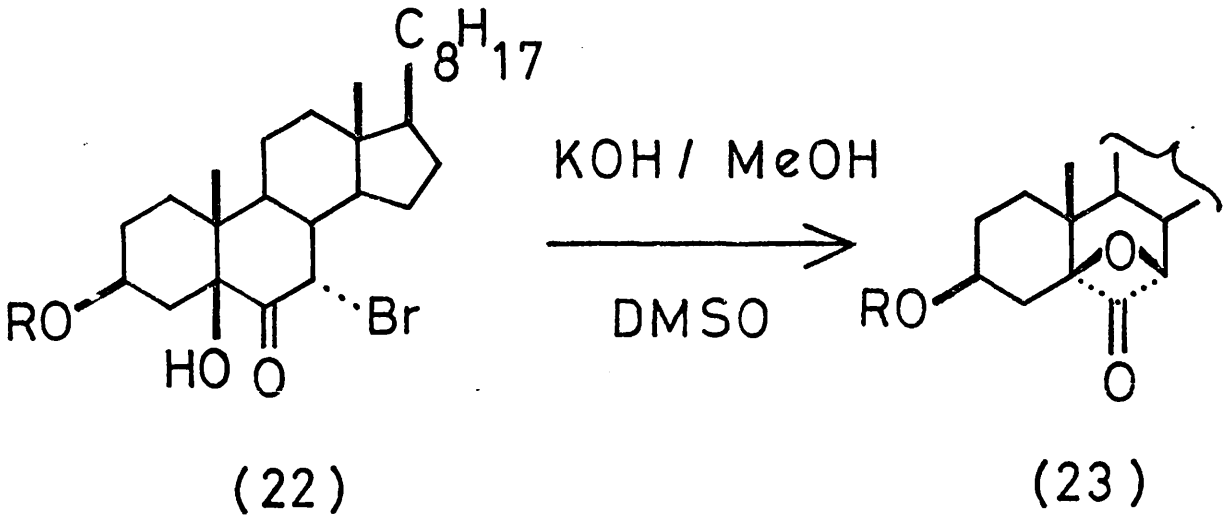


Fig.11: Synthesis of ring-B cholestane 3-oxetanones.

oxetanone formation is - 18 a.m.u. ; this is the only known dihydroxyacetone derivative which has a lower molecular weight than that of the parent corticosteroid, although aldosterone (19) may be stabilised for GLC as its 18→11:21 acetal (20) [84], where a similar molecular weight relationship exists.

The 17α, 21-oxido (or 17α, 21-anhydro) derivative possesses an ether linkage and a carbonyl group in a strained four-membered ring. This structural unit is known as a 3-oxetanone system. The first report of a 17α, 21-oxido steroid was in 1955, when Allen et al. claimed that an oxetanone of this type resulted from the acid-catalysed hydrolysis of the corresponding ethylene ketal.[98] This claim was proved erroneous, however, by two groups [99,100], who synthesised the true 17α, 21-oxido-20-one structure (17) and showed that it rearranged to a five-membered tetrahydrofuranone (18) when treated with acid (fig.8). During the period 1958-65, several preparations of 17α, 21-oxido steroids were reported, mainly by groups at Merck, Sharp and Dohme and at Upjohn, who observed moderate oral diuretic and anti-inflammatory activity in these compounds.[101-105] In all cases, the oxetanones were synthesised by internal displacement reactions involving the 17α-hydroxyl group and a leaving group (mesylate, tosylate, iodide or diazo function) at C-21 (fig. 10). Basic catalysts (potassium fluoride, silver phosphate, silver dihydrogen phosphate or potassium hydroxide) were employed in solvents such as dimethyl sulphoxide (DMSO), dimethylformamide (DMF), acetonitrile or 1,4-dioxan. The yields of oxetanones under these conditions were low (or not quoted). More recently, a synthesis of ring-B cholestane 3-oxetanones (23) was reported,[106] in which C-3 substituted 7α-bromo-5β-hydroxy-6-oxocholestanes (22) acted as substrates for oxetanone formation (fig. 11). In suitable cases, when the ring-A substituent occupied the 3β-position, yields in excess of 70% were obtained. However, in spite of the low yields of 17α, 21-oxido steroids quoted in published syntheses, it was decided to /

to adapt one of these procedures[104] as an initial route to compounds of this type. Following an investigation of their GLC and mass spectral properties, studies aimed at improving the yields of oxetanones from steroidal dihydroxyacetones were undertaken.

2.2 EXPERIMENTAL

PREPARATION OF OXETANONES (17α, 21-ANHYDRO DERIVATIVES) FROM STEROIDAL DIHYDROXYACETONES.

The general method is exemplified for the case of Reichstein's Substance S (17α, 21-dihydroxy-4-pregnene-3,20-dione):

2.2.1 Substance S 21-mesylate (17α, 21-dihydroxy-4-pregnene-3,20-dione 21-methanesulphonate) [107]

Substance S (100mg; 0.29 m mole) was dissolved in dry pyridine (1.4ml) and the solution cooled to 0°. Methanesulphonyl chloride (100μl) was then added dropwise and the reaction mixture held at 0° for 4h. Dilution with ice-water (10ml) produced a fine white precipitate, which was collected by filtration, washed with ice-water (2ml) and dried overnight in a vacuum desiccator. This afforded Substance S 21-mesylate (117 mg; 27.5 m mole) (Yield = 95%).

Analysis of the product by TLC, using as mobile phase ethyl acetate/light petroleum, b.p. 60-80°, (1:1 v/v), indicated the mesylate to be only slightly less polar than the starting material. (R_f values were : Substance S, 0.23; Substance S 21-mesylate, 0.27). After two recrystallisations from benzene/light petroleum, b.p. 60-80°, the mesylate had m.p. 170-171°. Infra-red: ν_{max} (KBr disc) 3490 cm^{-1} (m, broad), 1735 cm^{-1}(s), 1665 cm^{-1} (s), 1610 cm^{-1}(m), 1355 cm^{-1}(s), 1165 cm^{-1}(s) and 1040 cm^{-1}(s). (Found, C: 62.09; H: 7.58%. Calc. for $C_{22}H_{32}O_6S$: C: 62.22; H:7.61%.)

2.2.2 17α, 21-Anhydro Derivative of Substance S (17α,21-oxido-4-pregnene-3,20-dione) [104]

A suspension of anhydrous potassium fluoride (85mg) in dry redistilled dimethyl sulphoxide (2.2ml) was made into a slurry by stirring for 2h at 70°. Substance S 21-mesylate/

mesylate (92mg; 21.7 m mole) was then added, whereupon the reaction mixture turned deep yellow. Stirring was continued under anhydrous conditions overnight (16h), during which time the reaction mixture was maintained at 85-90°. The reaction was quenched by pouring into ice-water (25ml) and the resulting turbid solution extracted with ethyl acetate (3x25ml). The combined organic extracts were washed with water (1x20ml) and brine (1x20ml), then dried and evaporated *in vacuo*, affording a yellowish oil (70mg). Examination of this material by analytical TLC, using the same mobile phase as employed in (a) above, indicated the formation of two products with R_f values 0.46 and 0.57. GLC similarly revealed two peaks with $I^{225^\circ}_{SE-30}$ = 2505 (10%) and 2775 (90%). The corresponding values on Dexsil were $I^{275^\circ}_{DEXSIL}$ = 2900 and 3120). The early (minor) peak was identified as 4-androstene-3,17-dione by comparison of retention data and mass spectrum (molecular ion at *m/e* 286) with that of the authentic material. This compound would, no doubt, be formed in the flash heater zone of the gas chromatograph by thermal degradation of 21-fluoro-17α-hydroxy-4-pregnene-3,20-dione, which is the major by-product in this preparation. The later (principal) peak gave a mass spectrum compatible with the desired 17α,21-anhydro compound (molecular ion at *m/e* 328).

Preparative TLC, using as mobile phase ethyl acetate/light petroleum, b.p. 60-80°, (1:1 v/v), with a double elution, was used to separate the two reaction products. Elution of the upper band with ethyl acetate and evaporation *in vacuo* afforded the oxetanone (12mg) as a solid. Two recrystallisations from acetone/light petroleum, b.p. 60-80°, gave fine needles, m.p. 194-196°. Infra-red: ν_{max}(KBr disc) 1813 cm^{-1}(s), 1667 cm^{-1}(s), 1611 cm^{-1}(m) and 956 cm^{-1}(s). GLC analysis of this purified material, however/

however showed that incomplete separation of the oxetanone from the contaminating fluoride had been achieved. The impurity peak had nevertheless, diminished in size to 2-3% of that of the oxetanone peak.

Furthermore, vacuum sublimation of the recrystallised oxetanone using a 'cold finger' apparatus (125^{o}/0.03mm Hg) afforded material which still contained an impurity, as shown by TLC and GLC. (Found, C: 75.28; H: 8.54%. Calc. for $C_{21}H_{28}O_3$: C:76.79; H: 8.59%.)

A small sample (400µg) of the 17α,21-anhydro derivative of Substance S was converted to its 3,20-di-O-methyloxime (di MO), according to the method outlined in section 3.2.1.

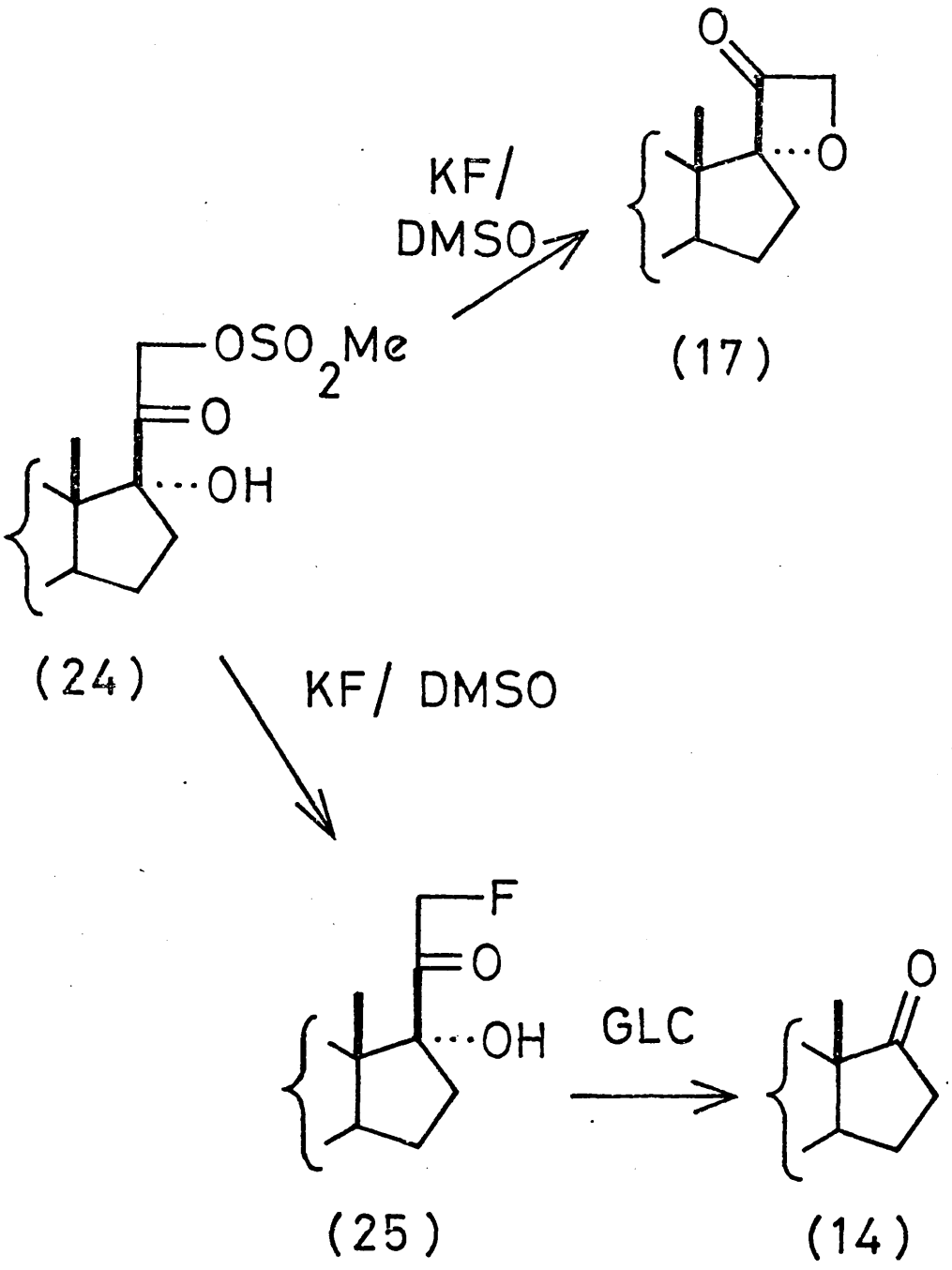


Fig. 12: Products obtained from reaction of dihydroxyacetone 21-mesylates with potassium fluoride in dimethyl sulphoxide.

2.3 RESULTS AND DISCUSSION

2.3.1 GENERAL RESULTS

Fourteen representative steroidal dihydroxyacetones were converted to their oxetanone derivatives by the Upjohn procedure,[104] in which cyclisation of the 21-mesylates (24) was effected by treatment with potassium fluoride in dimethyl sulphoxide (fig.12). In all cases, the corresponding 21-fluorides (25) were formed as by-products, resulting from the direct displacement of mesylate by fluoride ion. (The amount of 21-fluoride formed ranged from 5 to 30% of the total reaction product, as estimated by TLC and GLC.) In each case, examination of the reaction product by analytical TLC revealed two closely separated spots, the less polar of which corresponded to the oxetanone. Even in the most favourable instance (Substance S), repeated thin-layer chromatography failed to remove completely the contaminating 21-fluoride, and despite three recrystallisations and one vacuum sublimation, analytical purity was not achieved.

For the purpose of discussion, the steroids studied have been divided into two groups, as follows:-

Group 1: Non-fluorinated corticosteroids, with varying degrees of unsaturation in ring A and oxidation level at C-11 (Table 5).

Group 2: Fluorinated corticosteroids (Table 6). With the exception of 6α-fluoroprednisolone, these compounds all contained the 9α-fluoro-11β-hydrin group present in many anti-inflammatory corticosteroid drugs. Introduction of fluorine substituents adds to the polar character of corticoids, and thus to the difficulty of achieving satisfactory gas chromatographic behaviour. Indeed, the analysis of such compounds by gas phase methods has received/

Parent compound	Retention indices I^{225^o} SE-30	Retention indices I^{275^o} Dexsil-300GC	M^+	Base peak m/e>80	m/e values and relative abundances of characteristic ions. Fragment ions
Substance S (17α,21-Dihydroxy-4-pregnene-3, 20-dione)	2775	3120	328 (17)	286	270 (23) 269 (20) 258 (13) 244 (48) 242 (12) 229 (14) 148 (33) 136 (38) 124 (68)
Substance S - diMO (17α ,21-Dihydroxy-4-pregnene-3, 20-dione diMO)	2905	3080	386 (100)	386	371 (21) 355 (31) 324 (14) 315 (12) 153 (34) 152 (20) 151 (42) 137 (52) 125 (72)
Cortisol (11β,17α,21-Trihydroxy-4-pregnene-3, 20-dione)	2950	3365	344 (32)	302	329 (7) 326 (11) 311 (12) 286 (23) 284 (23) 274 (43) 242 (26) 227 (37) 189 (31) 163 (93) 145 (45) 136 (68) 123 (83)
Cortisone (17α ,21-Dihydroxy-4-pregnene-3, 11, 20-trione)	2830	3200	342 (3)	122	300 (48) 284 (8) 272 (6) 256 (7) 189 (13) 161 (15) 147 (20) 136 (18)
Prednisolone (11β,17α,21-Trihydroxy-1,4-pregnadiene-3, 20-dione)	2970	3420	342 (2)	95	300 (2) 284 (3) 283 (9) 265 (12) 147 (23) 135 (11) 134 (12) 123 (13) 122 (87) 121 (46)
Prednisone (17α,21-Dihydroxy-1, 4-pregnadiene-3, 11, 20-trione)	2850	3250	340 (10)	121	312 (9) 298 (91) 282 (13) 281 (10) 280 (11) 270 (14) 254 (23) 186 (17) 160 (59) 147 (64) 131 (35)
5β-Dihydro-S (DHS) (17α,21-Dihydroxy-5β-pregnane-3, 20-dione)	2665	-	330 (4)	288	315 (3) 272 (26) 271 (7) 244 (30) 229 (16) 174 (20) 147 (23) 133 (20) 122 (28) 119 (27) 107 (51) 95 (66)
5β-Dihydro-cortisone (DHE) (17α,21-Dihydroxy-5β-pregnane-3, 11, 20-trione)	2735	3090	344 (3)	302	287 (29) 286 (15) 274 (5) 191 (41) 163 (17) 149 (15) 147 (21) 135 (17) 124 (38) 122 (39) 121 (32) 109 (47) 107 (30)

Table 5: Retention index values and salient mass spectrometri c data for 17α ,21-anhydro derivatives of corticosteroids (Group 1

Parent compound	Retention indices. $I^{225^\circ}_{SE-30}$	$I^{275^\circ}_{Dexsil-300\ GC}$	M^+.	Base peak m/e>80	m/e values and relative abundances of characteristic ions. Fragment ions
9α-Flurocortisol (9α-Fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione)	2990	3400	362 (17)	320	344 (4) 342 (3) 304 (17) 300 (22) 292 (67) 258 (30) 173 (31) 163 (43) 136 (53) 123 (61)
9α-Fluoroprednisolone (9α-Fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione)	3020	3475	360 (0.6)	122	340 (15) 298 (6) 281 (15) 263 (7) 187 (9) 162 (23) 147 (15) 135 (14) 121 (67)
6α-Fluoroprednisolone (6α-Fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione)	2975	3455	360 (3)	139	340 (5) 318 (5) 301 (5) 298 (23) 283 (10) 281 (13) 263 (9) 147 (31) 140 (90) 134 (59) 121 (43)
Betamethasone (9α-Fluoro-16β-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione)	3075	3555	374 (0.4)	122	354 (9) 312 (13) 295 (15) 187 (10) 160 (12) 147 (13) 135 (17) 134 (10) 121 (61) 108 (28) 107 (46)
Dexamethasone (9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione)	3050	3530	374 (0.4)	122	354 (13) 312 (6) 295 (11) 187 (9) 160 (11) 147 (12) 135 (16) 134 (9) 121 (56) 108 (21) 107 (34)
6,7-Dehydrodexamethasone (9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4,6-pregnatriene-3,20-dione)	3035	3490	372 (7)	158	352 (2) 332 (22) 330 (24) 329 (93) 294 (10) 292 (66) 235 (18) 187 (34) 173 (25) 147 (23) 133 (28) 122 (46) 121 (57) 115 (31) 108 (46) 107 (33)
1,2-Dihydro-6,7-dehydrodexamethasone (9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-4,6-pregnadiene-3,20-dione)	3015	3455	374 (13)	332	346 (13) 314 (19) 299 (16) 297 (15) 189 (48) 160 (86) 147 (27) 133 (38) 123 (25) 122 (24) 115 (35) 109 (63) 108 (66) 95 (34)

Table 6: Retention index values and salient mass spectrometric data for 17α,21-anhydro derivatives of corticosteroids (Group 2).

received little attention, although Kelly chromatographed a fluorocorticosteroid as its dimethylsiliconide,[96,97] and Brooks and Lawson characterised a number of fluorocorticoid drugs as their MO-TMS and bismuthate oxidation derivatives.[108]

2.3.2 GLC PROPERTIES

Gas chromatographic retention data were recorded on two columns, SE-30 and Dexsil-300GC, and are presented in Tables 5 and 6.

For those compounds in Group1, good peak shapes were obtained, although the oxetanone peak was always preceded by that of the corresponding 17-ketosteroid, formed in the flash heater zone by thermal degradation of the aforementioned 21-fluoride (fig.12). Retention indices were, as expected, relatively low; the oxetanone from Substance S had a lower retention index (on SE-30) than any of the other derivatives commonly used to stabilise the side-chain for GLC (section 3.3).

The fluorinated oxetanones in Group 2 generally gave satisfactory GLC peaks, although in some cases tailing was observed. Peaks arising from the corresponding 17-ketosteroid were again present. In the two cases where a direct comparison could be drawn between fluorinated and non-fluorinated oxetanones (cortisol and prednisolone) the introduction of a 9α-fluoro substituent increased the retention index (on both SE-30 and Dexsil phases) by approximately 45 units.

2.3.3 MASS SPECTROMETRIC CHARACTERISTICS

Salient features of the mass spectra of the oxetanones studied are summarised in Tables 5 and 6, which show that molecular ions were obtained in all cases. The oxetanone derivatives undergo/

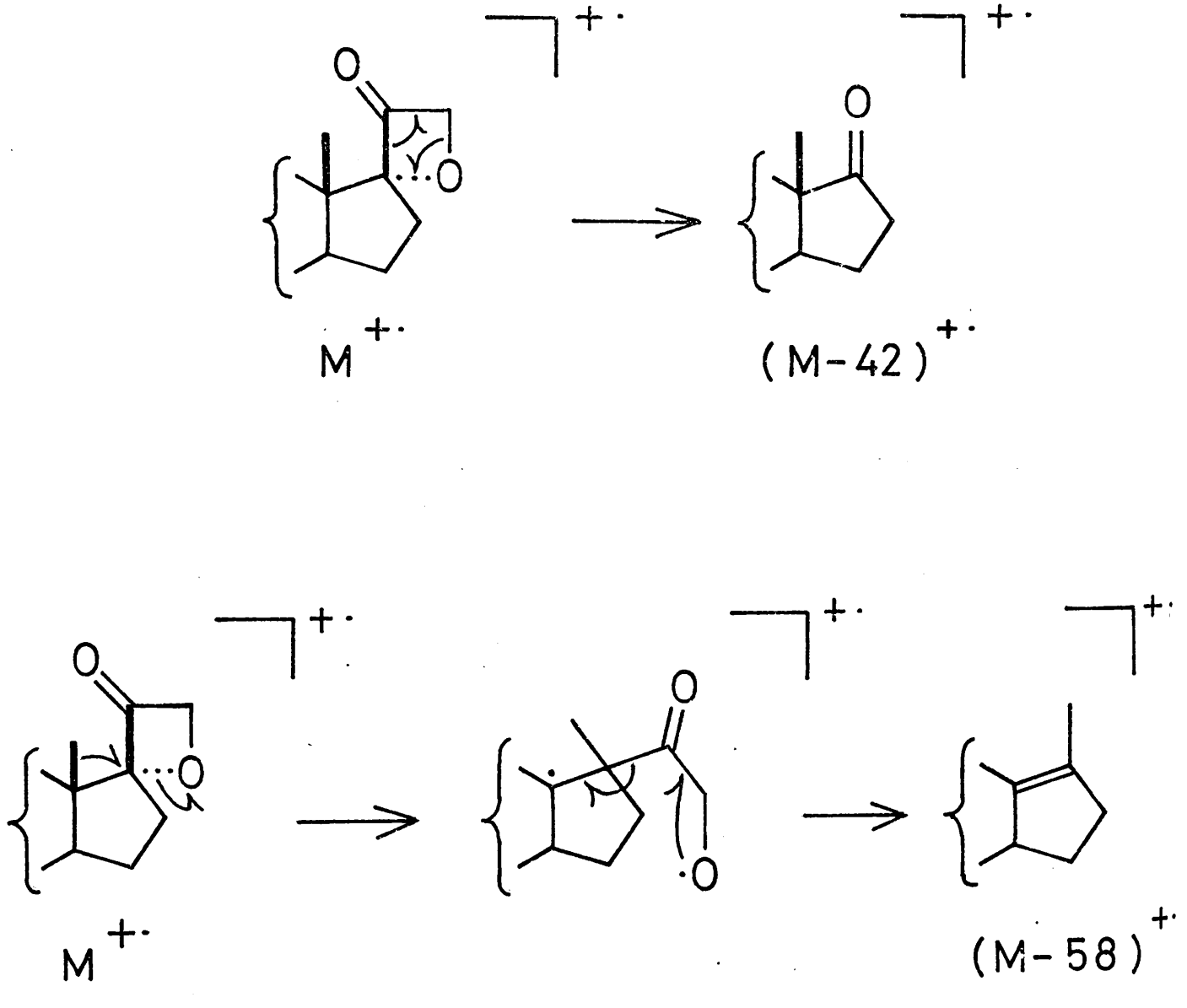


Fig. 13: Postulated fragmentations of the oxetanone ring.

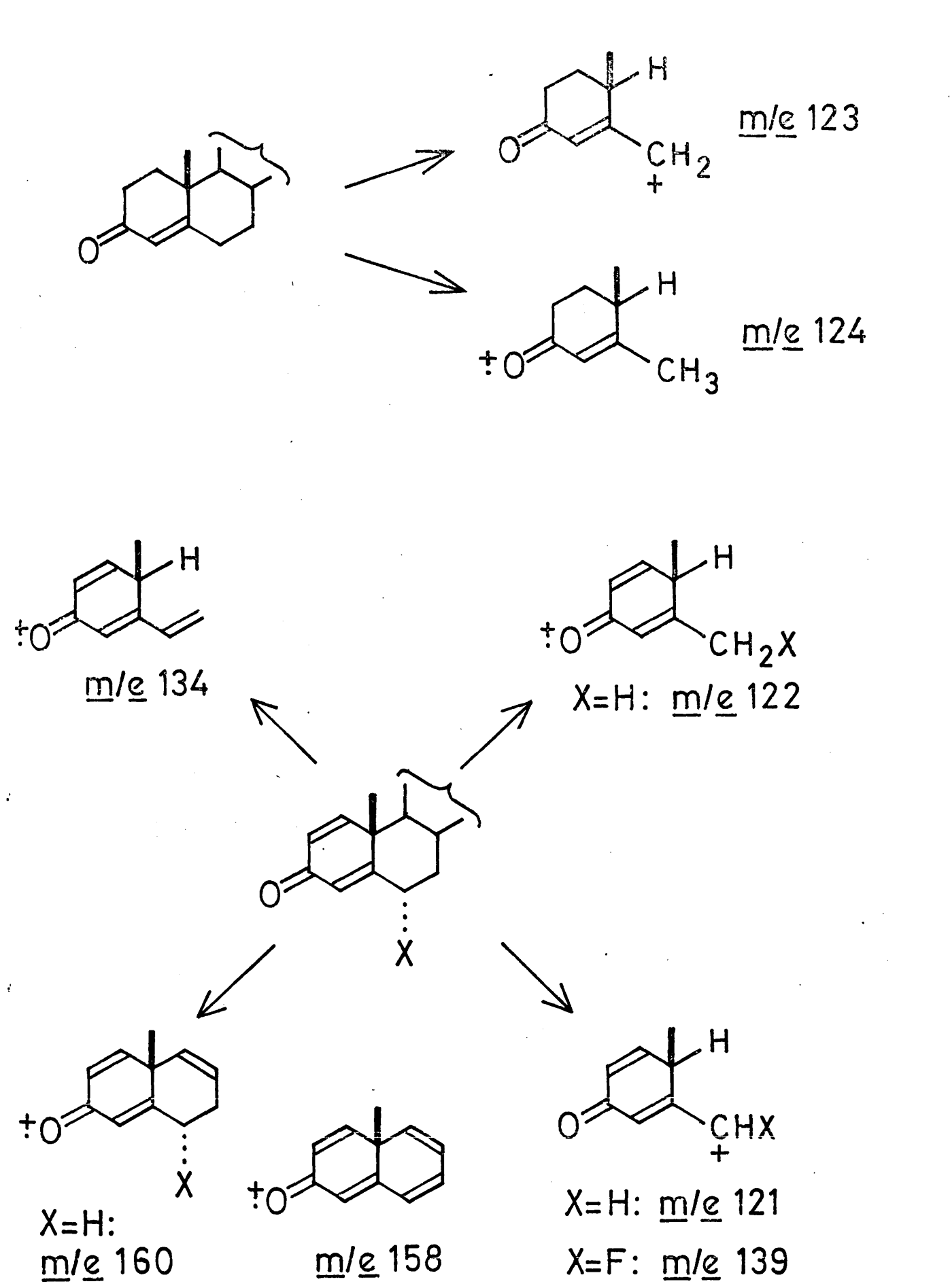


Fig. 14: Postulated representations of ions resulting from the mass spectral fragmentation of steroidal 4-en-3-ones and 1,4-dien-3-ones.

undergo distinctive fragmentations, notably the loss of ketene. This gave rise to prominent ions at m/e (M-42) (or at (M-HF-42) in the spectra of some of the fluorinated corticosteroids). Such ions afforded the base peak in six cases. The loss of 42 a.m.u. occurs in the spectra of Δ^4-3-ketosteroids, through scission of the C-1/C-2 and C-3/C-4 bonds,[109] although this process does not occur in ring-A saturated 3-ketones. However, the oxetanones derived from 5β-dihydro-S (DHS) and 5β-dihydrocortisone (DHE), both of which have a saturated ring A, exhibit the $(M-42)^{+\cdot}$ ion as their base peak. Clearly, the origin of this ion in such spectra cannot lie in ring A, but appears to derive from the oxetanone moiety itself (fig.13). Another interesting feature of the mass spectra of oxetanones in the occurrence of ions at m/e (M-58) or (M-59) (or both). These apparently result from extrusion of the side-chain (fig.13).

The characteristic fragments arising from the cleavage of ring B (at m/e 123 or 124 in 4-en-3-ones and at m/e 121 or 122 in 1,4-dien-3-ones) were prominent in most of the spectra, while 6α-fluoroprednisolone yielded the corresponding fluorine-containing ion at m/e 139 (fig.14). However, the base peak in the spectrum of 17α,21-anhydro cortisone, which contains the 4-en-3-one grouping, lay at m/e 122 and not at m/e 124; this feature is present in the spectrum of cortisone itself, and has been rationalised in terms of the absence of the 11α-proton, which is involved in a transfer process leading to the m/e 124 ion.[111,113]

Ions attributed to fragments containing rings A and B were present in the spectra of the 16-methyl corticosteroid derivatives. These ions were at m/e 160 in the spectra of betamethasone and its two isomers, and at m/e 158 in its 6,7-dehydro analogue (fig.14).

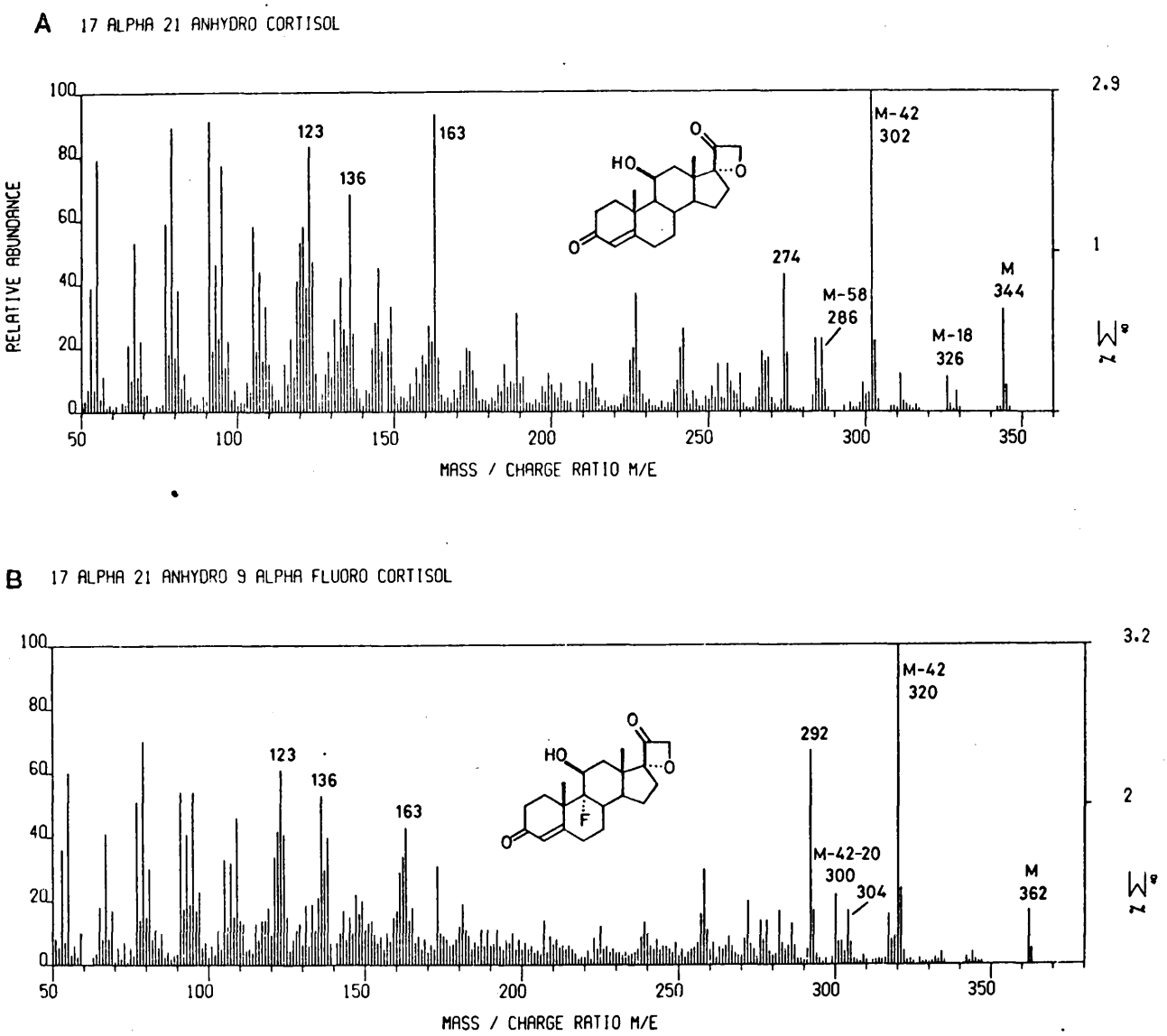


Fig.15: Mass spectra (70eV) of representative oxetanone derivatives:

(A) 17α,21-anhydro-cortisol;

(B) 17α,21-anhydro-9α-fluorocortisol.

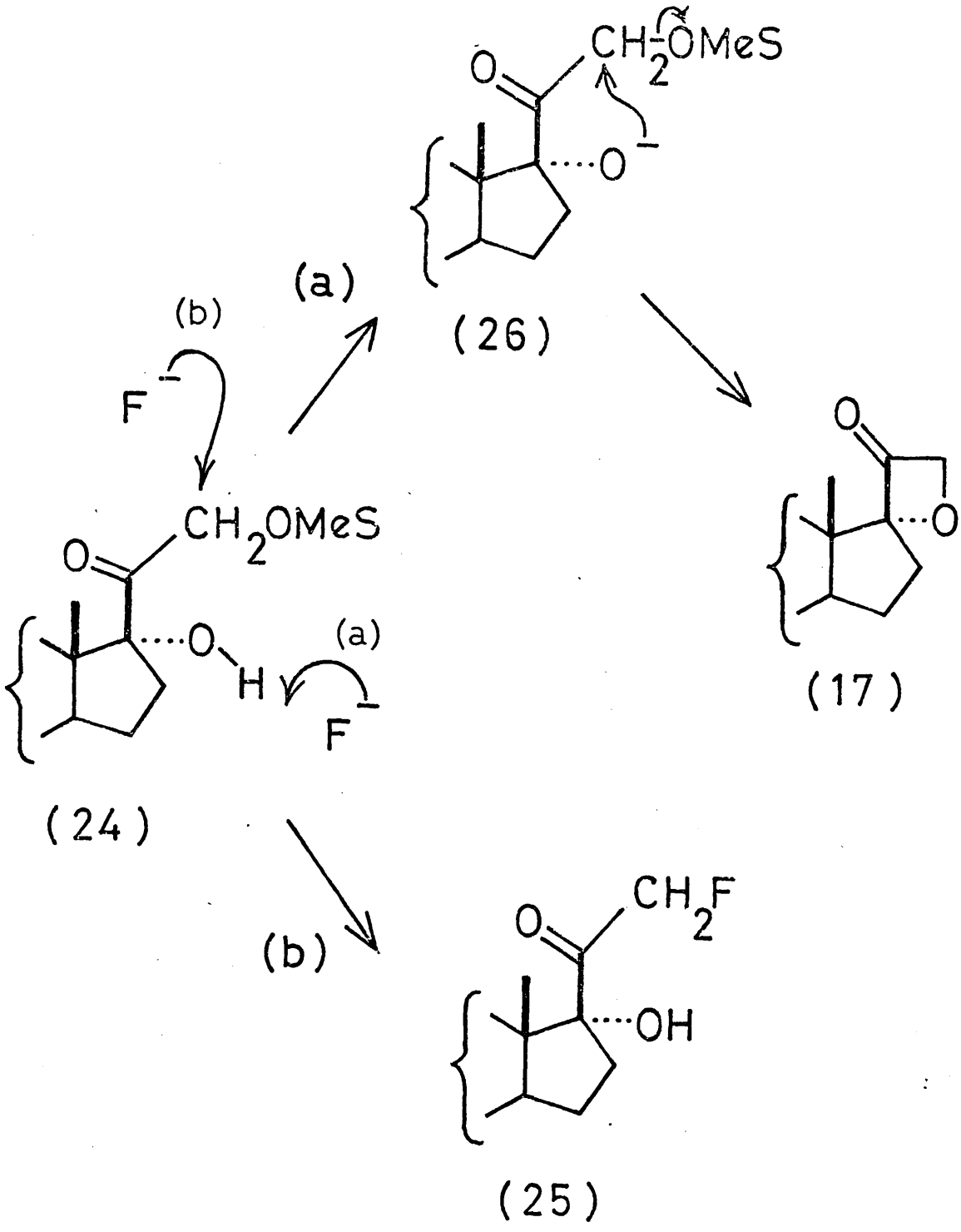


Fig. 16: Mechanism of cyclisation and displacement reactions of corticosteroid 21-mesylates.

Dexamethasone and betamethasone, differing only in the configuration of the 16-methyl group, gave practically identical mass spectra. Loss of HF was commonly encountered in the spectra of the fluorinated derivatives, giving rise to ions at m/e (M-20). The mass spectra of 17α,21-anhydro-cortisol and its 9α-fluoro analogue are shown in fig.15. The effect of introducing a fluorine substituent is evident in the mass shift of the major fragments.

The mass spectrum of the di-O-methyloxime (di MO) derivative of Substance S oxetanone (Table 5) furnished very useful complementary information. As expected, the molecular ion became much more abundant, and formed the base peak (at 70 eV). The high mass region contained ions typical of MO derivatives, at m/e (M-15) and m/e (M-31), resulting from losses of CH_3 and CH_3O radicals respectively. A fragment at m/e 315 (M-71) corresponded to the loss of 42 a.m.u. previously discussed, while the "ring A" ion had shifted to m/e 153.[114]

2.3.4 SYNTHETIC ASPECTS

The mechanism by which the dihydroxyacetone 21-mesylates are converted to oxetanone derivatives may be formally represented as shown in fig.16. Firstly, the base (in this case a fluoride ion) abstracts the proton from the 17α-hydroxyl group (process (a)) generating the oxy-anion (26). Secondly, an internal displacement reaction occurs, in which the leaving group (mesylate) is expelled, resulting in the formation of the spiro oxetanone system (17). In the competing reaction, the fluoride ion acts as a nucleophile, displacing the mesylate group by an S_N2 process (route (b)) and producing the dihydroxyacetone 21-fluoride (25). Therefore, to promote the cyclisation process - and inhibit the competing displacement reaction - two conditions must be satisfied:- (a) the base employed must possess low nucleophilicity but high basicity; and (b) the substituent at C-21 should be a moderately good leaving group (a very labile substituent would tend to undergo rapid /

BASE	SOLVENT	TEMP.	TIME	COMPOSITION OF PRODUCT (BY TLC AND GLC)
KF	DMSO	80°	18h	Oxetanone + 21-fluoride
KF	DMSO	80°	2h	As above (no starting material remaining).
KF	DMSO	58°	4h	Oxetanone, 21-fluoride and 21-mesylate.
KF	DMSO	25°	8h	21-mesylate only
KF	DMA	80°	18½h	Oxetanone + 21-fluoride
AgOMeS	DMSO	80°	18h	Oxetanone (?) + at least 4 other products.
KBF_4	DMSO	80°	17h	Complex mixture of products - no oxetanone
Pyridine	Pyridine	115°	1h	As above

Table 7: Effect of reaction conditions on the conversion of Substance S 21-mesylate to 17α ,21-anhydro S.

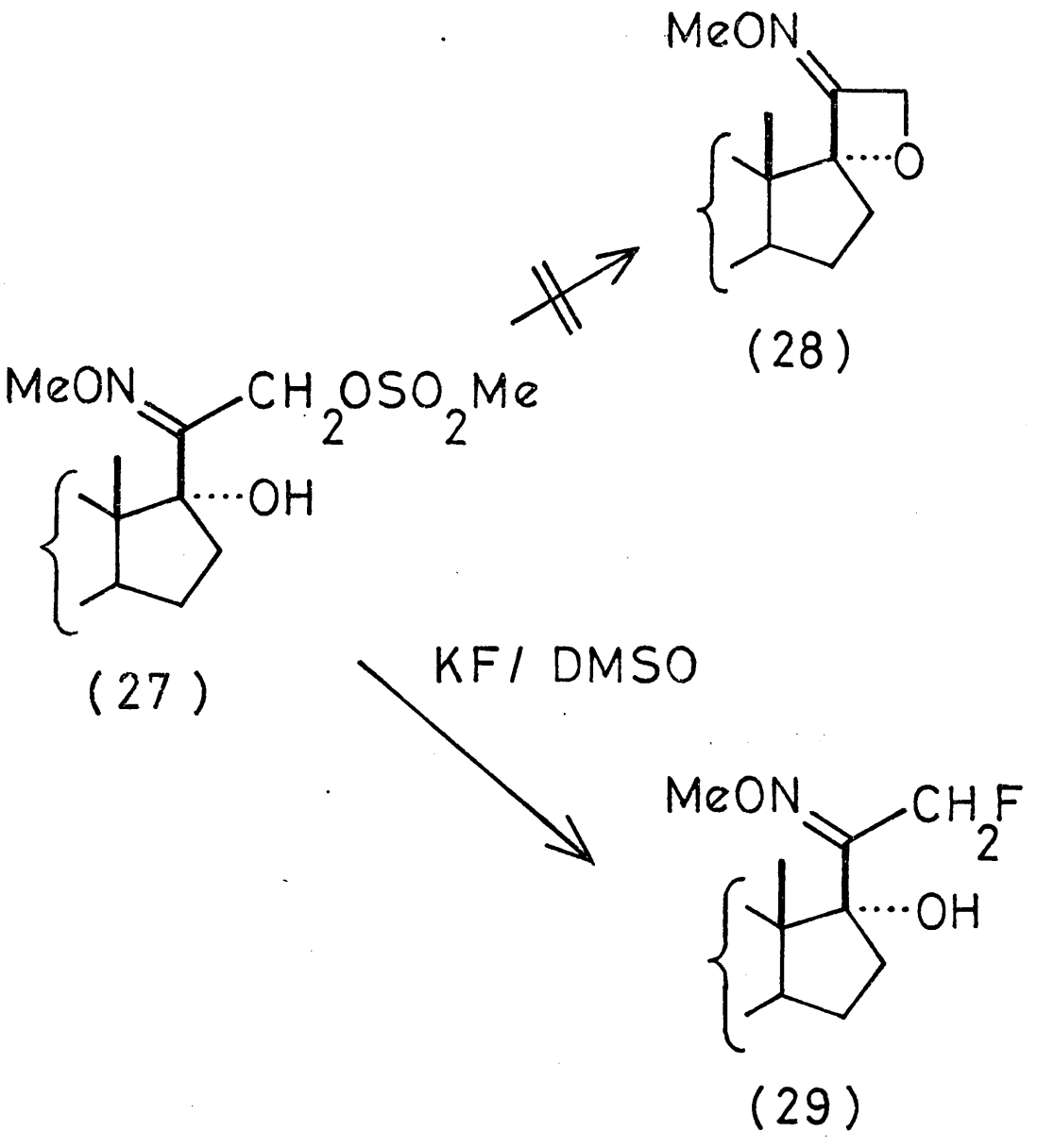


Fig. 17: Reaction of Substance S 3,20-diMO 21-mesylate with potassium fluoride in dimethylsulphoxide.

rapid S_N2 displacement). However, a feature which complicates the requirement for high basicity is that the use of strong bases can lead to undesirable D-homoannulation; this introduces a further limitation on the base chosen.

In an attempt to increase the yield of oxetanone, and at the same time reduce the amount of 21-fluoride formed, the effect of changing the following reaction parameters was studied: base, solvent, temperature and time. The results of these experiments, using Substance S mesylate as substrate, are summarised in Table 7, which shows that with the system potassium fluoride/dimethyl sulphoxide (DMSO) a much shorter reaction time (2h at 80^{o}) than that originally employed will suffice, although the same mixture of products is obtained. Dimethylacetamide (DMA) appeared to be interchangeable with dimethyl sulphoxide as solvent. The use of three different bases, all of low nucleophilicity, was investigated. These were fluoroborate ion (KBF_4^-), mesylate ion ($CH_3SO_3^-$) and pyridine (which also acted as solvent). The mesylate ion appeared attractive as a possible base, since any displacement at C-21 would regenerate the substrate for the cyclisation. However, all three proved unsatisfactory in effecting the desired reaction.

A slightly different approach was then investigated, in which the diMO derivative of Substance S 21-mesylate (partial structure 27) was used as a substrate for the cyclisation (fig.17). By derivatising the C-20 carbonyl function in this way, it was anticipated that its electron-withdrawing effect would be diminished. Consequently the susceptibility of the mesylate substituent at C-21 to direct displacement by fluoride ion might become electronically less favourable. Some degree of steric hindrance to approach of the fluoride ion at C-21 would also be expected from the -OMe group. It was hoped, therefore, that this substrate might lead to the diMO derivative of 17α,21-anhydro-S (28). However, this oxetanone derivative was not isolated from the reaction mixture, which yielded the diMO derivative of 21-fluoro-/

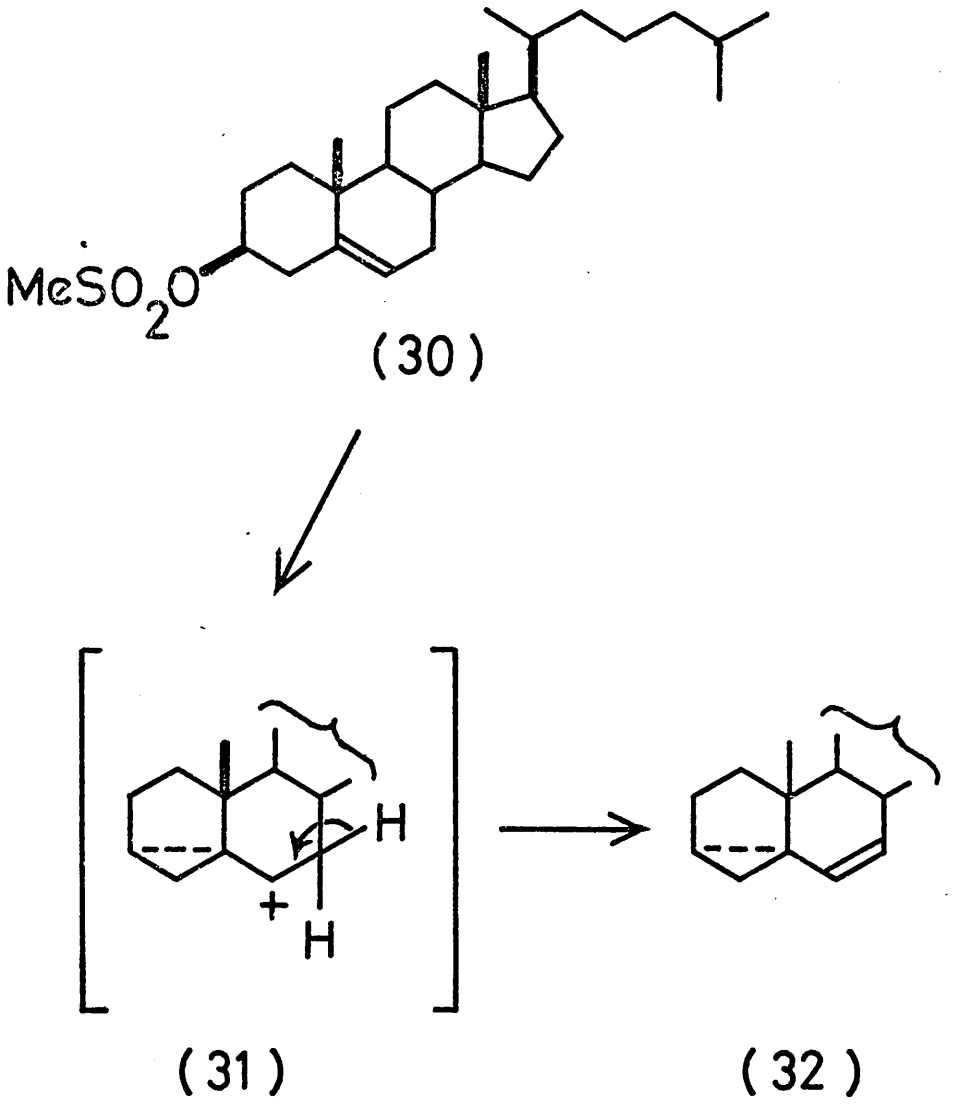


Fig.18: Thermal cyclisation of cholesteryl mesylate.

21-fluoro-17α-hydroxy-4-pregnene-3,20-dione (29) as the major product. This compound, interestingly enough, was stable towards GLC, and was characterised by GC-MS.

2.3.5 THERMAL CYCLISATION OF CORTICOSTEROID 21-MESYLATES

Following the observation that the 21-mesylate of Substance S gave, on injection onto a 1% SE-30 column at 225°, a single peak with the same retention index as that of Substance S oxetanone, the procedure was repeated for some other corticosteroid mesylates. Thus, cortisol, cortisone and prednisone mesylates also gave peaks with retention indices compatible with those of the corresponding 17α, 21-oxides. However, in these three examples, an additional (minor) peak was also present; retention data suggested that these additional components were the respective 17-ketosteroids.

On re-examination of Substance S 21-mesylate by GC-MS, the identity of the compound formed during gas chromatography – presumably in the flash heater zone – was confirmed as the oxetanone derivative by comparison of its mass spectrum with that obtained from an authentic sample. Thus, a thermal cyclisation was indicated, in which methanesulphonic acid was eliminated and the oxetanone derivative produced. An analogous process was observed by Vanden Heuvel *et al.*, who studied the gas chromatographic behaviour of mesylates and tosylates of certain sterols.[115] In the case of cholesterol mesylate (30) several peaks were obtained on the chromatogram, one of which corresponded to 3,5-cyclo-6-cholestene (32). It was suggested that this compound arose from a thermally-induced *i*-steroid reaction, whereby intramolecular displacement of the 3β-mesylate took place with homoallylic participation of the Δ^5-double bond. The intermediate carbonium ion (31) could not undergo solvation in the gas phase and was therefore saturated by loss of/

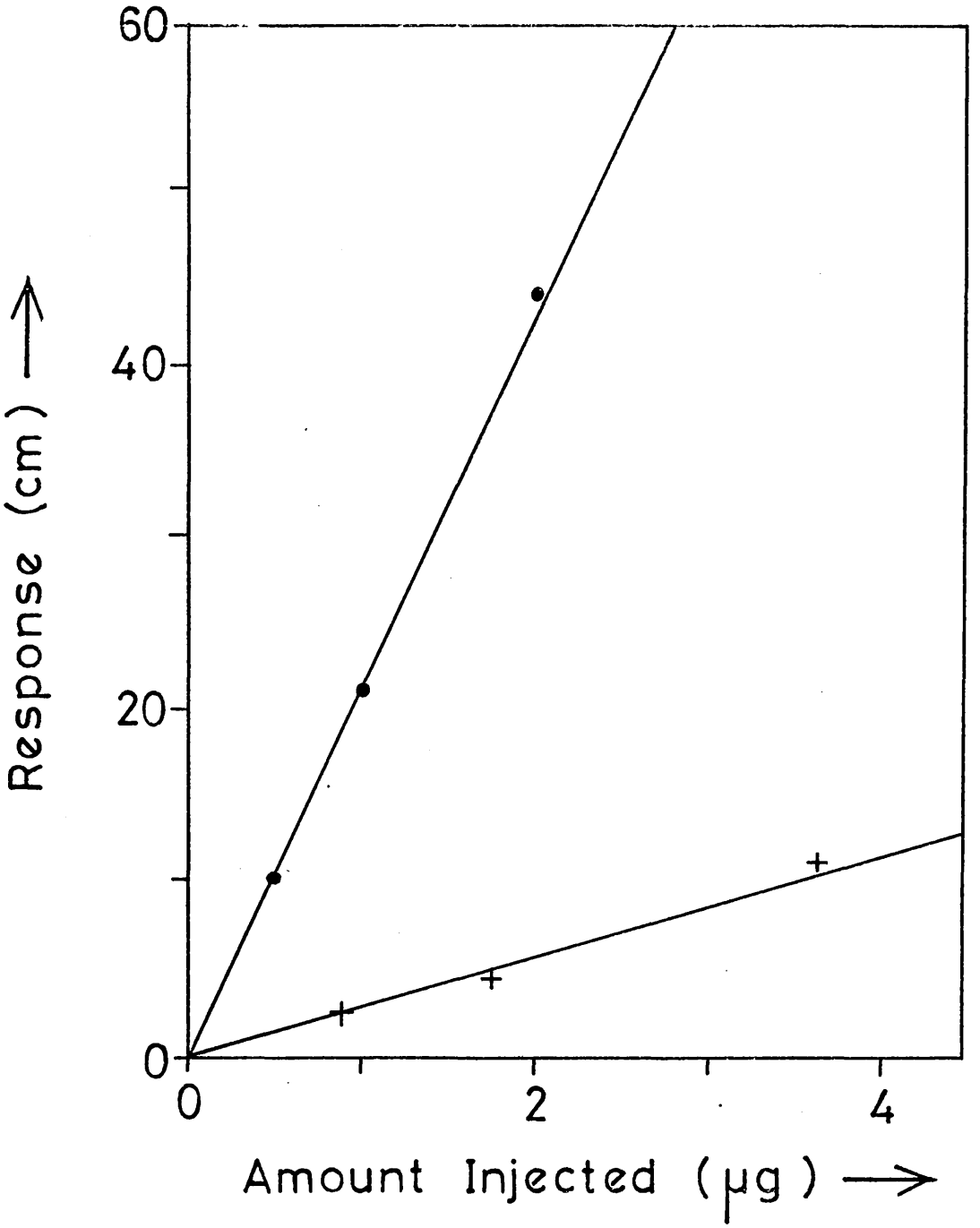


Fig. 18A: Flame ionisation detector response to derivatives of Substance S:-
• 17 21-anhydro S;
+ S 21-mesylate
Response measured as peak height, with adjustment for different amplifications.

of a proton from C-7.

Quantitative aspects of the thermal cyclisation (in the GLC flash heater zone) of Substance S 21-mesylate were investigated, and the results are summarised in fig. 18A. A linear relationship was demonstrated between peak height (under constant GLC conditions) and mass of the mesylate and its corresponding oxetanone. However, the detector response for Substance S oxetanone resulting from the pyrolysis of the 21-mesylate was only 16± 2% of that obtained from a corresponding mass of oxetanone. This result is very similar to that previously reported for the thermal degradation of steroidal dihydroxyacetones to their corresponding 17-ketones.[83-85)]

2.3.6 CONCLUSIONS

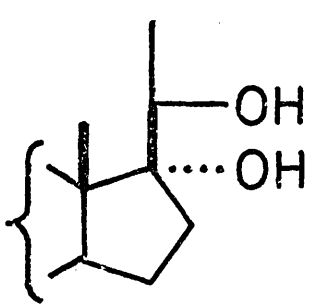
Oxetanones are potentially useful derivatives for the study of steroidal dihydroxyacetones by gas phase methods. They possess good gas chromatographic properties, affording satisfactory GLC peaks even in cases where the steroid bears several additional functional groups. Their retention indices are generally lower than for other types of corticosteroid derivative (excluding the side-chain oxidation products), as their molecular weights are eighteen units less than those of the parent steroids. Furthermore, oxetanones afford informative mass spectra, exhibiting molecular ions, and also characteristic fragments of high abundance in the upper mass region. Such fragments provide a means of detecting particular structural types at high sensitivity by "single ion monitoring" (section 3.3).

The chief problem currently impeding the application of these derivatives to the study of suitable corticosteroids lies in the unsatisfactory method of preparation, whereby interfering/

interfering 21-fluorinated compounds are formed by a competing reaction. It is hoped that a more practical preparative method will eventually emerge.

SECTION 3

A COMPARISON OF CORTICOSTEROID DERIVATIVES BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY.



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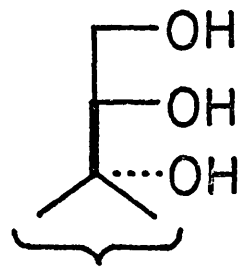

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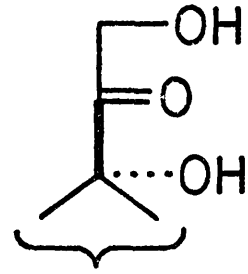

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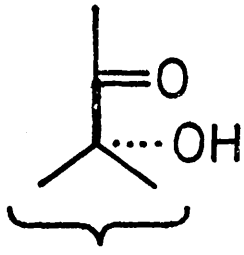

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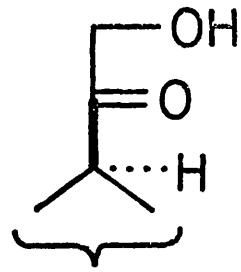

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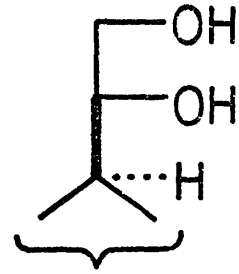


(34)

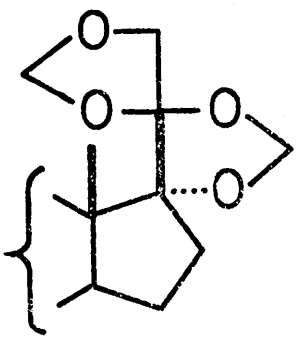
Fig.19: The six corticosteroid side-chains.

3.1 INTRODUCTION

The rapid increase, during the early 1960's, in the use of GLC in steroid analysis, was accompanied by a demand for suitable derivatives. Through enhancing the volatility, and reducing the polarity of steroids, such derivatives led to greatly improved chromatographic behaviour and to the feasibility of analysing complex mixtures of biological origin.

Trimethylsilyl (TMS) ethers, first described as derivatives for alcohols in 1957[116], were later applied in the steroid field by Luukkainen *et al.*[43] These derivatives, which had excellent GLC properties, rapidly found widespread use in steroid analyses, and were applied, *inter alia*, to the study of estrogens,[117-119] androgens[120-126] and bile acids.[127] Acetates were also found to be useful derivatives for hydroxysteroids,[128] although their retention times were somewhat higher than the corresponding TMS ethers. Trifluoroacetates, on the other hand, were found to have relatively short retention times,[129] and proved to be particularly useful when used as derivatives for electron-capture detectors.

However, considerable difficulty was experienced in the gas chromatography of certain steroids, notably those belonging to the corticosteroid family. These compounds comprise the adrenocortical hormones (fig.2), their immediate precursors and principal metabolites, and possess one of the six types of side-chain shown in fig.19. Under the conditions necessary for steroid GLC, thermal decompositions and rearrangements were frequently encountered, particularly with the 17-hydroxycorticosteroids (11-13, 15). For example, 20,17-ketols (15) could undergo D-homo steroid formation[130], while dihydroxyacetones (13) suffered side-chain cleavage in the flash heater zone, affording the corresponding 17-ketones.[81] (Some thermal/



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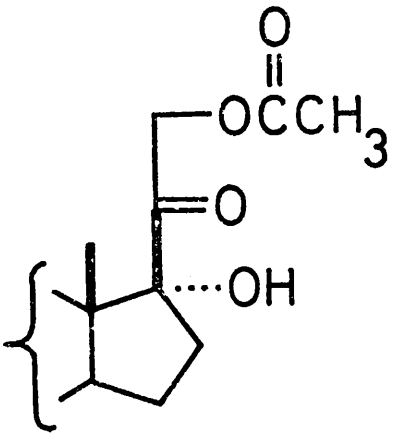

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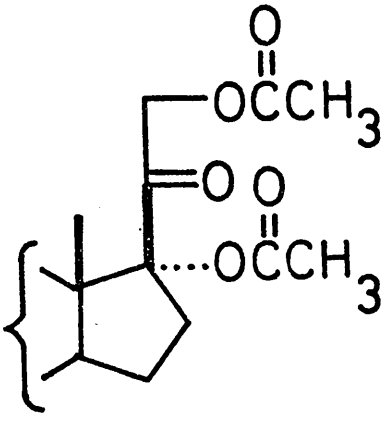

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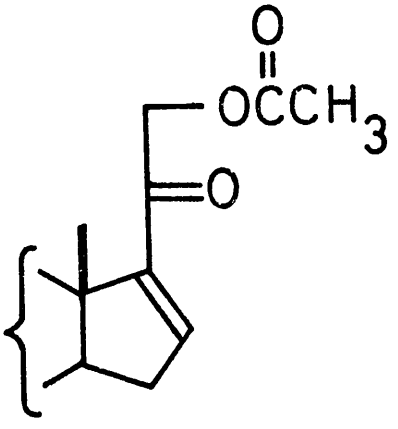

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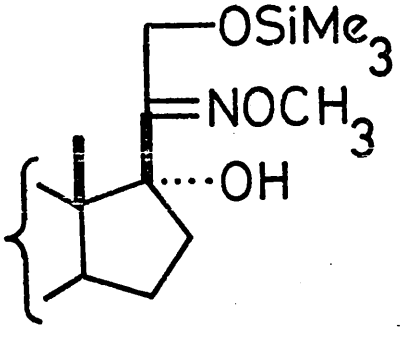

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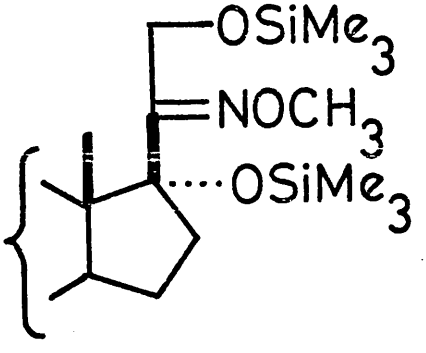
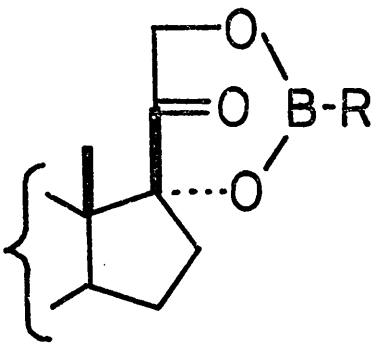


(40)

Fig. 20: Corticosteroid derivatives (I).

thermal D-homoannulation of the dihydroxyacetone structure has also been observed.[131]) Although chemical oxidation of corticosteroids afforded products which were amenable to GLC (section 2.1), various means of stabilising the side-chains through derivative formation were investigated.[132] Particular attention centred on the labile dihydroxyacetone grouping, for which the first derivative shown to possess gas chromatographic stability was the bismethylenedioxy (BMD) derivative (35) of Kirschner and Fales[133]. Gas chromatography of the cortisone derivative was accomplished, but the reaction was not found to be generally applicable. Cortisone 21-acetate (partial structure 36) was studied by Wotiz et al. and found to be somewhat more stable to GLC than cortisone itself.[134] However, some decomposition (8 to 14%[133]) and rearrangement were still evident. 17,21-diacetates (37) could be chromatographed intact,[135,136] although some elimination occurred on GLC to give the 21-acetoxy-Δ^{16}-20-one derivative (38).

Following the introduction of the O-methyloxime (MO) derivative for ketosteroids[137], it was shown by Gardiner and Horning that the 20-MO 21-TMS ethers (39) were effective in stabilising steroidal dihydroxyacetones.[54] These, and the corresponding 20-MO 17,21-diTMS ethers (40)[138] could be prepared in a quantitative fashion from microgram amounts of steroid[139], and possessed excellent GLC properties. In addition, the prior conversion of reactive ketone groups to their O-methyloximes prevented the uncontrolled formation of enol-TMS derivatives in the subsequent silylation step.[140] Isomers of the syn/anti type were observed for some MO derivatives,[54, 114, 141-144] although when the ketone group was located at C-17 or C-20, as is the case in most urinary/



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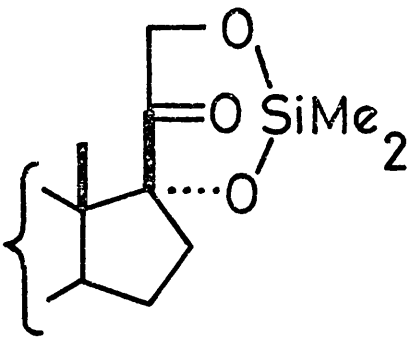

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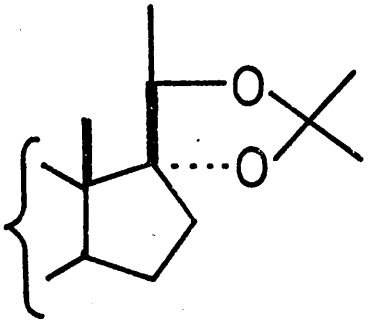

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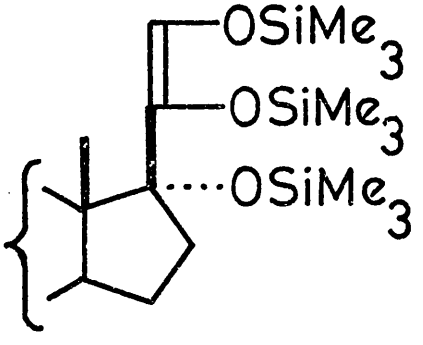


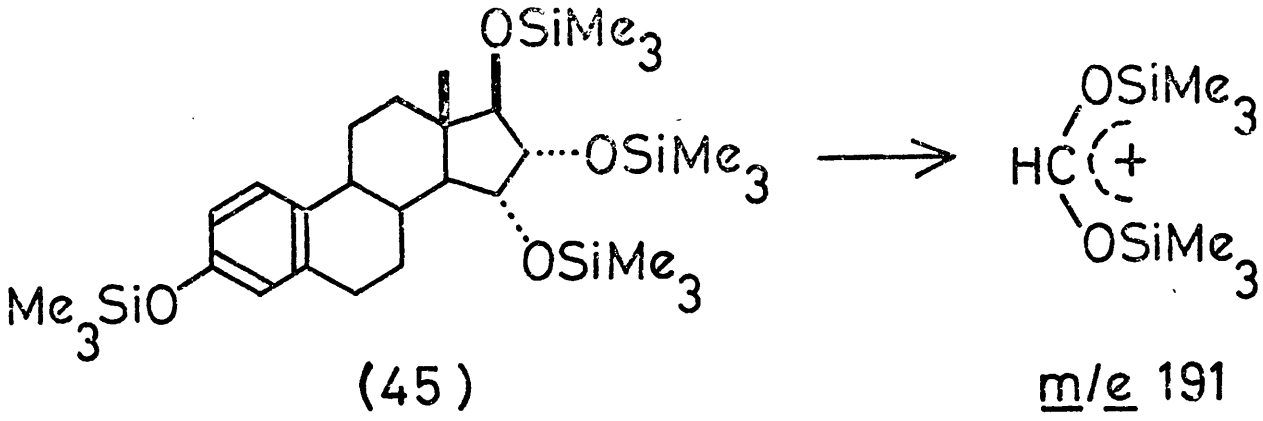
(44)

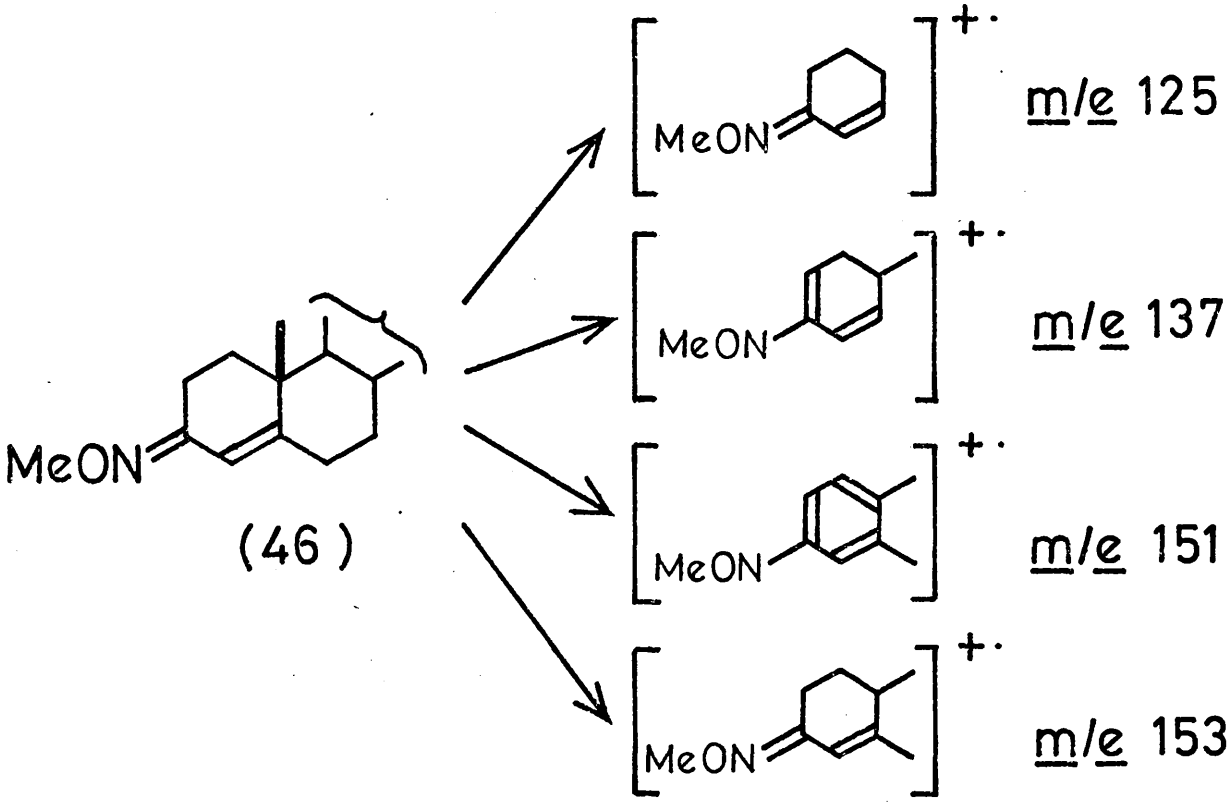
Fig. 21: Corticosteroid derivatives (II).

urinary metabolites, a single GLC peak was obtained.[145,146] Thus, TMS ethers and, where appropriate, MO-TMS derivatives could be successfully employed to stabilise the various types of corticosteroid side-chain, and thus to permit the GLC analysis of complex mixtures of these compounds, such as one encounters in urinary steroid extracts.

A slightly different approach to the problem involved the incorporation of the 1,2 - or 1,3-diol system present in most corticosteroid side-chains into a cyclic derivative. Conversion of the dihydroxyacetone structure (a 1,3-diol) to such a derivative was effected by treatment with a suitable boronic acid, and the resulting six-membered cyclic boronate ester (41; R= alkyl or aryl) found to be stable towards GLC.[94, 95, 147] 17, 20- and 20,21-diols formed analogous five-membered derivatives, while the unusually stable boronate ester obtained from 17,20,21-triol groupings has been shown to possess a six-membered ring, incorporating the oxygen functions at C-17 and C-21 (see appendix III). The cyclic dimethylsiliconide (42), introduced by Kelly,[96,97] was also shown to stabilise the dihydroxyacetone side-chain for GLC. Acetonides (43) have been prepared from steroidal 1,2-diol systems,[148] and have been shown to be useful derivatives for the gas chromatographic characterisation of natural, and drug metabolites possessing these structures.[149, 150] The use of boronate esters, dimethylsiliconides and acetonides has several advantages in the GLC of corticosteroids, notably the element of specificity involved in their formation; non-cyclic derivatives lack this feature.

More recently, TMS enol-TMS derivatives have been described, and shown to possess excellent gas chromatographic properties in favourable cases e.g. ring A - saturated metabolites with dihydroxyacetone or 20, 21-ketol side-chains.[151-154] In each type, enolisation of the 20-ketone affords a 20,21-double bond (cf. partial structure 44), and although formation of two/





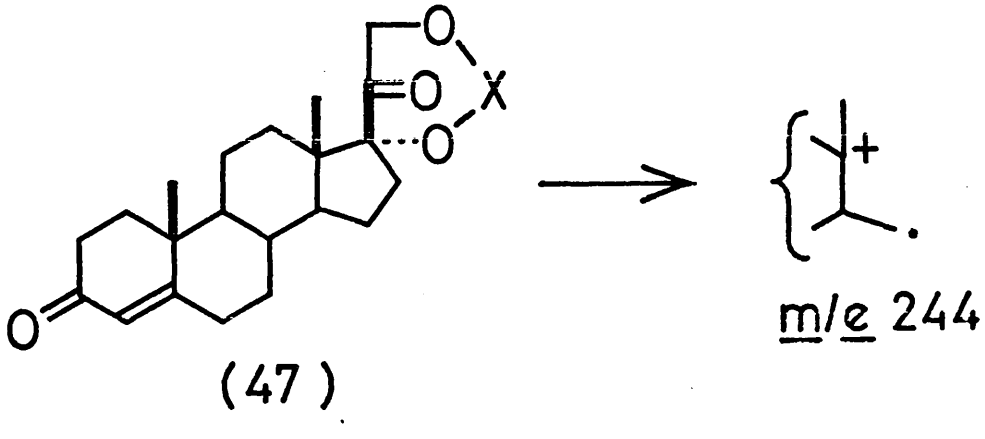


Fig.22: Characteristic fragmentations of steroid derivatives.

two geometrical isomers is possible, only a single GLC peak is observed.

With the advent of combined GC-MS, further criteria were required of steroid derivatives, namely that they should yield readily interpretable and structurally informative mass spectra. Fortunately, the same derivatives as used for GLC have frequently been found to be of value in GC-MS. This has certainly been true of TMS ethers,[155] which almost invariably afford spectra with clear molecular ions, or indirect evidence thereof (e.g. loss of trimethylsilanol from the molecular ion frequently occurs to give a peak $(M-90)^{+\cdot}$ [52]). Fragmentation generally proceeds via facile α-cleavage, and ions characteristic of the environment of the silyl group are often obtained in high relative abundance. Thus the ion at m/e 191 is frequently encountered in the mass spectra of compounds with vicinal TMS groups, and arises by a rearrangement process.[156] This fragment has been employed for the determination of estetrol (45) by "single ion monitoring".[64] The identification of ions in the spectra of TMS ethers may be facilitated by the use of perdeuterio-TMS derivatives, when the silyl-containing fragments are revealed by an appropriate shift in mass.[157]

In O-methyloximes, the nitrogen atom stabilises the molecular ion, and reduces the extent of fragmentation in the high mass region. Characteristic losses of 15 a.m.u. (methyl radical) and 31 a.m.u. (methoxyl radical)[158,159] from the molecular ion are observed in the spectra of MO derivatives, while O-methyloximes of Δ^4-3-ketosteroids (partial structure 46) afford "ring A" fragments at m/e 125, 137, 151 and 153 (fig.22).[114] The cyclic boronate esters and dimethylsiliconides also show well-defined molecular ions, and in the case of the dihydroxyacetone derivatives (47), formation of the most abundant fragments (common to both types) involves loss of the heterocyclic ring through scission of the C-13/C-17 and C-15/C-16 bonds (fig.22).[96,160]

Although plentiful GLC and mass spectrometric data are available for individual corticosteroid derivatives, little material of a comparative nature has been published. In the survey described below, the gas chromatographic and mass spectrometric properties of a number of representative derivatives have been compared, and aspects of their preparation and stability are discussed. (Some products of side-chain degradation are also included for comparison.) The technique of "single ion monitoring" has been applied to the quantitative estimation of five derivatives of Reichstein's Substance S and results on the linearity of response and lower limits of detection are reported.

3.2 EXPERIMENTAL

PREPARATION OF DERIVATIVES

Derivatives were formed by conventional methods, normally using 0.4mg (ca. 1μm mole) of steroid, except in the case of the 17α,21-anhydro derivative of Substance S, which was prepared as outlined in section 2.2.

The derivatives studied may be divided into three groups:-

Group A - Single Derivatives:- those prepared by a "one-step" procedure.

Group B - Mixed Derivatives:- those prepared by a "two-step" procedure, each step derivatising a different type of functional group.

Group C - Derivatives arising from the oxidative cleavage of the corticosteroid side-chain.

3.2.1 Group A - Single Derivatives.

O - Methyloximes.[161] The steroid (400μg) was placed in a Pyrex glass tube and methoxyamine hydrochloride (2-4mg) added. The mixture was dissolved in dry pyridine (100-200μl), the tube stoppered, and allowed to stand at room temperature overnight. (Alternatively, the reaction could be carried out in 30min by heating at 60-70°.) The solvent was then evaporated under a stream of nitrogen, and the residue treated with ethyl acetate (2ml) and a 4% H Cl - 10% Na Cl solution (2ml). The aqueous layer was extracted with ethyl acetate (2x2 ml), and the combined ethyl acetate extracts washed with a 5% $NaHCO_3$-10% NaCl solution (3x2ml) and dried. Evaporation of this final solution yielded the O-methyloxime, which was taken up in ethyl acetate (200μl) for GLC. Samples were stored as ethyl acetate solutions at 5°.

Note: Keto groups at C-11 do not form methyloximes under these conditions.

Trimethylsilyl (TMS) ethers. Three different procedures were used;/

used, according to the degree of steric hindrance of the hydroxyl group (s) to be silylated:

(i) Unhindered hydroxyl groups (e.g. at C-3, C-6, C-20 and C-21)[162] The steroid (400μg) was treated with 200μl of N,O-bis-(trimethylsilyl)-acetamide (BSA) or N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). (Where necessary, one or two drops of dry pyridine were added to effect solution of the steroid). The reaction was found to be complete in a few hours at room temperature, or in 15 min on heating to 60-70°. The reagent was then evaporated under nitrogen and the product taken up in ethyl acetate (200μl) for GLC. Samples were stored as ethyl acetate solutions at 5°.

(ii) Moderately hindered hydroxyl groups (e.g. at C-11β)[162] In this case, a catalyst, trimethylchlorosilane (TMCS) was required. Thus the steroid (400μg) was treated with 200μl of a mixture of BSA and TMCS (2:1) and allowed to stand overnight at room temperature. The TMCS and excess BSA were evaporated under nitrogen, and the residue was re-extracted into BSA (200μl) for GLC.

(iii) Highly hindered hydroxyl groups (e.g. at C-17α in the pregnane series)[161] Again, a catalysed reaction was necessary, using a silylating mixture comprising N-trimethylsilyl-imidazole (TSIM), BSA and TMCS (3:3:2). The steroid (400μg) was placed in a screw-capped, Teflon-lined, "Reacti-Vial" (Pierce Chemical Co., Rockford, Illinois, U.S.A.) and the above silylating solution (200μl) added. The tube was then heated for 4h at 150°, and the reagents were evaporated under nitrogen. The residue was taken up in BSA (200μl) for GLC.

Note: Many derivatives of this type were found to be highly susceptible to hydrolysis unless stored in a silylating reagent: BSA was found convenient for this purpose.

Deuterium-labelled trimethylsilyl ethers. These were prepared, for unhindered hydroxyl groups, by the use of d_{18}-N,O-bis-(trimethylsilyl)-acetamide (Merck, Sharp and Dohme of Canada, Ltd.) The method was the same as that described in (i) above.

Methylboronate[95] The steroid (400μg) was treated with 300μl of a standard solution of methylboronic acid in ethyl acetate (0.5mg/ml) - this corresponded to approx. two molar equivalents of reagent. The mixture was left at room temperature for 5-10min and an aliquot used directly for GLC.

Note: In cases where a single, free hydroxyl group was present at some other position in the molecule, only one equivalent of reagent was used to prevent formation of a linear boronate ester at such a site.

A small-scale preparation of Substance S methylboronate was carried out as follows:- Substance S (4μg) was mixed with a solution of methylboronic acid (4 molar equiv.) in ethyl acetate (6μl), and kept in a closed tube at room temperature for 15 min. An aliquot ($\frac{1}{4}$) was used for GLC, which indicated that the boronate had been formed in 85% yield, as judged by the use of a reference sample. The remainder was diluted to 100μl, and 2μl (equivalent to 60ng of Substance S) was used for "single ion monitoring" (section 3.3.4).

Dimethylsiliconide[97] The steroid (400μg) was placed in a Pyrex tube (3x$\frac{3}{8}$") with a constriction in the neck. The sample was then dissolved in a mixture of dry benzene and acetone, and the resulting solution taken to dryness under nitrogen. (This procedure ensured that the steroid was quite anhydrous). 200μl of a solution of dimethyldiacetoxysilane (2%) and triethylamine (2%) in dry hexane was then added, and the tube briefly flushed out with nitrogen and sealed off in a flame. After a period of 4h at 45° the tube was broken open and the mixture injected directly into the gas chromatograph.

Note: Both the derivatives and the reagent, dimethyldiacetoxysilane, were found to be extremely susceptible to hydrolysis.

Consequently, fresh solutions of reagent were prepared at regular intervals and stored in tightly capped vials under anhydrous conditions.

Acetonides[150] Anhydrous conditions were also necessary for the effective preparation of acetonides. Thus, the steroid (400μg) was placed in a screw-capped Pyrex tube (4x½") and dried by the method described above for dimethylsiliconide preparation. The sample was then dissolved in dry acetone (2ml) containing p -toluenesulphonic acid (2mg), anhydrous calcium chloride (50mg) was added, and the mixture shaken for 4h. The calcium chloride was removed by filtration, and the filtrate treated with two drops of pyridine. This solution was taken to dryness under nitrogen and the residue extracted with dry benzene (20ml). This extract was then washed successively with N sodium hydroxide (2ml) and distilled water (2ml), and the combined washings were back-extracted with benzene (15ml). Finally, the combined benzene extracts were dried and evaporated in vacuo to yield the acetonide, which was taken up in ethyl acetate (200μl) for GLC.

3.2.2 Group B- Mixed Derivatives

O-Methyloxime-trimethylsilyl ethers (MO-TMS)[161] These derivatives were prepared by a two-step process, in which the ketone groups were first converted to their O-methyloximes, as outlined above, and the hydroxyls subsequently derivatised using the appropriate silylating method.

O-Methyloxime dimethylsiliconides (MO-dimethylsiliconides) Steroid O-methyloximes, prepared in the normal manner, were converted to this mixed derivative using dimethyldiacetoxysilane, as described above. Anhydrous conditions were again found to be essential for this reaction.

O-Methyloxime heptafluorobutyrates (MO-HFB)[163] The steroid O-methyloxime (200µg) was placed in a "Reacti-Vial" and dissolved in acetonitrile (60µl). N-Heptafluorobutyryl-imidazole (HFB-imidazole; 100µl) was added, and the mixture held at room temperature for 30 min. The reaction mixture was then extracted with hexane (3x150µl) and the combined hexane extracts were evaporated to dryness under nitrogen. The residue was extracted with ethyl acetate (100µl) at -5°, and this solution used for GLC.

Oxetanone O-methyloxime (Oxetanone-MO) A sample of the 17α,21-anhydro derivative (oxetanone) from Reichstein's Substance S (section 2.2) was converted to its di-O-methyloxime by the normal procedure.

O-Methyloxime methylboronate (MO-methylboronate). Treatment of steroid O-methyloximes with a slight excess of methylboronic acid, as outlined above, resulted in smooth conversion of suitable diol groupings to the corresponding MO-methylboronates.

Methylboronate-trimethylsilyl ethers (Methylboronate-TMS). In some instances, boronate ester formation yielded a derivative still retaining one or more free hydroxyl groups. In favourable cases, where the boronate moiety was relatively stable (e.g. boronates from pregnane 17,20,21-triols), these hydroxyls could subsequently be derivatised using the appropriate silylating method. Thus, 17α, 20β, 21-trihydroxy-4-pregnen-3-one formed a 17α, 21-methylboronate (see Appendix III), which was then converted to the 17α,21-methylboronate 20β-trimethylsilyl ether by overnight reaction with BSA at room temperature.

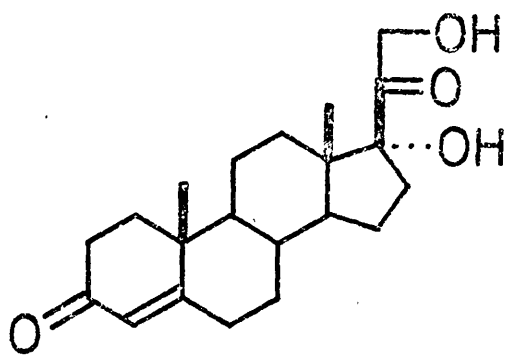
3.2.3 GROUP C - Side-chain Oxidation Products

Oxidation with Periodic Acid[86] The steroid (400µg) was placed in a 10ml Pyrex centrifuge tube and 400µl of a solution of periodic acid in 50% aqueous dioxan (10mg/ml) added. The tube was corked and the reaction allowed to proceed for 3h at room temperature. The reaction mixture was then diluted with distilled water (2ml) and extracted with methylene chloride (3x1ml). Emulsification of the /

the two phases was accomplished by agitating the stoppered centrifuge tube for approx. 20 sec. on the Micromixer. The emulsion was broken by a brief centrifugation, and the (lower) methylene chloride layer withdrawn with a Pasteur pipette. The combined extracts were evaporated to dryness and the residue taken up in ethyl acetate (400µl).

Where the substrate for the oxidation was a 20,21-ketol or a 17α,21-dihydroxy-20-one, a carboxylic acid function resulted at C-20. In this case, the acid was methylated by treating the above ethyl acetate solution with an excess of a freshly prepared ethereal solution of diazomethane (prepared from bis-(N-methyl-N-nitroso)-terephthalamide [164]). A persistent yellow colour indicated the methylation was complete. Finally, the reaction mixture was taken to dryness under nitrogen and the residue re-dissolved in ethyl acetate for GLC.

Oxidation with Sodium Bismuthate.[26,91] The steroid (400µg) was placed in a screw-capped Pyrex tube (10cm x 1cm), together with sodium bismuthate (4mg) and 50% aqueous acetic acid (400µl). The tube was wrapped in aluminium foil to exclude light, and shaken at room temperature for 3 h. A saturated solution of sodium bicarbonate (1.0ml) was then added, and the resulting mixture filtered into a clean centrifuge tube. The product was extracted (in a similar manner to that outlined above for the periodic acid cleavage) using dry methylene chloride (3x1ml), the combined extracts washed, first with N sodium hydroxide (1.0ml) and then with water (2.0ml), and finally dried and evaporated. The residue was taken up in ethyl acetate (200µl) for GLC.



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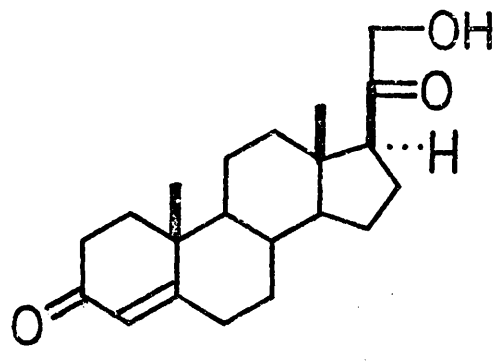

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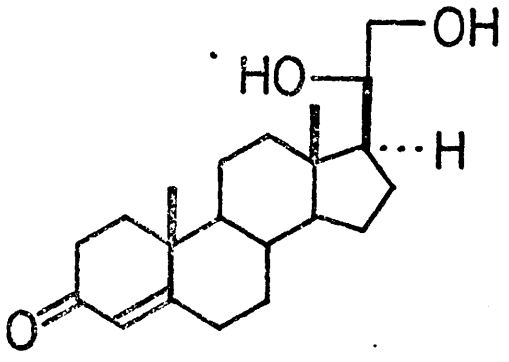

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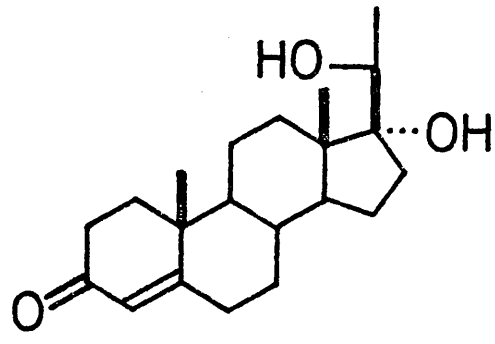

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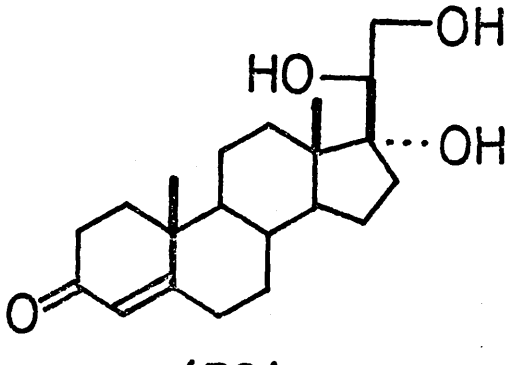

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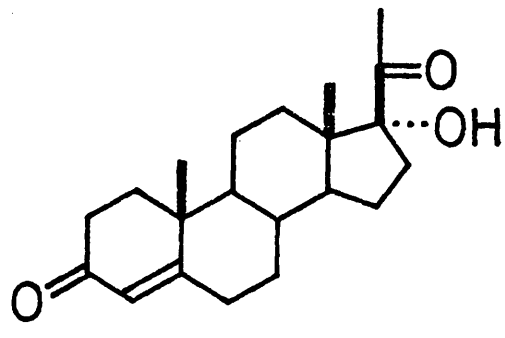


(53)

Fig.23: Representative corticosteroids studied in section 3.

3.3 RESULTS AND DISCUSSION

The following steroids, which were used in this study, were taken to be representative of the six types of corticosteroid, classified according to their side-chain:-

(i) Dihydroxyacetone:

Reichstein's Substance S (17α,21-dihydroxy-4-pregnene-3,20-dione; 48).

(ii) 20,21-Ketol:

Deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione; 49)

(iii) 20,21-Diol:

20β,21-Dihydroxy-4-pregnen-3-one (50).

(iv) 17, 20-Diol:

17α,20β-Dihydroxy-4-pregnen-3-one (51).

(v) 17,20,21-Triol:

17α,20β,21-Trihydroxy-4-pregnen-3-one (52).

(vi) 20,17-ketol:

17α-Hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione; 53).

The structures of these compounds are given in fig.23.

3.3.1 PREPARATION OF DERIVATIVES

MO TMS Ethers. The results confirm the general efficacy of the two-stage procedure[54], whereby reactive ketonic groups are converted to O-methyloximes and these products are trimethylsilylated, with or without reaction of the 17α-hydroxyl group.[138,152,161,162,165] Thus study was limited to 11-deoxysteroids, but conditions have already been well established for the trimethylsilylation of 11β-hydroxycorticosteroids.[152,162] 11-Ketones are highly resistant to methoximation,[166] while their conversion to enol-TMS ethers occurs only slowly even under vigorous conditions.[152,161]

Methylboronates. The preparation of these derivatives was effected by mixing the steroid with methylboronic acid in an organic solvent.[95] This extreme simplicity allowed the formation of methylboronates from as little as 4μg of steroid diol. (The presence of additional/

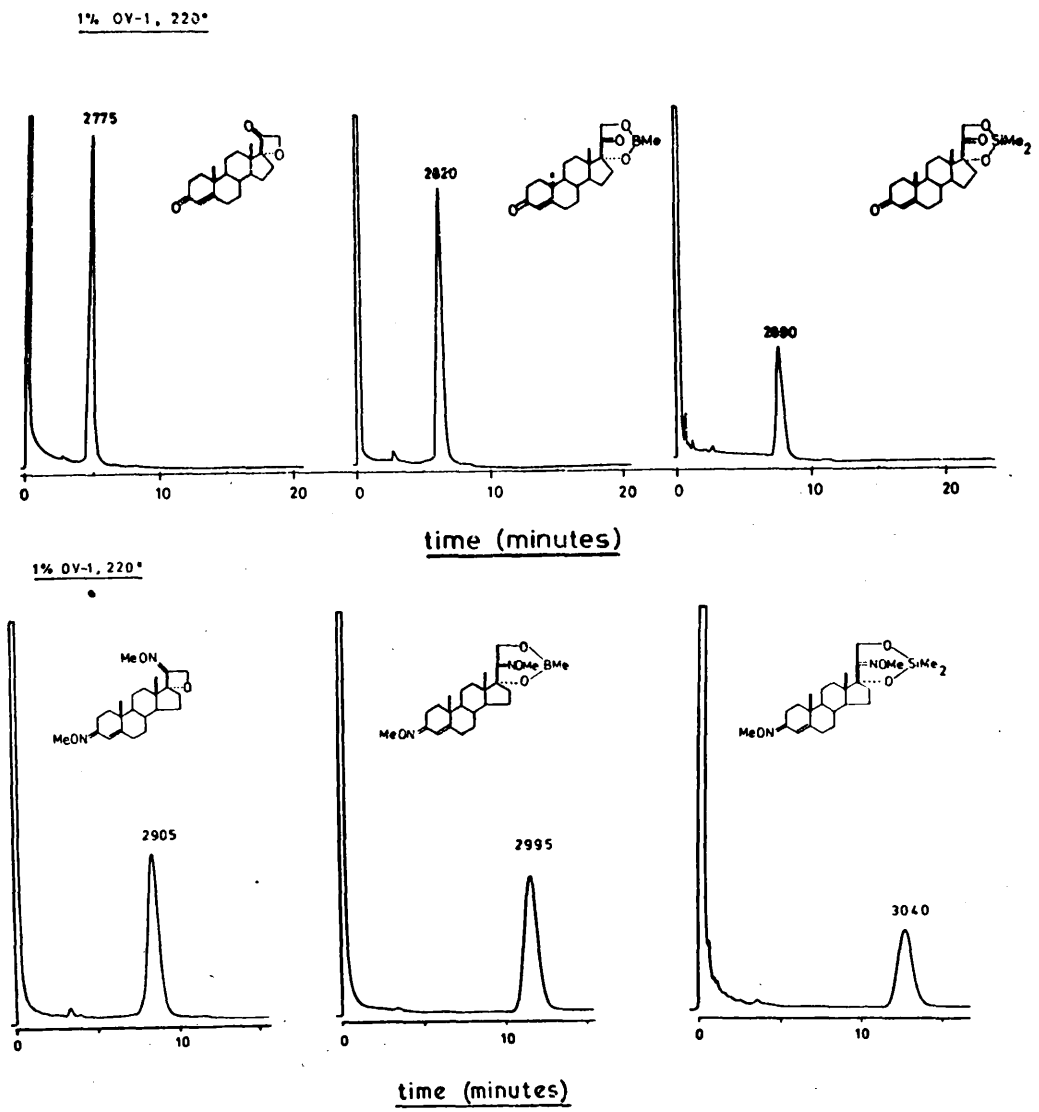


Fig. 24: Gas chromatograms of representative single and mixed derivatives of Substance S.

additional hydroxyl groups complicates the reaction.[95])

Dimethylsiliconides. These derivatives were satisfactorily obtained only from a dihydroxyacetone and from a 17, 20-diol: conditions suitable for the formation of a 20,21-dimethylsiliconide [97] were not found. Both the reagent (dimethyldiacetoxysilane) and the derivatives were extremely susceptible to hydrolysis. This finding contrasts with the reported stability towards hydrolysis of dimethylsiliconides from 16α, 17α-dihydroxycorticosteroids.[167,168])

Acetonides of Diols. The method of Bailey [150], where p-toluenesulphonic acid is employed as a catalyst, was found satisfactory for the small-scale preparation of acetonides from both 17,20- and 20,21-diols. The use of perchloric acid as a catalyst [148] would doubtless be superior for the formation of 17,20-diol acetonides in larger quantity. The acetonide obtained from the 17, 20,21-triol is assumed to be the 20,21-derivative.[148-150])

Oxetanones from Steroidal Dihydroxyacetones. These derivatives were prepared from large amounts (100mg) of steroid, as outlined in section 2.2.

Side-Chain Degradation Products. Oxidation with periodic acid[85]) or sodium bismuthate [91] resulted in the formation of an aldehyde or carboxylic acid function at C-20, or removal of the side-chain to yield the corresponding 17-ketone. Carboxylic acids were methylated with diazomethane for gas chromatography.

3.3.2 GAS CHROMATOGRAPHIC PROPERTIES

Gas chromatographic retention indices for the various derivatives are listed in Tables 8-14. Satisfactory peaks were obtained in all instances; chromatograms of representative single and mixed derivatives of Substance S are illustrated in fig.24. It should be noted that although the Dexsil-300GC column has been/

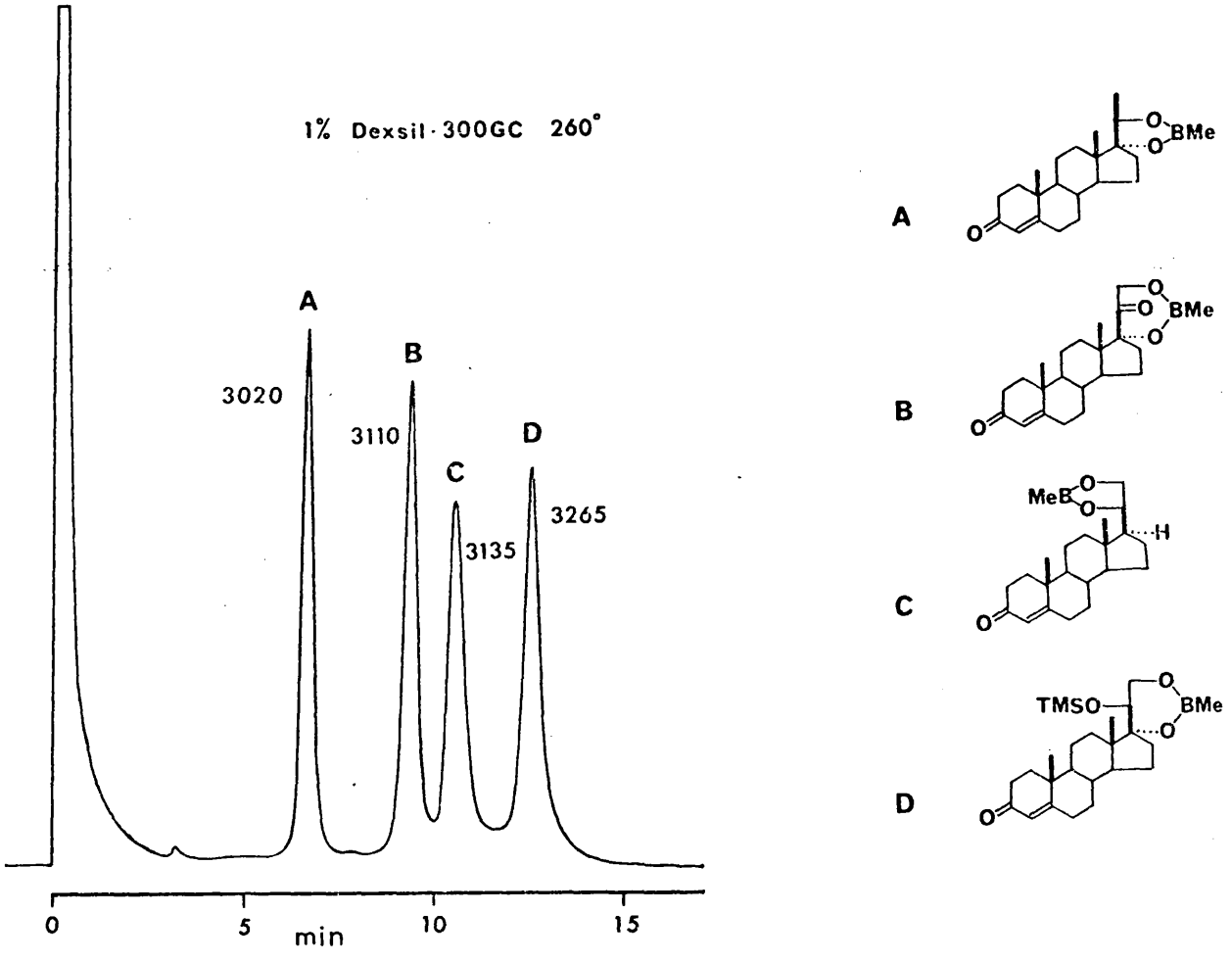


Fig. 25: Separation of four corticosteroid types as their boronate ester derivatives.

been found suitable [66] for certain steroidal tertiary alcohols, it would not be generally applicable to compounds containing free hydroxyl groups, since adsorption and/or decomposition has been observed in such cases.

While the retention data obtained are insufficient for detailed correlations, several general effects may be observed:-

(i) Methoximation of carbonyl groups increases the retention on OV-1 (cf. fig.24) but decreases it on Dexsil.

(ii) Among the derivatives formed without side-chain degradation, the methylboronates have the lowest retention indices, with the exception of the oxetanone from Substance S (on OV-1) and the MO triTMS ether of 17α,20β,21-trihydroxy-4-pregnen-3-one (on Dexsil). The latter exception arises because of the cumulative effects of the three TMS groups and the O-methyloxime function in reducing the retention time on the more polar phase.

(iii) Trimethylsilylation of the 17α-hydroxyl group is advantageous in reducing the retention times to values which are comparable (on Dexsil) with those of methylboronates.

(iv) It is evident that the only practical derivatives for the analytical separation of all six corticosteroid types are the MO TMS ethers. For the four types containing α or β-diol groupings, the methylboronates provide a possible alternative with the advantage of lower retention times (cf. fig.25): thus the 4-pregnen-3-ones possessing the dihydroxyacetone, 20β,21-diol, 17α,20β-diol and 17α,20β,21-triol side-chains yield MO (full-) TMS ethers with I_{OV-1} 3070, 3140, 2985, and 3160 respectively, while the corresponding methyl boronates have I_{OV-1} 2820, 2850, 2795 and 2970.

No marked differences were noted at the 1 μg level in the response of the flame ionisation detector to the various derivatives. The important question of the lower limits of detection is discussed below in relation to mass spectroscopic data (section 3.3.4).

3.3.3 MASS SPECTROMETRIC CHARACTERISTICS

Salient features of the mass spectra of the compounds studied are cited in Tables 8-14. Ions below m/e 80 have been disregarded in assignment of base peaks. Molecular ions were generally prominent, although two derivatives failed to give one, namely the MO-HFB derivative obtained from Substance S (Table 8) and the 20β,21-diol diTMS ether (Table 10.) In the former case, an intense ion at m/e (M-18) was obtained, indicating loss of the 17α -hydroxyl group and presumptive formation of the corresponding 16-dehydro derivative. This elimination may have occurred in the ion source, but it is more likely (from the low retention index value) that dehydration took place during derivative preparation. No molecular ion was obtained from the 20β,21-diol diTMS ether because of the supervention of α-cleavage of the TMS ether. Similar dominance of ether fragmentation accounted for the low abundance of the characteristic "ring A" ion of m/e 124 (cf. fig.14) in the TMS ethers of the 20,21-diol, 20,21-ketol and in the 20,21-diTMS ether of the 17,20,21-Triol. The fragmentations of other types of side-chain derivative competed more evenly with those characteristic of the nucleus: compounds retaining the 4-en-3-one group yielded ions of m/e 124, and the corresponding O-methyloximes gave ions at m/e 125, 137, 151, and 153[114] (fig.22). The mass differences between the various derivatives, and their individual fragmentation modes, together provide a wide range/

Compound	Retention Indices I^{250^o} OV-1	I^{275^o} Dexsil-300GC	$M^{+\cdot}$	Base Peak, m/e>80	m/e values and relative abundances of characteristic ions. Fragment ions
3,20-diMO 21-TMS	3165	3290	476 (9)	386	445 (65) 373 (44) 355 (85) 125 (52)
3,20-diMO 21-d_9-TMS	3155	3270	485 (9)	386	454 (67) 373 (44) 355 (85) 125 (50) 120 (73)
3,20-diMO 17,21-diTMS	3070	3135	548 (31)	517	445 (21) 427 (43) 273 (46) 125 (28) 103 (68)
17,21-dimethylsiliconide	2890	3170	402 (92)	91	387 (11) 374 (69) 359 (61) 244 (77) 242 (72) 229 (87) 143 (90) 124 (29)
17,21-methylboronate	2820	3110	370 (69)	244	355 (11) 243 (26) 229 (42) 124 (29) 91 (59)
17,21-anhydro-derivative (oxetanone)	2775	3120	328 (17)	286	270 (23) 269 (20) 258 (13) 244 (48) 242 (12) 229 (14) 148 (33) 136 (38) 124 (68)
3,20-diMO methylboronate	2995	3165	428 (72)	273	413 (4) 397 (27) 366 (6) 258 (6) 153 (16) 125 (17) 91 (25)
3,20-diMO dimethylsiliconide	3040	3220	460 (55)	429	445 (3) 398 (10) 273 (46) 170 (26) 158 (22) 153 (10) 151 (17) 143 (13) 125 (12)
3,20-diMO oxetanone	2905	3080	386 (100)	386	371 (21) 355 (31) 324 (10) 153 (34) 137 (52) 125 (72) 91 (42)
MO-HFB *	2925	2975	600 (0)	125	582 (84) 567 (9) 551 (29) 169 (24) 153 (71) 137 (90) 131 (71) 119 (21) 100 (43) 91 (38) 69 (131)

* See text.

Table 8: Retention index values and salient mass spectrometric data for derivatives of Reichstein's Substance S (17α,21-dihydroxy-4-pregnene-3,20-dione; 48).

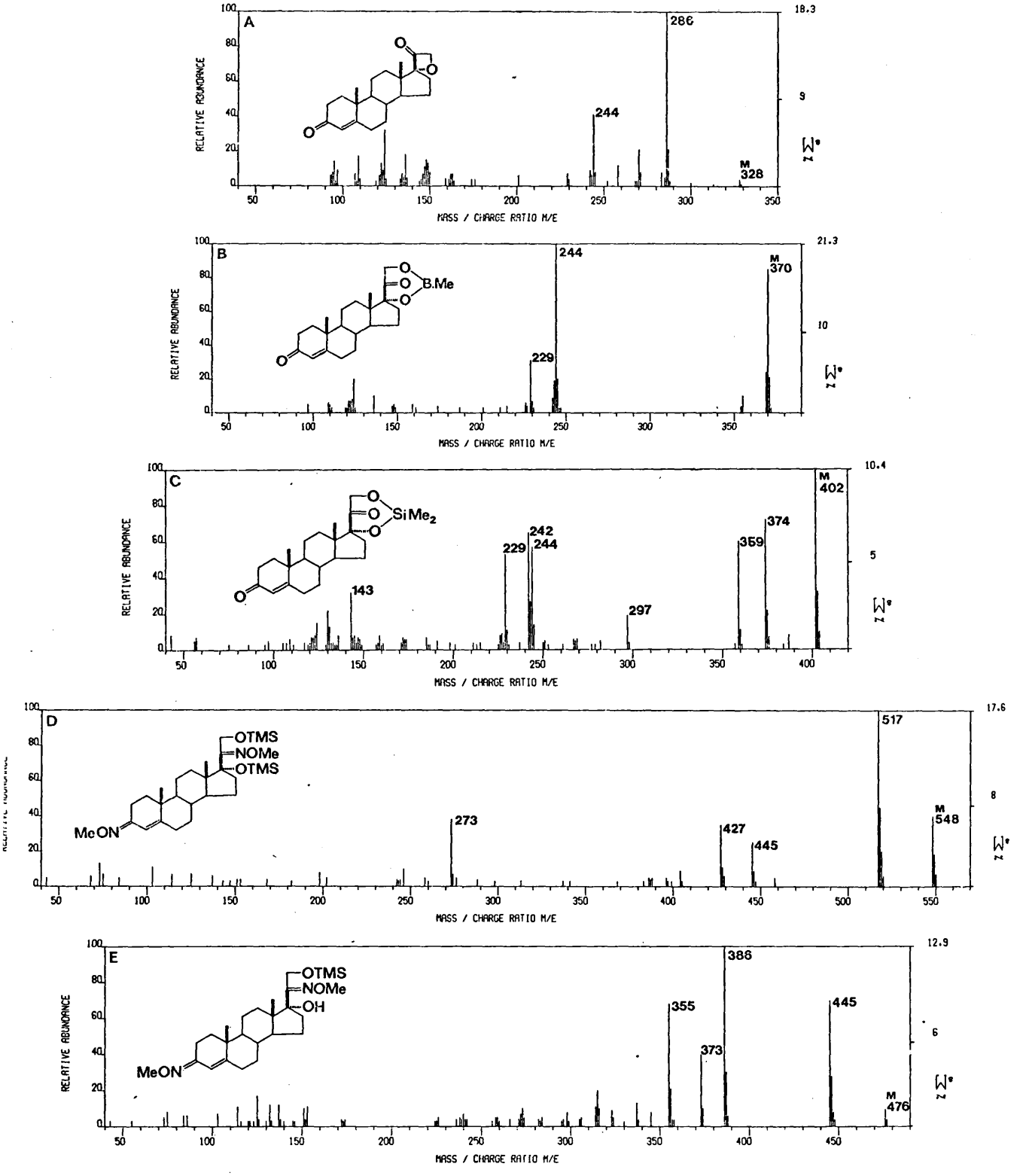


Fig.26: Mass spectra (22.5eV) of derivatives of Substance S:(A) 17,21-anhydro derivative,(B) 17,21-methylboronate,(C) 17,21-dimethylsiliconide,(D) 3,20-diMO 17,21-diTMS,(E) 3,20-di-MO 21-TMS.

range of mass spectroscopic ions suitable for analytical characterisation.

DIHYDROXYACETONE (Table 8) Mass spectra of the five principal types of single derivative of Substance S are shown in fig.26. The di-MO mono TMS ether and its d_9 analogue gave closely similar fragmentation patterns (with the expected mass shifts): the base peak in each case was at m/e 386, representing loss of the trimethylsilanol moiety. The diMO diTMS ether, in contrast, gave no significant peak at m/e (M-90); the dominant fragmentation proceeded via loss of a methoxyl radical. The characteristic[165] ion at m/e 273 doubtless comprises rings A, B and C with C-14 and C-18, and corresponds to the well-known fragment of this type (m/e 244; fig.22) observed for cyclic boronates[94,160] and dimethylsiliconides[96] with the added 29 mass units due to the MO group. The dimethylsiliconide gave a particularly intense molecular ion: the base peak at m/e 91 is structurally insignificant. A notable feature of this spectrum is the peak at $(M-28)^{+\cdot}$: a similar ion was recorded by Kelly[96] for cortisol 11-dimethylsilyl ether 17,21-dimethylsiliconide. The nature of this elimination has not yet been firmly established, but it probably represents loss of CO. No corresponding peak occurs in the spectrum of the methylboronate, which in other respects parallels the dimethylsiliconide. The mass spectrum of Substance S oxetanone is described elsewhere (section 2.3.3). Previously reported mass spectra related to those discussed above include those of tetrahydro-S MO diTMS ether,[169] cortisol dimethylsiliconide[96], the diMO (full-) TMS ethers of cortisol,[152] cortisone[152] and dexamethasone,[170], and MO-TMS derivatives of various fluorinated cortiscosteroid drugs.[108]

Compound	Retention Indices $I^{250^{o}}_{OV-1}$	$I^{275^{o}}_{Dexsil-300GC}$	$M^{+\cdot}$	Base Peak, m/e>80	m/e values and relative abundances of characteristic ions: Fragment ions
21-TMS	3005	3320	402 (2)	299	387 (54) 271 (90) 253 (54) 147 (48) 143 (49) 103 (54)
3,20-diMO 21-TMS	3030	3130	460 (100)	460	429 (73) 286 (41) 273 (43) 188 (42) 175 (31) 125 (45) 103 (68)
20,21-methylboronate	2935	3075	354 (18)	110	339 (3) 124 (8) 91 (10)

Table 9: Retention index values and salient mass spectrometric data for derivatives of Deoxycorticosterone (DOC; 21-hydroxy-4-pregnene-3,20-dione; 49).

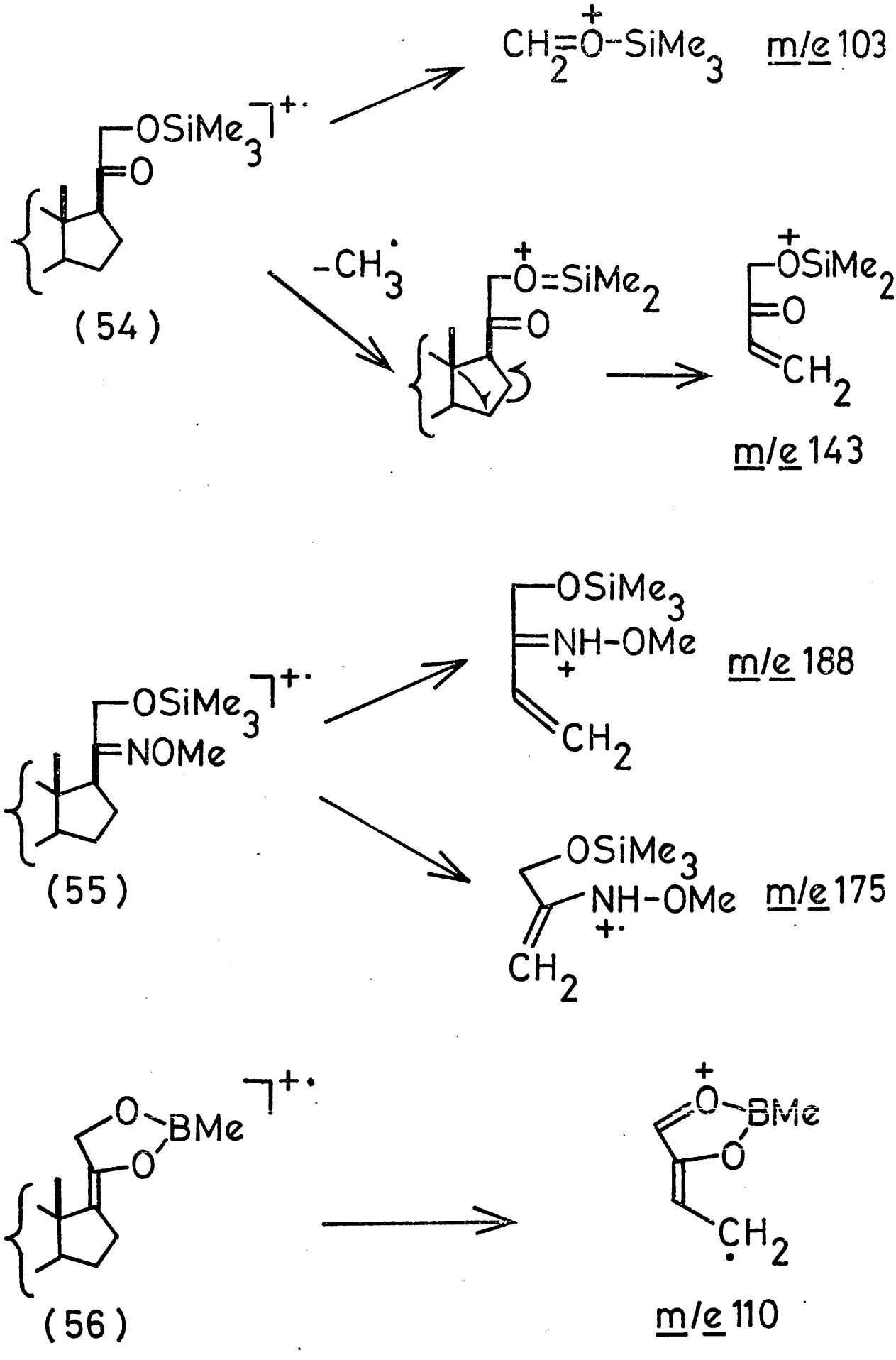


Fig. 27: Fragmentations of 20,21-ketol derivatives.

The methylboronate and the corresponding 3,20-diMO methylboronate "mixed" derivative afforded similar mass spectra in that the major mode of fragmentation in each case was the cleavage of ring D and expulsion of the boronate ring. However, in the diMO, ions characteristic of the O-methyloxime grouping were also prominent, the net result being a superimposition of fragmentation patterns. A similar effect was encountered in the spectra of the diMO dimethylsiliconide and the diMO oxetanone. The mass spectrum of the diMO HFB derivative was dominated by the typical "ring A" fragments (fig.22), and contained prominent fluorine-containing ions at m/e 69 (CF_3^+), 119 ($C_2F_5^+$) and 169 ($C_3F_7^+$).

20,21-KETOL (Table 9) The 21-TMS ether of deoxycorticosterone (partial structure 54) underwent the expected major α-cleavage of the C-20/C-21 bond, with charge retention on either of the fragments. This led to ions at m/e 103 (fig.27) and m/e (M-103); such ions are typical of TMS ether derivatives of primary alcohols [116] such as 21-hydroxysteroids,[171,172] although they have also been observed in the spectra of TMS ethers of steroids with vicinal hydroxyl groups.[173] The abundant ion at m/e 143 is noteworthy: an ion of this mass was observed in about 30% relative abundance in the spectrum of 11β,21-dihydroxy-5α-pregnane-3,20-dione diTMS ether.[171] The mode of fragmentation may be as depicted in fig.27. The spectrum of the diMO TMS ether (partial structure 55), in agreement with data previously reported,[152,171] included strong peaks at $(M-174)^{+\cdot}$ and $(M-187)^+$ and quasi-complementary peaks at m/e 175 and m/e 188 - the latter representing the side-chain with one and two ring D carbon atoms, respectively, as proposed by Gustafsson and Sjövall[171] (fig.27). The 20,21-methylboronate, for which evidence[95] favours a $\Delta^{17(20)}$-structure (56), yielded a spectrum dominated by the "boronate" fragment at m/e 110. This feature is characteristic of the boronates of 20,21-ketols, which all afford prominent ions at m/e (95+R), where 'R' is the alkyl group attached to boron.[160] Boronate derivatives of other types of cortiscosteroid side-chain,/

Compound	Retention Indices $I^{250^o}_{OV-1}$	$I^{275^o}_{Dexsil-300GC}$	$M^{+\cdot}$	Base Peak, m/e>80	m/e values and relative abundances of characteristic ions: Fragment ions
20,21-diTMS	3095	3285	476 (o)	283	461 (1) 373 (34) 265 (6) 159 (18) 147 (27) 129 (18) 103 (18)
3-MO 20,21-diTMS	3140	3245	505 (8)	312	490 (3) 475 (36) 460 (6) 402 (15) 372 (25) 282 (55) 280 (63) 153 (5) 147 (41) 103 (20)
20,21-methylboronate	2850	3135	356 (27)	124	314 (31) 271 (18) 229 (11) 173 (14) 85 (36)
20,21-acetonide	2890	3165	372 (0.7)	357	297 (31) 279 (8) 173 (17) 159 (17) 157 (18) 124 (19) 101 (80) 72 (50) 43 (95)

Table 10: Retention index values and salient mass spectrometric data for derivatives of 20β,21-dihydroxy-4-pregnen-3-one (50).

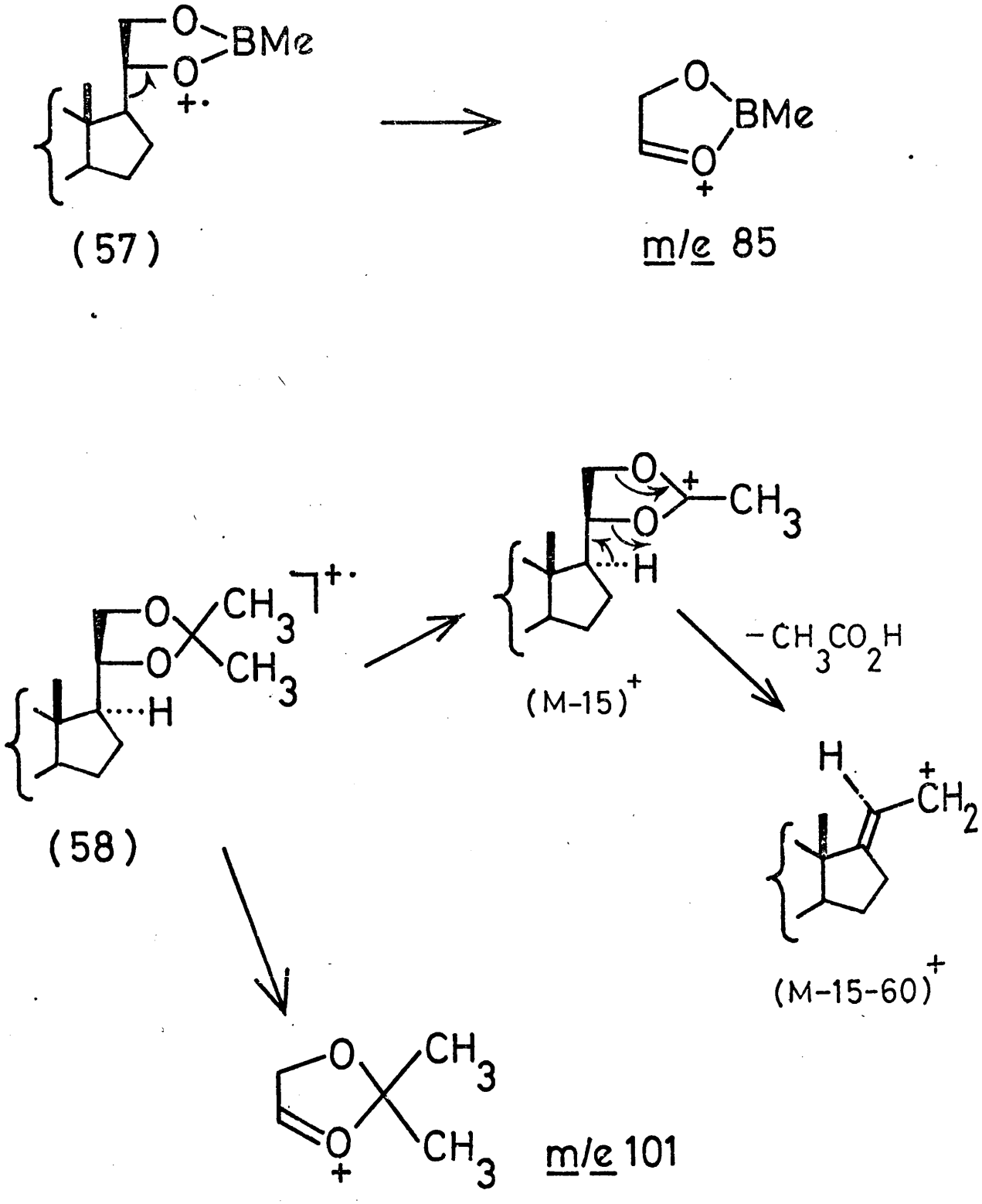


Fig.28: Fragmentations of 20β,21-diol derivatives.

side-chain, on the other hand, give spectra with abundant nuclear fragments, and fewer boron-containing ions.[160]

The three derivatives compared here illustrate well the manner in which the base peak can be directed to various regions of the spectrum.

20,21-DIOL (Table 10) The diTMS ether of 20β,21-dihydroxy-4-pregnen-3-one gave no discernible molecular ion because of the predominance of α-cleavage and elimination of trimethylsilanol, affording the base peak, $(M-193)^+$. Methoximation of the 3-ketone stabilised the molecular ion, as expected, and a peak at m/e 505 ($M^{+\cdot}$) was evident in the MO di TMS spectrum. A more balanced fragmentation occurred with the methylboronate (partial structure 57) leading to the "ring A" ion (m/e 124) as base peak, and to prominent ions $M^{+\cdot}$ and $(M-42)^{+\cdot}$, the latter arising through loss of ketene from ring A (cf. fig.14). Fragmentation of the boronate ring afforded the ion at m/e 85 (fig.28). The acetonide (partial structure 58) exhibited yet another mode of fragmentation, giving the $(M-15)^+$ ion as base peak; this ion is formulated as a tertiary carbonium ion, stabilised by the two neighbouring oxygen atoms of the acetonide ring[174] (fig.28). Loss of the elements of acetic acid (60a.m.u.) from the $(M-15)^+$ion [174] affords the prominent peak at m/e 297, while the intense ion at m/e 101 is doubtless due to the dimethyldioxolenium ion $C_5H_9O_2^+$, paralleling the fragment of m/e 85 from the boronate. The ion at m/e 101 is prominent in the spectrum of 20α-dihydroprednisolone acetonide recorded by Bailey,[150] and in the spectrum of the 20,21- acetonide from a pregnanetetrolone studied by Gustafsson and Sjövall.[171]

17,20-DIOL (Table 11). The mass spectra of the monoTMS ether, the MO monoTMS ether and the MO diTMS ether were all dominated/

Compound	Retention Indices $I^{250^\circ}_{OV-1}$	$I^{275^\circ}_{Dexsil-300GC}$	$M^{+\cdot}$	Base Peak m/e>80	m/e values and relative abundances of characteristic ions — Fragment ions
20-TMS	3015	3250	404 (30)	287	386 (16) 359 (29) 329 (30) 314 (9) 244 (9) 229 (23) 157 (69) 124 (17) 123 (18) 121 (16) 117 (82) 91 (26)
3-MO 20-TMS	3035	3180	433 (37)	117	418 (3) 402 (7) 388 (5) 343 (4) 316 (96) 157 (36) 153 (14) 125 (15)
3-MO 17,20-TMS	2985	3060	505 (5)	388	475 (3) 415 (4) 358 (10) 298 (32) 267 (28) 157 (6) 153 (3) 125 (4) 117 (34)
Methylboronate	2795	3020	356 (100)	356	341 (5) 314 (12) 245 (14) 229 (25) 125 (70) 124 (63) 111 (38)
Dimethylsiliconide	2840	3220	388 (50)	157	373 (11) 143 (40)
Acetonide	2870	3120	372 (66)	141	357 (21) 314 (36) 297 (40) 242 (19) 124 (12) 123 (15) 91 (25) 86 (55)

Table 11: Retention index values and salient mass spectrometric data for derivatives of 17α,20β-dihydroxy-4-pregnen-3-one; 51).

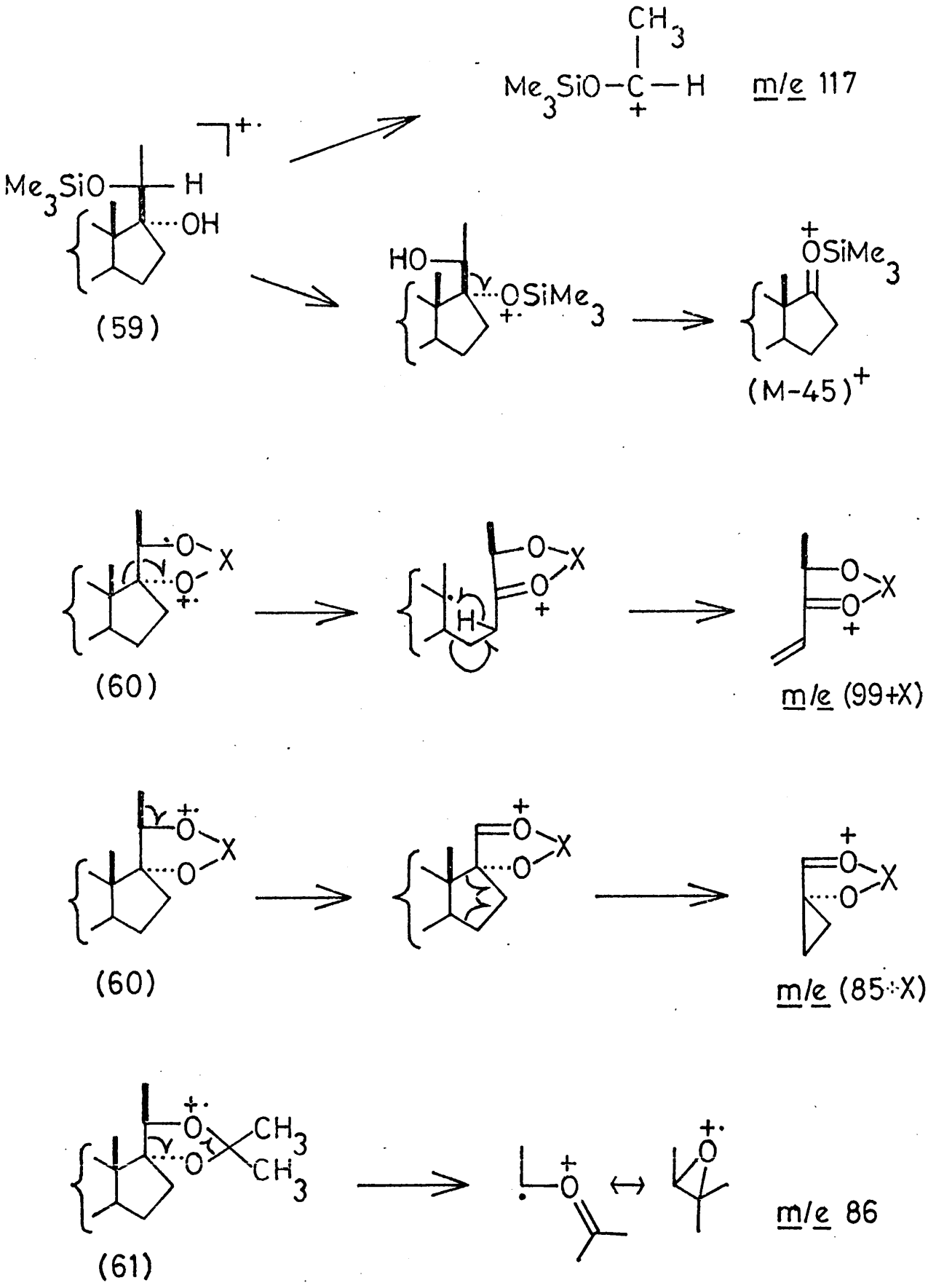


Fig. 29: Fragmentations of 17α,20β-diol derivatives.

dominated by ions arising from the fragmentation of the C-17/C-20 bond, with charge retention in either fragment. Such cleavage gives rise to ions at m/e 117 and m/e (M-117)[116,175,-179] (fig.29). The spectra of the two monoTMS ethers reveal ions at m/e (M-45). This feature has been rationalised[180] in terms of a rearrangement whereby the trimethylsilyl moiety is first transferred to the 17α-hydroxyl group. This is followed by cleavage of the C-17/C-20 bond, with the expulsion of the radical species CH_3CHOH. The three cyclic derivatives exhibited individual breakdown patterns, although each afforded an intense ion comprising the side-chain moiety, at m/e (99+X). In addition, the methylboronate and dimethylsiliconide gave strong peaks at (85+X); these could arise by cleavage at C-13/C-17 and C-15/C-16 with hydrogen transfer to the nuclear fragment, although an alternative mechanism[160] is illustrated in fig.29. The prominent ion at m/e 86 ($C_5H_{10}O^{+\cdot}$) in the acetonide spectrum evidently arises from the 1,3-dioxolane ring; a possible mode of formation is shown in fig.29.

17,20,21-TRIOL (Table 12) The diTMS ether (partial structure 62) yielded the base peak at m/e 116, characteristic of such derivatives[175], and an intense ion $(M-133)^+$. This loss of 133 a.m.u. has been noted by Rosenfeld[180] and by Aringer et al.[152], although a satisfactory explanation of its origin was not presented. A proposed mechanism for the formation of the $(M-133)^+$ ion is given in fig.30. (It should be borne in mind that, in view of the ease of migration of TMS groups, structures such as (62) may not be correct - see also comments below on the methylboronate-TMS ether "mixed" derivative. Some information on this problem might be gained by "mixed" silylation studies.[181]). In the spectra of the 3-MO 20,21-diTMS ether and the 3-MO 17,20,21-triTMS ether, the base peaks were due to the corresponding ion from direct cleavage/

Compound	Retention Indices $I^{250^{o}}_{OV-1}$	$I^{275^{o}}_{Dexsil-300GC}$	$M^{+\cdot}$	Base Peak m/e	m/e values and relative abundances of characteristic ions: Fragment ions
20,21-diTMS	3220	3425	492 (8)	116	389 (28) 371 (21) 359 (95) 124 (6) 103 (30)
3-MO 20,21-diTMS	3255	3385	521 (10)	315	503 (4) 491 (80) 418 (13) 328 (7) 171 (23) 153 (14) 125 (23) 103 (51)
3-MO 17,20,21-triTMS	3160	3205	593 (3)	388	490 (22) 298 (27) 267 (21) 266 (15) 243 (34) 191 (28) 147 (54) 125 (8) 103 (17)
Methylboronate	2970	3245	372 (100)	372	330 (10) 287 (48) 269 (31) 245 (23) 124 (59)
Acetonide	3015	3310	388 (6)	287	345 (4) 330 (10) 313 (7) 269 (18) 229 (13) 124 (24) 101 (19) 59 (55) 43 (66)
Methylboronate 20-TMS	3015	3265	444 (28)	103	429 (8) 414 (13) 354 (21) 341 (9) 267 (45) 124 (14)

Table 12: Retention index values and salient mass spectrometric data for derivatives of 17 α,20 β,21-trihydroxy-4-pregnen-3-one (52).

Compound	Retention Indices $I^{250^{o}}_{OV-1}$	$I^{275^{o}}_{Dexsil-300GC}$	$M^{+\cdot}$	Base Peak m/e 80	m/e values and relative abundances of characteristic ions Fragment ions
3,20-diMO	2960	3110	388 (23)	357	325 125 86 (8) (11) (21)
3,20-diMO 17-TMS	2910	3010	460 (32)	429	370 339 273 200 188 172 170 158 156 (4) (22) (33) (16) (20) (19) (24) (27) (23)

Table 13: Retention index values and salient mass spectrometric data for derivatives of 17 α-hdroxyprogestèrone (17 α-hydroxy-4-pregnene-3,20-dione; 53).

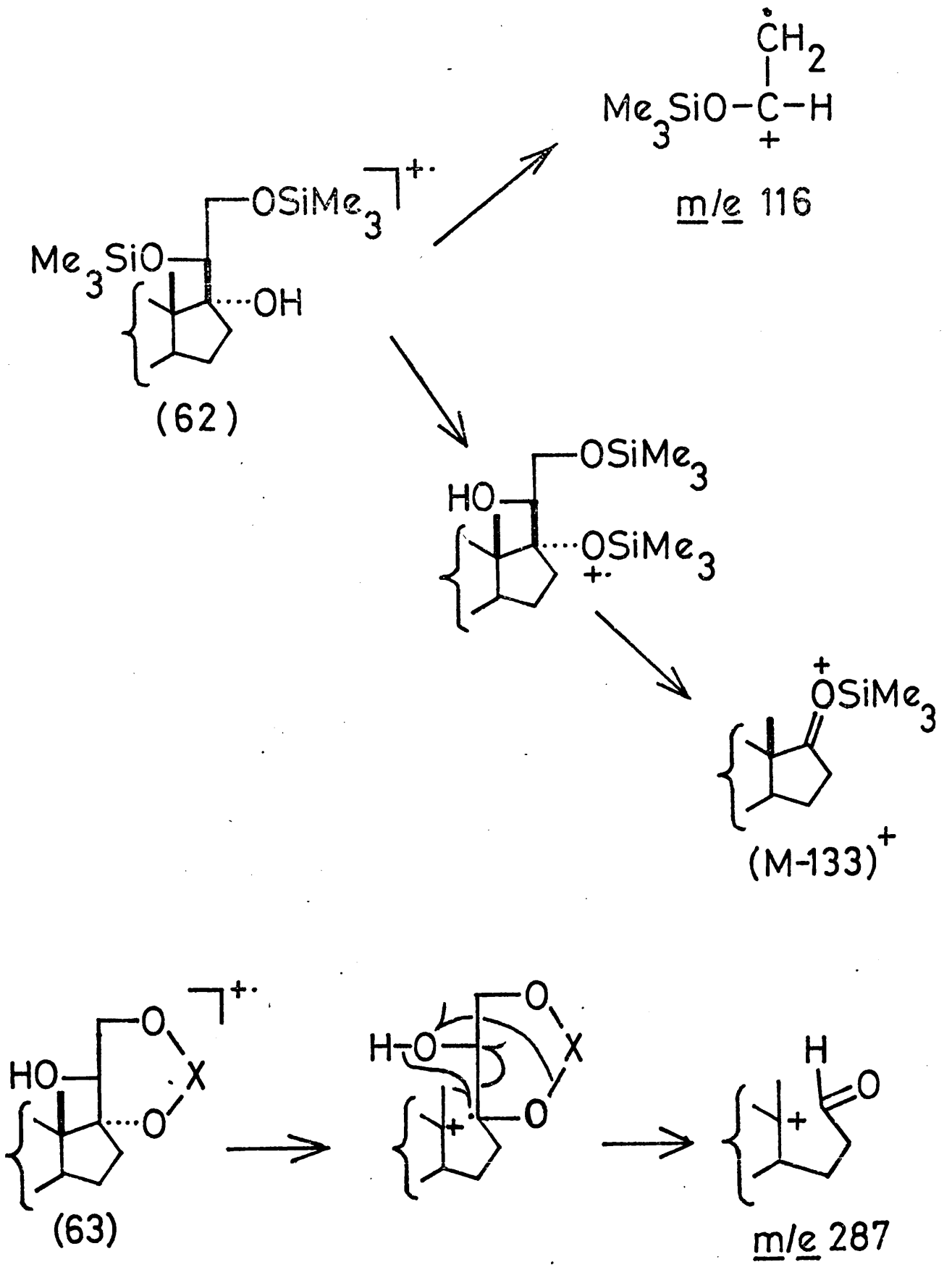


Fig.30: Fragmentations of 17α,20β,21-triol derivatives.

cleavage of the C-17/C-20 bond.

As in previous examples, the cyclic derivatives displayed modes of fragmentation markedly different from those of TMS ethers. The methylboronate gave an intense molecular ion together with the "ring A" ion (m/e 124), and a nuclear fragment (m/e 287) resulting from the loss of the (rearranged)boronate side-chain[160]. Boronates from 17,20,21-triols are formulated as six- membered esters, involving the 17- and 21-hydroxyl groups[95,147](Appendix III). Trimethylsilylation of the triol boronate thus yields the methylboronate 20-TMS ether "mixed" derivative. However, the spectrum of this compound had, as its base peak, m/e 103, an ion normally characteristic of TMS ether derivatives of primary hydroxyl groups (cf. fig.27). It is therefore proposed that the side-chain rearranges to a 17,20-methylboronate 21-TMS ether structure, which then undergoes scission of the C-20/C-21 bond to give the m/e 103 fragment. In the acetonide, the aforementioned "nuclear" fragment (m/e 287) dominated the spectrum, while prominent ions in the low-mass region at m/e 43 and m/e 59 may be ascribed[174]to the species $CH_3C\equiv O^+$ and $(CH_3)_2C=\overset{+}{O}H$, respectively, deriving from the acetonide ring.

20,17-KETOL (Table 13). The spectra of the diMO and diMO TMS ether both gave as base peaks $(M-31)^+$. The latter spectrum also contained a series of even-mass ions comprising the side-chain MO group rather than the ring A MO group:[114,146] detailed assignments would require mass measurements combined with a study of labelled derivatives. The characteristic ion at m/e 273 [165]has been mentioned above with respect to the dihydroxyacetone analogues.

No stable cyclic derivatives were obtained from/

Compound	Retention Indices $I^{250^o}_{OV-1}$	$I^{275^o}_{Dexsil-300GC}$	$M^{+\cdot}$	Base Peak, m/e >80	m/e values and relative abundances of characteristic ions Fragment ions
Methyl 17α-hydroxy-3-oxo-androst-4-ene-17β-carboxylate free	2805	3100	346 (100)	346	331 287 286 245 244 229 148 136 124 (15) (10) (6) (28) (62) (53) (26) (20) (34)
3-MO	2840	3040	375 (100)	375	360 344 273 153 151 137 125 (8) (23) (30) (51) (42) (51) (62)
3-MO 17-TMS	2850	2985	447 (46)	273	416 153 137 125 91 (12) (21) (17) (21) (20)
Androst-4-ene-3,17-dione free	2505 (230°)	2900	286 (100)	286	271 244 201 150 149 148 124 (9) (62) (25) (25) (24) (43) (77)
3,17-diMO	2615 (230°)	2800	344 (100)	344	329 313 281 153 151 137 125 (9) (52) (14) (31) (36) (52) (76)
Methyl 3-oxoandrost-4-ene-17β-carboxylate free	2730	3000	330 (44)	124	315 299 288 245 147 (5) (8) (48) (25) (55)
3-MO	2745	2935	359 (88)	125	344 328 268 153 151 137 (8) (33) (8) (62) (50) (77)
3-Oxcandrost-4-ene-17β-carboxaldehyde free	2660	2990	300 (66)	124	285 282 258 244 229 215 (8) (7) (62) (41) (14) (30)
3,20-diMO	2750	2900	358 (100)	358	343 327 273 153 151 137 125 (7) (37) (23) (49) (39) (54) (80)

Table 14: Retention index values and salient mass spectrometric data for side-chain degradation products and their derivatives.

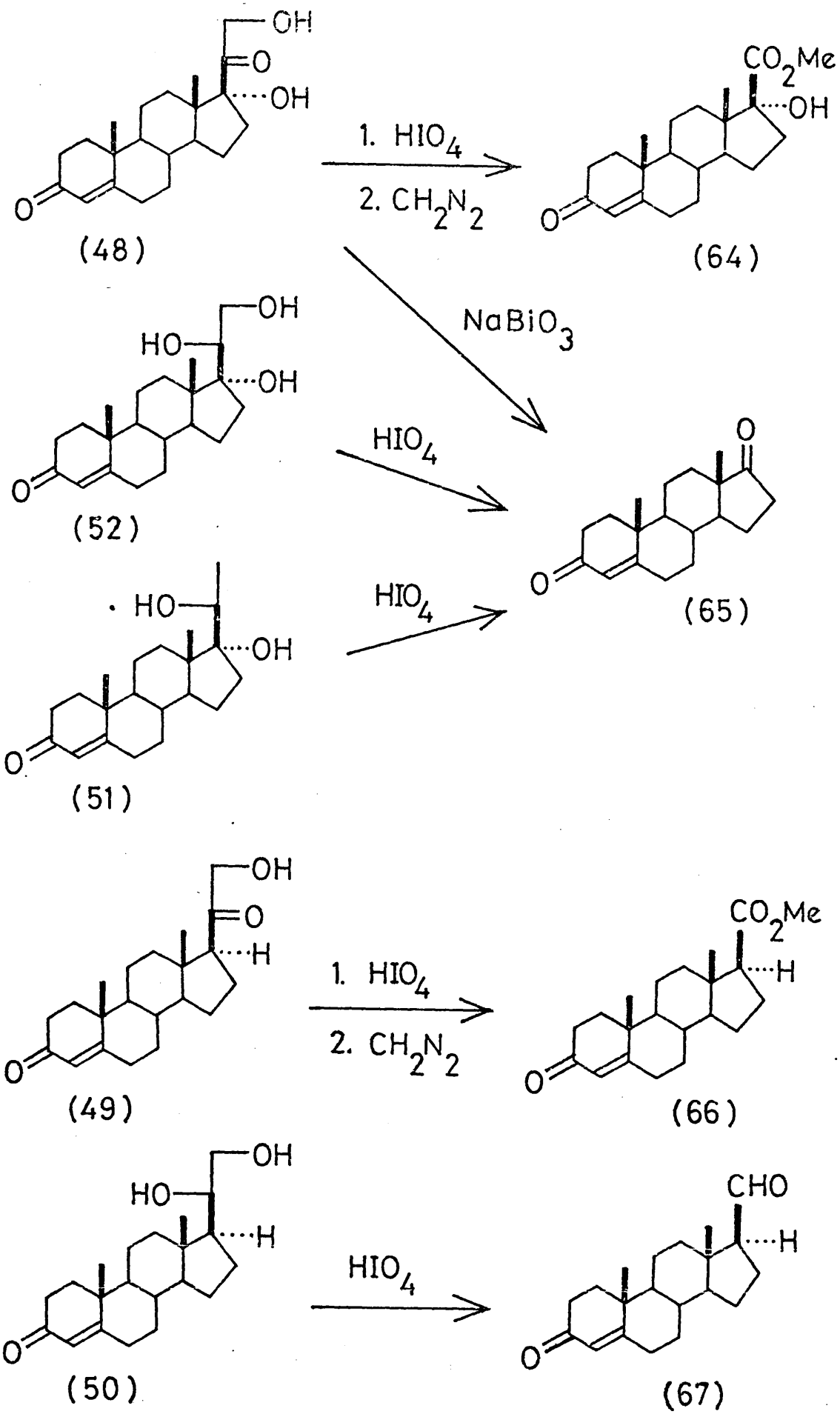


Fig. 31: Routes to the side-chain degradation products.

from 17α-hydroxyprogesterone. This finding agrees with the observed resistance of 20,17-ketol groupings towards enolisation to give a $\Delta^{20(21)}$-structure.[152] Enolisation of the isomeric 20,21-ketols occurs much more readily, resulting in the more favourable $\Delta^{17(20)}$-double bond (see above).

SIDE-CHAIN DEGRADATION PRODUCTS (Table 14). Each corticosteroid type, with the exception of the 20,17-ketol, underwent side-chain cleavage on treatment with periodic acid or sodium bismuthate (fig.31); etiocholenic acid derivatives were methylated prior to study by GC-MS. The mass spectra of these oxidation products yielded abundant molecular ions, while the major fragmentations were those characteristic of the 4-en-3-one group[111] (cf. fig.14). The aldehyde (67) afforded two ions, at m/e 244 and m/e 229, which apparently resulted from cleavage of ring D, and an ion $(M-85)^+$, presumed to comprise rings B,C and D, analogous to the fragment at m/e (M-85) in the spectrum of testosterone[111] (fig.32). Similar ions at m/e 244 and m/e 229 were present in the spectrum of the 17α-hydroxyetiocholenic acid derivative (64), which also afforded a peak at m/e (M-59) corresponding to loss of the carbomethoxy group via α-cleavage. The 17-deoxy analogue (66) yielded a prominent ion at m/e 147, which may be formulated[111] as a fragment containing rings C and D, plus C-7.

The spectra of the MO and MO-TMS derivatives of the side-chain oxidation products were dominated by the well-defined[114] "ring A" ions typical of Δ^4-3-ketosteroid O-methyloximes (fig.22). Molecular ions were again abundant, and the "nuclear" fragment m/e 273 [165] was evident in most cases.

3.3.4 DETECTION OF CORTICOSTEROIDS BY "SINGLE ION MONITORING".

The detection of particular compounds by means of a/

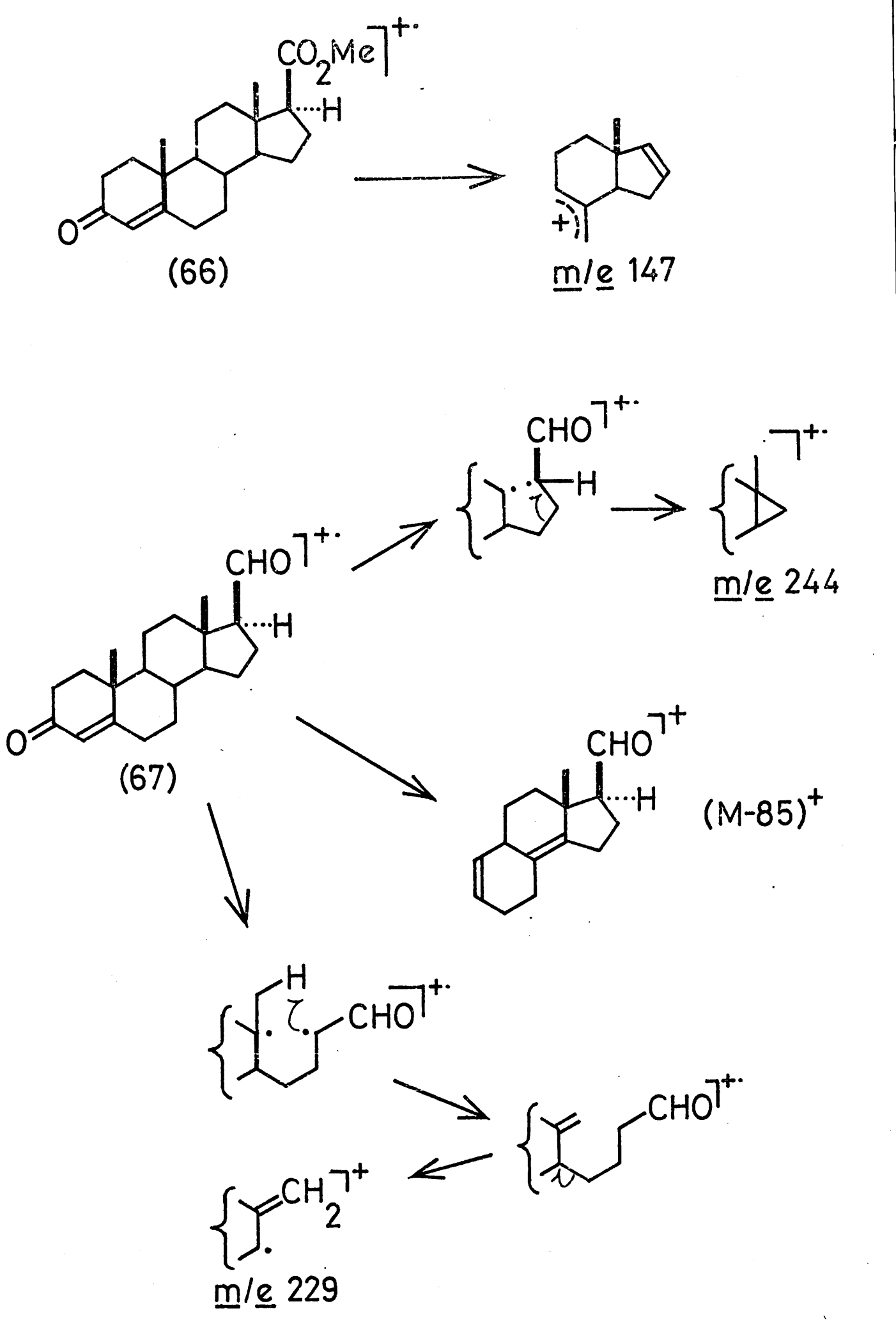


Fig. 32: Fragmentations of side-chain degradation products.

a small set of characteristic ions in their mass spectra is now a well-established technique.[58,60,61,73,182] In suitable instances, the very simple technique of focusing at a single m/e value may afford a satisfactory means of detecting and estimating steroids at low concentrations.[63,66,183,184] The success of such procedures depends critically upon a number of factors, the relative importance of which must be assessed experimentally. The principal considerations in respect of corticosteroids (for which derivative formation is a prerequisite) are as follows:-

(i) The ease, reliability, selectivity and completeness of derivative formation.

(ii) The stability of the derivatives.

(iii) Their retention times, and the quality of the chromatographic peaks.

(iv) The degree to which the derivatives may be lost by adsorption or destruction during gas chromatography.

(v) The character of the ions to be selected for monitoring. (The choice of fragment will depend on the structural features that it is desired to detect).

(vi) The nature of probable interference by ions from other compounds in the sample, and its avoidance - as far as possible - by optimal selection of the sample preparation method, type of derivative, stationary phase and chromatographic conditions.

(vii) The m/e values at which similar interference may arise from impurities constantly or frequently present in the "background" of the GC-MS system. (In some cases, sample fragment ions coincide with prominent "background" peaks, and thus prove unsuitable for single ion monitoring purposes. Consequently, a different derivative, yielding an ion of more suitable/

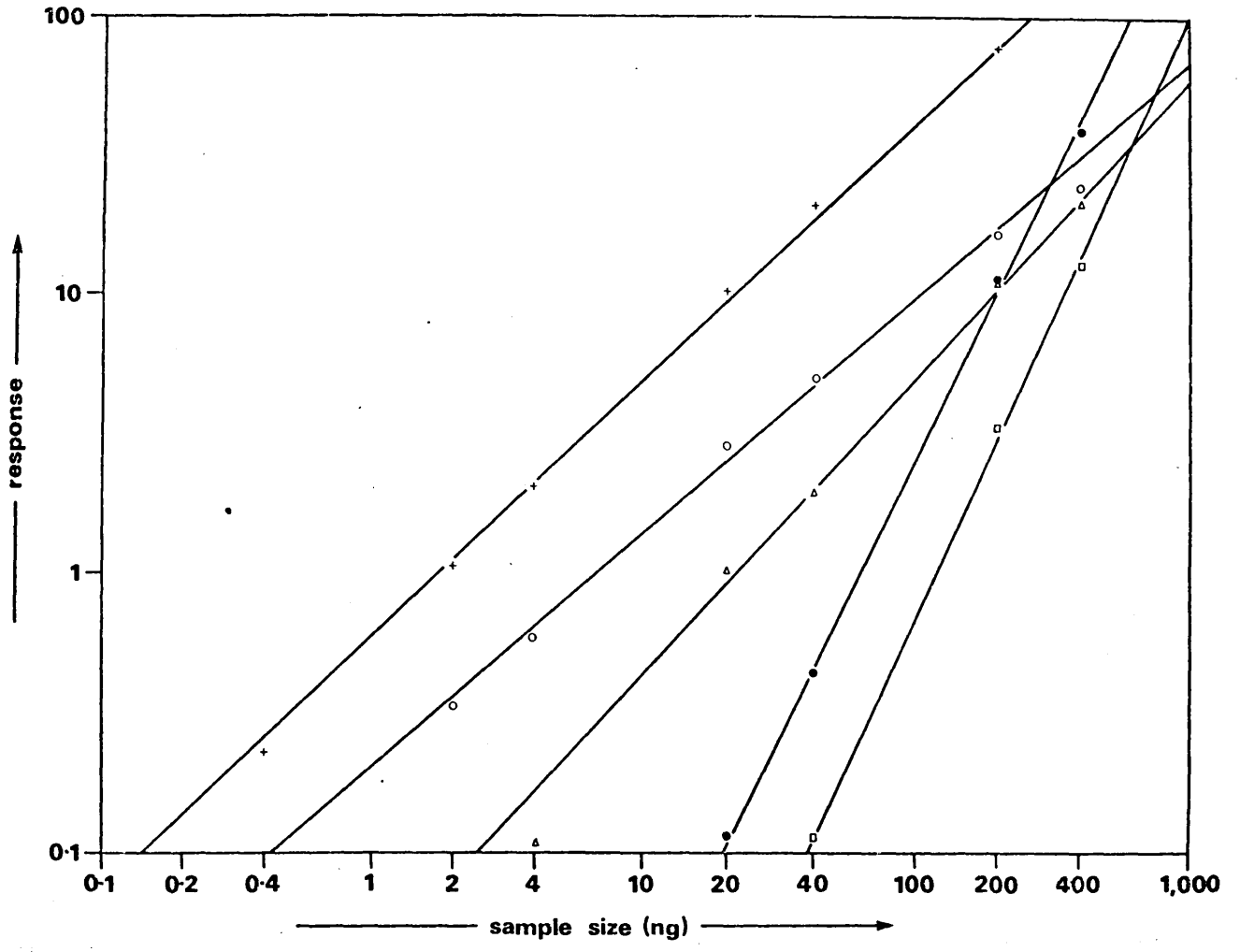


Fig.33: "Single ion monitor" response to derivatives of Substance S.
+17,21-anhydro derivative (m/e 286); □ 17,21-methylboronate (m/e 370);
○ 17,21-dimethylsiliconide (m/e 402); △ 3,20-diMO 17,21-diTMS
(m/e 548); ● 3,20-diMO 21-TMS (m/e 386).
Electron multiplier voltage 1.7-3.7kV.
Response measured as peak height, with adjustment for different
amplifications (Logarithmic scales).

suitable m/e value, may have to be employed. O-rings, septa and solvent impurities contribute to the "background", as does "bleed" from the GLC column - this latter source of interfering ions may be avoided to some extent by the use of alternative stationary phases, although most of the high temperature phases used for steroid work are silicones which give a number of common ions.)

(viii) The relationship of the actual m/e values selected for monitoring to the degree of mass resolution, as well as to the sensitivity of the electron multiplier system in the region of the spectrum concerned.

In this survey, particular attention was given to five representative derivatives of Reichstein's Substance S. In each instance, ions of even mass were selected for monitoring: the majority of strong "background"peaks are of odd mass. For the diMO TMS ether, the base peak $(M-90)^{+\cdot}$ was chosen; for the diMO diTMS ether, dimethylsiliconide and methylboronate, the molecular ions were used; and for the oxetanone, the base peak $(M-42)^{+\cdot}$ was employed. In each instance, the lower limit of detection was explored, with the results summarised in fig.33. All the derivatives were satisfactorily applicable for detection and estimation of quantities above 100 ng. Below this level, marked differences in behaviour were noted, and only the oxetanone remained clearly detectable at the 400 pg level. It appeared probable that the loss of response was due to adsorption of the derivatives on the chromatographic column.

It may be observed that the derivatives which were most easily prepared (methylboronate; diMO 21-TMS) were also those most susceptible to loss at low sample sizes, whereas the more/

more difficulty accessible oxetanone and dimethylsiliconide yielded the best results under these conditions. The derivative of choice with respect to convenience of preparation and range of detectable concentration would appear to be the diMO diTMS. This result is in agreement with the current trend towards the use of fully-silylated derivatives for the gas-phase characterisation of steroids.[152,161,162] The introduction of O-benzyloximes (BO) and BO-TMS ethers[185,186] ranks as an important complementary development in this area, while the use of other O-alkyloximes is discussed in sections 4 and 5.

The data in Tables 8-14 suggest that derivatives suitable for detection by single ion monitoring should be obtainable from all types of corticosteroid. In certain instances, apparently suitable peaks might be inappropriate: for example, m/e values of 124,125 and 157 represent ions which are relatively abundant in the "background" spectra of the instrument used in this work at the temperatures (for OV-1 and Dexsil) required for steroid derivatives.[66] Untoward coincidences of this kind can be circumvented either by selecting an entirely different derivative, or by using simple homologues to shift the masses of the desired ions without any undue alteration to the fragmentation mode. Examples include d_9-TMS ethers, chloromethyl (dimethyl)silyl ethers, O-ethyloximes and other O-alkyloximes, O-trimethylsilyloximes and O-benzyloximes.

The extent to which mass spectrometric detection is likely to prove useful in the analysis of urinary corticosteroids is by no means fully explored. Single ion monitoring has been found suitable for the study of certain steroidal drug metabolites, and is particularly effective for steroids which occur in unconjugated/

unconjugated form, such as Dianabol (17β-hydroxy-17α-methyl-1,4-androstadien-3-one) and its 6β-hydroxylated metabolite.[187] Recently, a procedure for the estimation of both cortisol and estriol im plasma or urine has been developed, in which single ion monitoring is employed to detect prominent fragment ions in the diMO-triTMS ether and tri ($\underline{d}_9$)TMS ether derivatives, respectively.[188]

3.3.5 CONCLUSIONS

The results of this survey indicate that the majority of derivatives which have been developed for the gas chromatography of corticosteroids also have qualities useful for GC-MS. The range of compounds examined is, of course, very limited, and some derivatives of proved utility - particularly the enol-TMS ethers [151-154] - have not received attention, while others await evaluation. Examples of known derivatives of steroidal dihydroxyacetones which have not, apparently, been applied to gas-phase studies are the 17,21-formals,[189] acetonides,[190] other 17,21-acetals,[191] and orthoesters.[192,193]

The variety of fragmentation modes displayed by the different classes of derivative is of obvious value in the identification of corticosteroids. In addition, ions characteristic of individual compounds (or common structural features) are frequently produced, and permit the detection of their parent molecules by monitoring the ion current at a single $\underline{m/e}$ value or a few such values. The practicability of the mass spectrometric estimation of corticosteroids in the sub-nanogram range is clearly foreshadowed by the data presented here.

SECTION 4

O-ALKYLOXIMES AS DERIVATIVES FOR THE STUDY OF KETOSTEROIDS BY GAS CHROMATOGRAPHY

4.1 INTRODUCTION

A variety of derivatives have been employed for the study of ketosteroids by gas chromatography. These include thioketals,[194] enol esters,[195] hydrazones and oximes. Enol-trimethylsilyl ethers (enol-TMS ethers) have been shown to be suitable derivatives for corticosteroid metabolites with 20,21-ketol or dihydroxyacetone side-chains,[151-154] although 4-en-3-one groupings may give rise to a mixture of products,[154] while only partial reaction occurs with 17-ketosteroids.[138,154] N,N-Dimethylhydrazones may be readily prepared from reactive ketone groups,[196] although they have been shown to lack stability on exposure to light and air; the pentafluorophenylhydrazone has been applied to the estimation of estrone, using GLC with electron capture detection.[197]

The derivative of choice in most cases, however, has been a substituted oxime. Simple (unsubstituted) oximes are of limited value due to their high polarity, although they may be readily converted to the non-polar O-trimethylsilyl oximes (TMS oximes) or O-acetyl oximes.[175] TMS oximes were first applied in the carbohydrate field by Sweeley *et al.*,[198] and later shown to be suitable for the gas chromatography of ketosteroids.[146,175,199] O-Methyloxime (MO) derivatives, originally introduced by Fales and Luukkainen in 1965[137], have proved to be of great use owing to their ease and completeness of formation, stability and excellent GLC properties (see section 3). The "mixed" MO-TMS derivatives of Gardiner and Horning[54] have been widely applied to the study of urinary steroid profiles by gas chromatography and mass spectrometry.[55,200-202] The retention increments (on non-selective stationary phases) associated with the formation of MO derivatives or O-ethyloximes (EO)[146] are small, and do not lead to gas chromatographic group separations of/

of ketosteroids from related hydroxysteroids and steroids possessing unreactive keto groups.[54] The analogous O-benzyloximes (BO derivatives)[185,186] readily afford complete separations of this type because of their much longer retention times. However, the large retention increments accompanying BO formation preclude the analysis of most diketonic steroids; furthermore, the late elution of BO derivatives of C_{21} ketosteroids leads to some difficulties because of the abundance of "background" ionisation in mass spectrometry.

In the present investigation, aimed at extending further the range of substituted oximes, four O-alkylhydroxylamines have been selected which afford oximes with retention times (and molecular weights) intermediate between those of MO and BO derivatives: these are O-sec-butoxyamine, O-isobutoxyamine, O-n-pentoxyamine and O-isopentoxyamine. It was envisaged that the new alkyloximes would complement the established derivatives, by preserving to some extent the group separating power of the benzyloximes but also allowing (like the methyloximes[114]) the elution of diketone derivatives. The availability of a range of O-alkylhydroxylamine reagents would permit the recording, from complex mixtures of hydroxy- and ketosteroids, of comparative steroid profiles which should simplify interpretations based on gas chromatography - mass spectrometry.

4.2 EXPERIMENTAL

4.2.1 PREPARATION OF O-ALKYLOXIME DERIVATIVES

Analytical samples of O-alkyloximes were prepared in pyridine solution, using 0.5mg of reference steroid, according to the general procedure of Sakauchi and Horning[161] (cf. O-methyloximes; section 3.2). Reactive hydroxyl groups were (in some cases) subsequently converted to trimethylsilyl (TMS) derivatives, according to the method of Chambaz and Horning[162] (section 3.2).

Pregnenolone O-isopentyloxime (from 40mg of pregnenolone) was obtained in 95% crude yield as a product giving only a single GLC peak, and, after two recrystallisations from ethanol/water, gave flaky crystals, m.p. 116-117° (Found, C:77.67; H:10.78; N:3.45%. Calc. for $C_{26}H_{43}O_2N$: C:77.75; H:10.79; N:3.49%.).

N.M.R. (100MHz) gave:- 9.34τ singlet (3H (C_{18} Me-group); 9.07τ 1:1 doublet (J=6Hz, 6H (two terminal Me-groups in isopentyl chain)); 8.97τ singlet (3H (C_{19} Me-group)); 8.19τ singlet (3H (C_{21} Me-group)); 5.93τ 1:2:1 triplet (J=6.5Hz, 2H (methylene protons α to oxygen function in isopentyl chain)); 4.62τ 1:1 doublet (broad, J=5Hz, 1H (C_6)).

Progesterone (60mg) was similarly converted to its di-O-isopentyloxime in 92% crude yield. After three recrystallisations from methanol, it had m.p. 86-88° (Found, C:76.58; H:10.82; N:5.98%. Calc. for $C_{31}H_{52}O_2N_2$: C:76.81; H:10.81; N:5.78%.)

SYNTHESIS OF DEUTERIUM-LABELLED REAGENTS

4.2.2 Synthesis of Isopentoxyamine-1-d_1 Hydrochloride

(i) Isoamyl alcohol-1-d_1. To a stirred suspension of lithium aluminium deuteride (1.092 g; 28.8 m mole) in anhydrous/

anhydrous ether (40 ml) under a nitrogen atmosphere, was added a solution of isovaleraldehyde (5.006 g; 58.2 m mole) in anhydrous ether (10 ml). Complete addition took 35 min, and was accompanied by gentle reflux of the reaction mixture. Work-up afforded 5.8 ml (4.736 g; 52.6 m mole) of isoamyl alcohol-1-$\underline{d}_1$. (Yield = 90%). Infra-red:- ν_{max} at 3350cm^{-1} (s, broad) (ν(O-H)); 2170cm^{-1} (s) (ν(C-D)). N.M.R. (100 MHz) gave :- 9.08τ 1:1 doublet (J=6Hz, 6H (two Me groups)); 8.5τ triplet (J=7Hz, 2H (C_2)); 8.31τ multiplet (1H (C_3)); 6.96τ singlet (1H (-O$\underline{H}$)); 6.41τ 1:2:1 triplet (J=7Hz, 1H (C_1)).

(ii) Isoamyl bromide-1-$\underline{d}_1$. This was prepared according to the method of Wiley et al.[203]. To a stirred solution of dry triphenylphosphine (11.88 g; 45.4 m mole) in dry dimethylformamide (50 ml) held under a nitrogen atmosphere was added 5.0 ml (4.08g; 45.4 m mole) isoamyl alcohol-1-$\underline{d}_1$. Bromine (2.4 ml: 1.03 molar equivalent) was then added dropwise, the temperature of the reaction mixture being maintained below 50°. Two successive distillations at atmospheric pressure yielded isoamyl bromide-1-$\underline{d}_1$, b.p. 117-120° (2.9 ml; 3.28 g; 21.4 m mole). (Yield=47.3%). Infra-red:- ν_{max} at 2235 cm^{-1}(m) (ν(C-D)). N.M.R. (100MHz) gave:- 9.07τ 1:1 doublet (J=6Hz, 6H (two Me-groups)); 8.25τ 1:1 doublet (broad, J=6Hz, 3H (C_2 and C_3)); 6.62τ 1:2:1 triplet (J=6Hz, 1H (C_1)).

(iii) Isoamyl 1-$\underline{d}_1$-benzhydroxamate[204]. Benzhydroxamic acid (2.686 g; 19.6 m mole) was dissolved in AnalaR methanol (25 ml), and the resulting solution vigorously stirred while a solution of sodium hydroxide/

hydroxide (0.784 g; 19.6 m mole) in water (2.6 ml) was added. Isoamyl bromide-1-$\underline{d}_1$ (3.0 g; 19.7 m mole) was then added, and stirring continued for 24 h. The reaction mixture was kept at room temperature for 3 days, after which it was evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate/water (10 ml : 10 ml), the organic layer washed with water (3x5 ml), and the washings back-extracted with ethyl acetate (5 ml). The combined organic extracts were dried, filtered and evaporated under reduced pressure, to yield the benzhydroxamate (1,557 g; 7.49 m mole) as a pale yellow oil. (Yield=38%.) Infra-red:- ν_{max} at 3475cm^{-1}(w) and 3410cm^{-1}(w) (ν(N-H)); 3025cm^{-1} (w) (ν(aryl-H)); 2155cm^{-1}(w) (ν(C-D)); 1650-1705cm^{-1}(s) (ν(C=O)). N.M.R. (100MHz) gave:- 9.11τ 1:1 doublet (J=6Hz, 6H (two Me-groups)); 8.44τ 1:1 doubled (broad, J=6Hz, 3H (C_2 and C_3)); 6.05τ 1:2:1 triplet (J=7Hz, 1H (C_1)); 2.66τ multiplet (3H ($\underline{m}$ and $\underline{p}$-aromatic protons)); 2.27τ 1:1 doublet (J=8Hz, 2H ($\underline{o}$-aromatic protons)).

(iv) $\underline{\text{Isopentoxyamine-1-d}_1\text{ hydrochloride}}$.[204] The crude benzhydroxamate (1.50 g; 7.21 m mole) obtained above was treated with 15 ml of a mixture of 12N-HCl/methanol (1:3), and the resulting clear solution refluxed for a period of 3 h. The solvent was evaporated on a rotary evaporator, yielding an oil, which slowly solidified. This residue was treated with ether (15 ml), and the solid product collected by filtration, washed well with more ether (15 ml) and dried under vacuum, affording a colourless, amorphous solid (0.779 g; 5.5 m mole), (Yield= 76.6%), which on recrystallisation from ethanol/ether, gave large translucent plates, double m.p. 115-117^{o} and/

and 143-145°. Infra-red:- $\nu_{max}^{(CH\ Cl_3)}$ at 2165cm^{-1}(w) (ν(C-D)). N.M.R. (100 MHz) gave:- 9.18τ1:1 doublet (J=5Hz, 6H (two Me-groups)); 8.44τ 1:1 doublet (broad, J=6Hz, 3H (C_2 and C_3)); 5.67τ 1:2:1 triplet (J=6Hz, 1H (C_1)).

4.2.3 Synthesis of Isopentoxyamine-1-d_2 Hydrochloride.

(i) Isovaleric acid. A mixture of isovaleraldehyde (7.144g; 83 m mole) and AnalaR acetone (5ml) was placed in a 50ml two-necked flask, equipped with pressure - equilibrated dropping funnel and thermometer. The mixture was stirred magnetically and cooled in an ice-bath to approx. 10°. Jones reagent (20ml; prepared by dissolving 13.36g chromium trioxide in a mixture of conc. sulphuric acid (11.5ml) and water (20ml), and making the resulting solution up to 50ml with water) was then added dropwise, maintaining the temperature of the reaction mixture below 30°. Complete addition took 2h, after which the reaction product was poured into water (50ml) and extracted with ether (3x50ml). The combined ether extracts were then treated with 1N sodium bicarbonate (2x50ml), and the bicarbonate washings were acidified by careful addition of dilute (2N) hydrochloric acid (40ml). The resulting cloudy solution was ether-extracted (3x150ml) and the combined ether extracts finally dried and evaporated in vacuo to yield isovaleric acid (4.4g; 43 m moles; Yield=52%).

Note: The acid isolated contained some dissolved Cr^{III} salts, as evidenced by a pale green coloration.

(ii) Methyl isovalerate. Isovaleric acid (4.4g; 43 m mole) was dissolved in anhydrous ether (20ml), and the solution placed in a 500 ml round-bottom flask. A solution of /

of diazomethane (approx. 60 m mole) in anhydrous ether (250ml) was then added in portions, with swirling, and the final solution evaporated to give the product as a pale green liquid (the colour arising from the aforementioned chromium salts). The crude ester was finally distilled, using a "Quickfit" micro-distillation apparatus, and the methyl isovalerate collected as a clear liquid (Yield=1.966g; 17 m mole; 40%).

(iii) Isoamyl alcohol-1-d_2. This compound was obtained by the reduction of methyl isovalerate with lithium aluminium deuteride, in a similar fashion to that previously described for the preparation of the mono-deuterated alcohol from isovaleraldehyde (section 4.2.2). The isoamyl alcohol-1-d_2 (0.77g) so obtained had the following constants: Infra-red:- ν_{max} at 3380cm^{-1} (broad, s) (ν(O-H)); 2170cm^{-1}(m) and 2115cm^{-1}(s) (ν(C-D)). N.M.R. (100MHz):- 9.05τ 1:1 doublet (J=6Hz, 6H (two Me-groups)); 8.50τ 1:1 doublet (broad, J=7Hz, 2H (C_2)); 8.31τ singlet (broad, 1H (O-H)); 8.27τ multiplet (J=6Hz, 1H (C_3)).

(iv) Isoamyl bromide-1-d_2. Bromination of isoamyl alcohol-1-d_2 (0.764g) was carried out according to the method previously described (section 4.2.2). In this case, however, the reaction product was distilled, and the distillate which had been collected below 150^o was treated with water (1ml) and extracted with light petroleum, b.p. 30-40^o (4x10ml). The combined petroleum extracts were washed with water (5ml) and brine (5ml), then dried and evaporated to yield isoamyl bromide-1-d_2 (0.397g) as a colourless liquid. Infra-red:- ν_{max} at 2290cm^{-1}(w) and 2180cm^{-1} (m) (ν(C-D)).

(v) Isoamyl 1-d_2-benzhydroxamate. Condensation of isoamyl/

isoamyl bromide-1-$\underline{d}_2$ (0.393g; 2.56 m mole) with benzhydroxamic acid (0.354g; 2.58 m mole) was carried out as outlined above (section 4.2.2). Work-up afforded the benzhydroxamate ester (0.254g; 1.21 m mole) in 48% yield. Infra-red:- ν_{max} at 2230cm^{-1}(m), 2165cm^{-1}(m) and 2120cm^{-1} (m) (ν(C-D)).

(vi) Isopentoxyamine-1-$\underline{d}_2$ hydrochloride. Using the same procedure as described above (section 4.2.2), isoamyl-1-$\underline{d}_2$-benzhydroxamate (0.254g; 1.21 m mole) was hydrolysed to give isopentoxyamine-1-$\underline{d}_2$ hydrochloride, which crystallised from ethanol/ether as colourless plates (yield= 53.5mg; 0.39 m mole; 32%). N.M.R. (100MHz) gave:- 9.21τ 1:1 doublet (J=5Hz, 6H (two Me-groups)); 8.47τ unresolved multiplet, 3H ($C_2$and C_3).

4.2.4 Pregnenolone-isopentyloxime(-1-$\underline{d}_1$)-TMS and (1-$\underline{d}_2$)-TMS.

These derivatives were prepared as indicated above (section 4.2.1) using the appropriate labelled reagents. They gave single gas chromatographic peaks ($I^{250^o}_{OV-1}$ = 3130), and mass spectra consistent with the incorporation of one and two deuterium atoms, respectively ($M^{+\cdot}$: m/e 474,475) (see Appendix II).

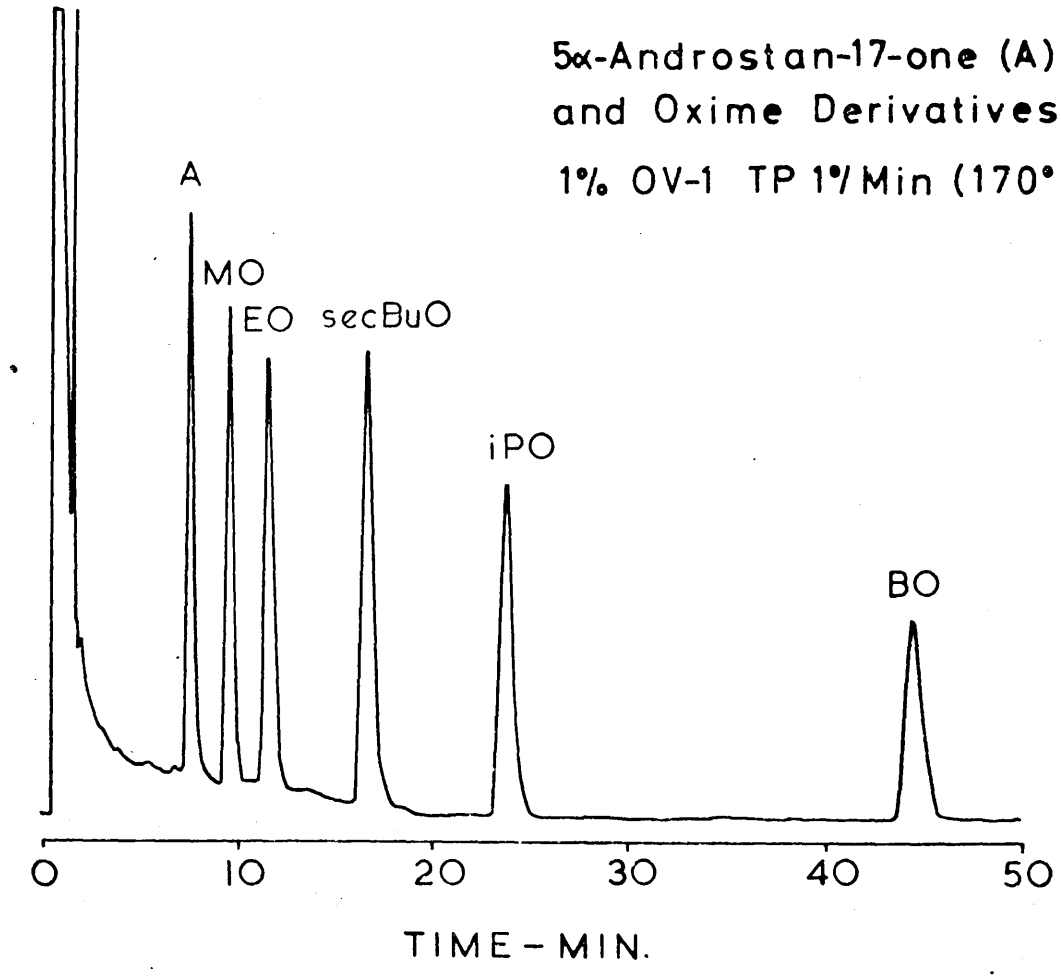


Fig. 34: Separation of 5α-androstan-17-one (A) and its O-methyloxime (MO), O-ethyloxime (EO), O-sec-butyloxime (secBuO), O-isopentyloxime (iPO) and O-benzyloxime (BO) with a 9ft 1% OV-1 column by temperature programming at 1°/min from 170°. Retention indices are: 2195, 2275, 2335, 2470, 2600 and 2980 respectively.

4.3 RESULTS AND DISCUSSION

4.3.1 PREPARATION AND GLC PROPERTIES OF O-ALKYLOXIME DERIVATIVES

All four O-alkylhydroxylamine hydrochlorides reacted with a number of representative ketosteroids to give the expected derivatives, provided the reagent was present in large excess (20: 1 or greater). Reaction could be affected either at 70° for 4 hr, or overnight at room temperature; the latter procedure was found convenient. Under these conditions, no unreacted starting material could be detected by GLC.

The resulting oximes were found to have good gas chromatographic properties and possessed the expected increments in retention time relative to the corresponding free ketones: these were approximately 270 retention index units for the O-butyloximes and approximately 410 units for the O-pentyloximes, with a 1% OV-1 phase. (The increments on the OV-17 phase were less, being about 200 and 365 retention index units, respectively.) These properties are illustrated in fig.34, where five oxime derivatives of 5α-androstan-17-one are compared.

Initial experiments indicated that the two isomeric O-butyloximes had very similar properties to each other, as also had the corresponding O-pentyloximes (Tables 15 and 16). Two of the reagents, sec-butoxyamine hydrochloride and isopentoxyamine hydrochloride, were selected for more detailed investigation. Their reactions were examined with representative ketosteroids possessing 3-one, 4-en-3-one, 16-one, 17-one and 20-one groupings. Quantitative conversion (as judged by GLC) to the O-alkyloxime derivatives was observed under the conditions cited above. 3α-Hydroxy-5β-androstane-11,17-dione yielded only the 17-mono isopentyloxime/

Parent Steroid	Free Ketone		O-Isobutyloxime		O-sec-Butyloxime	
	OV-1	OV-17	OV-1	OV-17	OV-1	OV-17
5α-Androstan-17-one	2195	2555	2495	2745	2470	2695
5α-Pregnan-20-one	2395	2685	2720	2905	2685	2865
17β-Hydroxy-5-androstan-3-one	2195	2515	2500	2755	2465	2710
Testosterone	2560	3020	2780/2800	3200	2755/2770	3165
Testosterone TMS ether	2655	2895	2905/2925	3130	2870/2880	3100
Pregnenolone	2625	3055	-	-	2905	3245
Progesterone	2710	3215	-	-	3195/3220	3545
4-Androstene-3,16-dione	2550	3125	-	-	3045/3070	3435/3455
3β-Hydroxy-5-androsten-16-one	2475	2865	-	-	2715	3100/3120
3β-Hydroxy-5-androsten-16-one TMS ether	2550	2795	-	-	2830	3060/3080

Table 15: Retention index values* of steroid O-butyloxime derivatives.

* Retention index values were measured at 210° (I up to 2400), 220° (I 2400-2900) and 240° (Iover 2900), with respect to n-alkanes co-injected with the derivatives. Columns were 6 ft in length and packings coated with 1% stationary phase.

Parent Steroid	O-n-Pentyloxime		O-Isopentyloxime	
	OV-1	OV-17	OV-1	OV-17
5α-Androstan-17-one	2640	2905	2600	2855
5α-Pregnan-20-one	2850	3070	2825	3015
17β-Hydroxy-5α-androstan-3-one	2635	2915	2595	2870
Testosterone	2950	3375	2890/2920	3335
Testosterone TMS ether	3050/3070	3300	3015/3035	3260
Pregnenolone	-	-	3040	3400
Progesterone	-	-	3415/3445	3860
4-Androstene-3,16-dione	-	-	3330/3355	3745/3755
3β-Hydroxy-5-androsten-16-one	-	-	2850/2865	3255/3280
3β-Hydroxy-5-androsten-16-one TMS ether	-	-	2980	3215/3235

Table 16: Retention index values* of steroid O-pentyloxime derivatives.

* Retention index values were measured at 210° (I up to 2400), 220° (I 2400-2900) and 240° (I over 2900), with respect to n-alkanes co-injected with the derivatives. Columns were 6 ft in length, and packings coated with 1% stationary phase.

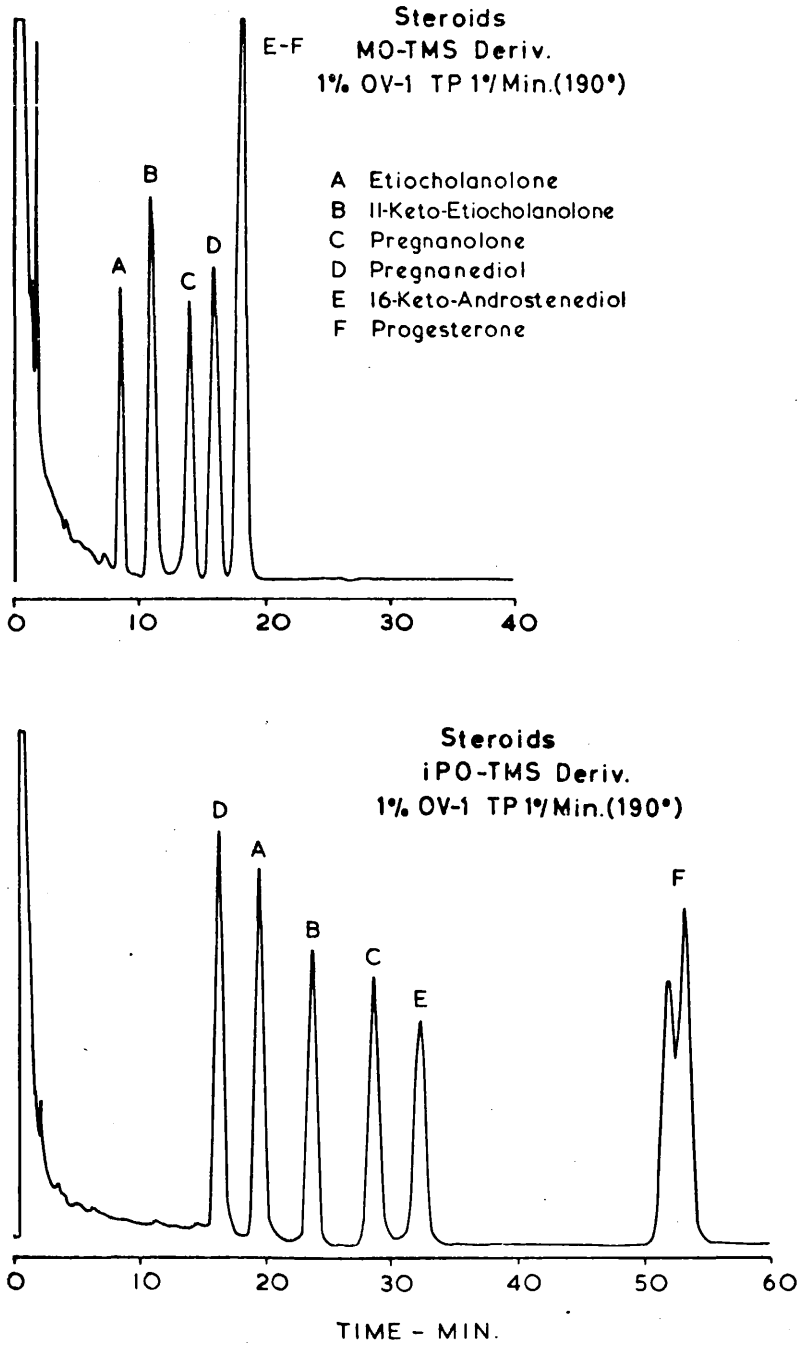


Fig. 35: Separation of steroids as TMS and MO-TMS derivatives (upper), and the same mixture as TMS and iPO-TMS derivatives (lower). The chromatogram was obtained on a 9ft 1% OV-1 column with temperature programming at 1^{o}/min from 190^{o}.

isopentyloxime, as indicated by mass spectrometry of the corresponding TMS ether ($M^{+\cdot}$ at m/e 461). The formation of syn/anti isomers was observed in some cases (see Tables), notably with 4-en-3-ones, and with 16-ones unsubstituted at C-17. Similar results have been reported earlier for the MO derivatives.[145] Steroids with a 3-one 17-one or 20-one structure, however, gave rise to only one GLC peak on derivative formation, although Dray and Weliky [114] reported the formation of two MO isomers from 17 β-hydroxy-5α-androstan-3-one and its 5β-epimer.

The usefulness of the isopentyloximes (iPO) in effecting chromatographic separation of mono- and diketosteroids from hydroxysteroids is illustrated in fig. 35. The upper chromatogram (a) shows a mixture of steroids as MO-TMS derivatives, and the lower (b) the same mixture as iPO-TMS derivatives, showing the improved separation from pregnanediol diTMS.

4.3.2 MASS SPECTROMETRIC CHARACTERISTICS

All the derivatives studied by mass spectrometry gave molecular ions in moderate abundance. It was expected that the fragmentation modes of the O-alkyloximes would parallel the well-known processes observed for MO-TMS derivatives, and comparison of the representative mass spectra in figs.36 and 37 confirmed this in some respects e.g. in the characteristic loss of the N-alkoxy radical, affording a prominent peak at m/e (M-31) in spectra of methyloximes, [158,159] at m/e (M-73) in spectra of butyloximes and at m/e (M-87) for pentyloximes. The O-alkyloximes from 20-ketosteroids yielded the expected ions by cleavage of ring D[114,146]; thus the ion at m/e 244 in the mass spectrum of 21-hydroxypregnenolone iPO diTMS (fig.36, 'B') comprises the side-chain together with the oxime grouping, C-16 and C-17, and corresponds to the fragment at m/e 188 in the MO diTMS derivative (cf. fig.36, 'A'). Similarly, in/

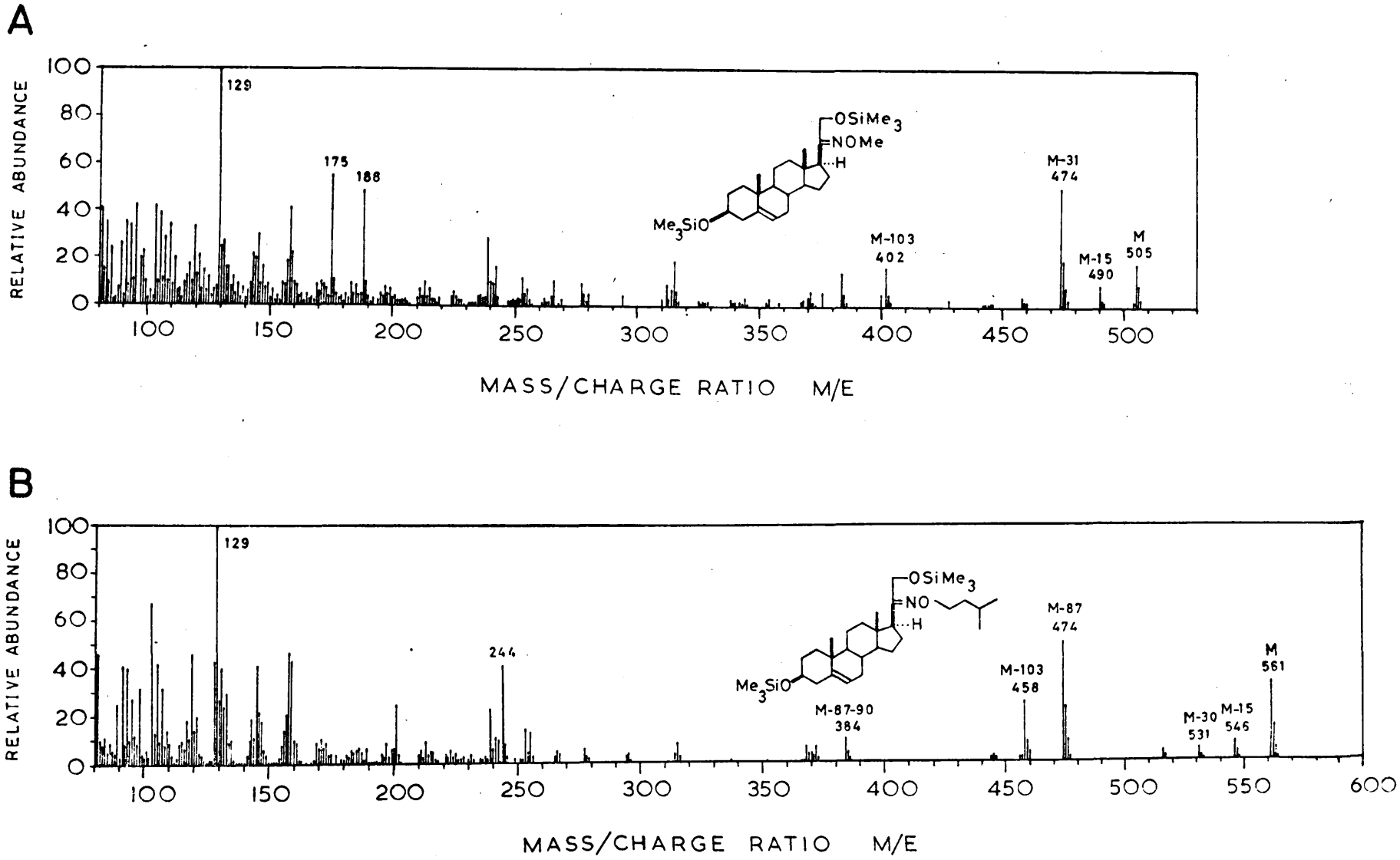


Fig.36: Mass spectra (70eV) of (A) 21-hydroxypregnenolone MO diTMS, and (B) 21-hydroxypregnenolone iPO diTMS.

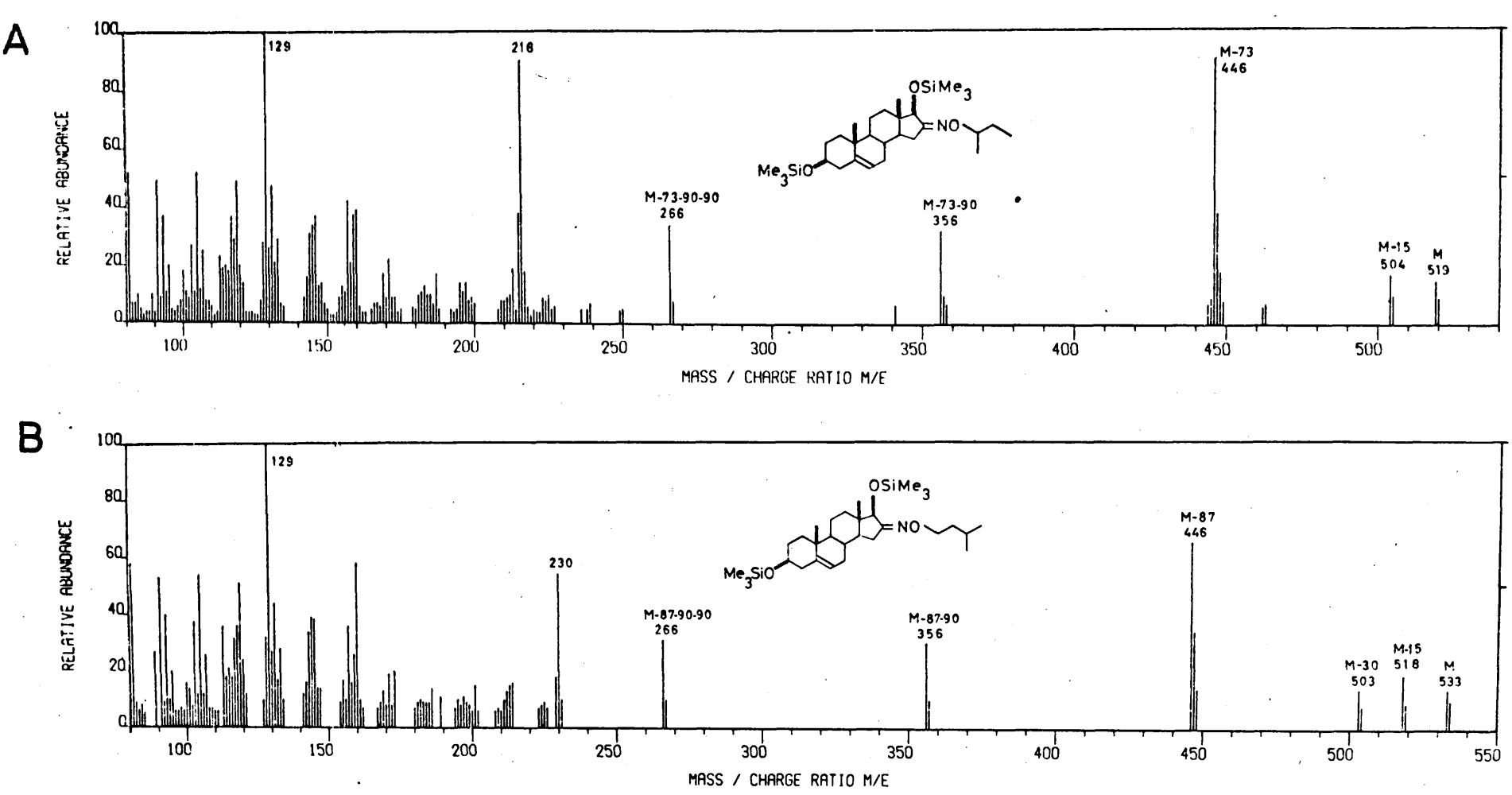


Fig.37: Mass spectra (70eV) of (A) 3β,17β-dihydroxy-5-androsten-16-one secBuO diTMS and (B) 3β,17β-dihydroxy-5-androsten-16-one iPO diTMS.

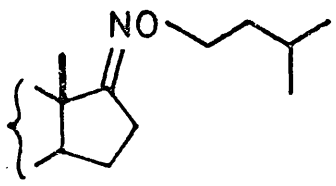
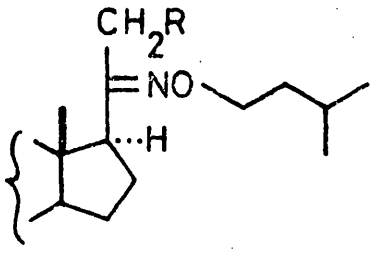
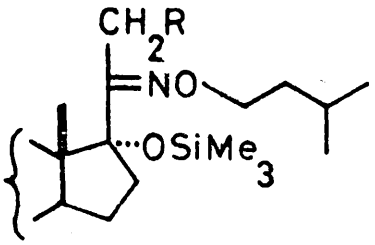
Structural type	Number of examples	$[M-30]^{+\cdot}$ %	$[M-44]^{+\cdot}$ %
	9	23–70	9–30
	8	1–17	0–3
	7	0	0

Table 17: Occurrence of $(M-30)^{+\cdot}$ and $(M-44)^{+\cdot}$ ions in the spectra of steroid O-isopentyloxime derivatives.

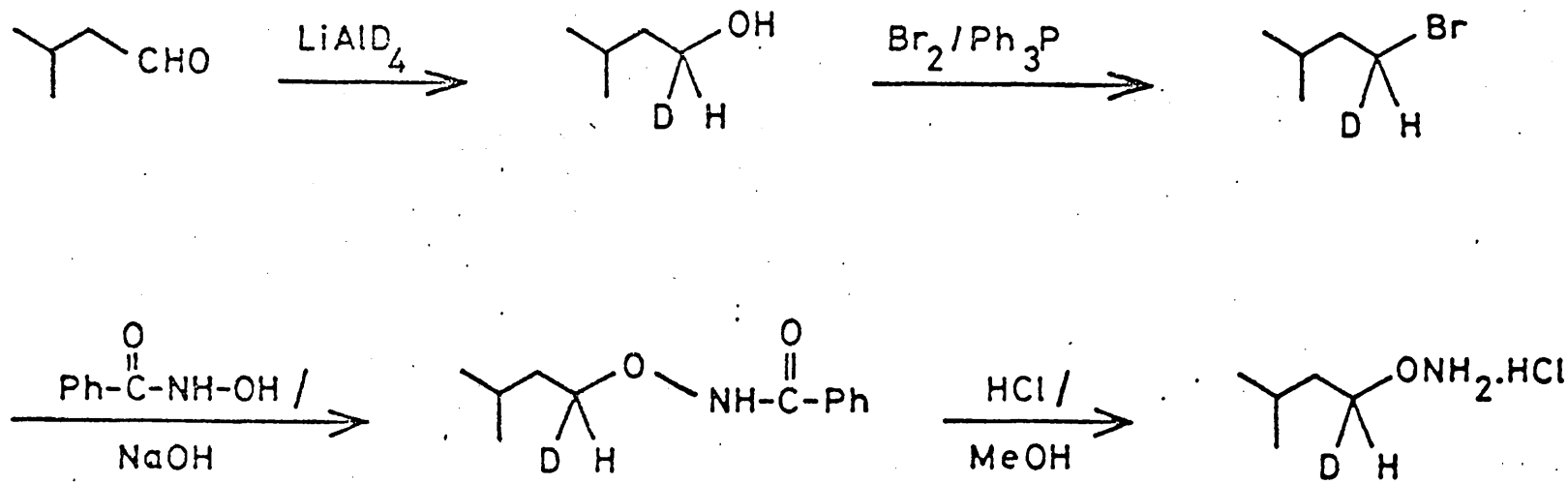
Abundances are expressed as percentages of respective base peaks.

in the spectra of the two O-alkyloxime derivatives of 3β, 17β - dihydroxy-5-androsten-16-one depicted in fig.37, abundant ions retaining the alkyloximino group are present at m/e 216 and m/e 230. These fragments, which comprise C-15, C-16 and C-17 (with their substituent groups), arise from scission of the C-13/C-17 and C-14/C-15 bonds, with hydrogen transfer from the nucleus; a corresponding ion at m/e 174 occurs in 95% relative abundance in the spectrum of the MO diTMS derivative (not illustrated), and a similar ion at m/e 145 appears in the spectrum of the diTMS ether.[205] The various oximes thus afford some common and some distinctive fragment ions, both types being useful in the recognition of ketosteroids in the analysis of mixtures by GC-MS.

The mass spectrum of the iPO - diTMS ether of 3β,17β - dihydroxy-5-androsten-16-one (fig.37, 'B') revealed a prominent ion at m/e 503, corresponding to the loss of 30 mass units from the molecular ion. Examination of other data indicated that $(M-30)^{+\cdot}$ ions were absent from the spectra of sec-butyloximes and isobutyloximes, but were formed by many isopentyloxime and n-pentyloxime derivatives. A further complicating feature was noted in the occurrence of $(M-44)^{+\cdot}$ and $(M-45)^{+}$ ions, as exemplified in fig.36, 'B'. The incidence and abundance of $(M-30)^{+\cdot}$ and $(M-44)^{+\cdot}$ ions were markedly dependent on the structures of the ketonic substrates, as indicated in Table 17. 17-Oxosteroid iPO derivatives yielded strikingly prominent ions, especially at $(M-30)^{+\cdot}$; 20-oxosteroid isopentyloximes unsubstituted in ring D gave lesser amounts; and in 16α- or 17α-trimethylsilyloxy-20-oxosteroid iPO derivatives, no ions of either type were detected.

High resolution mass spectrometry gave the exact mass of the $(M-30)^{+\cdot}$ ion from pregnenolone iPO (molecular weight: 401)/

A



B

Similarly :

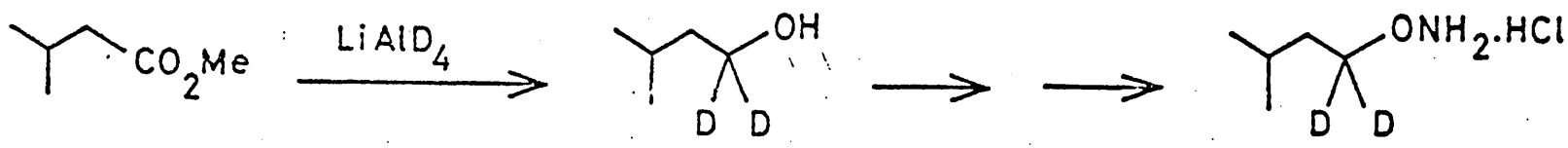
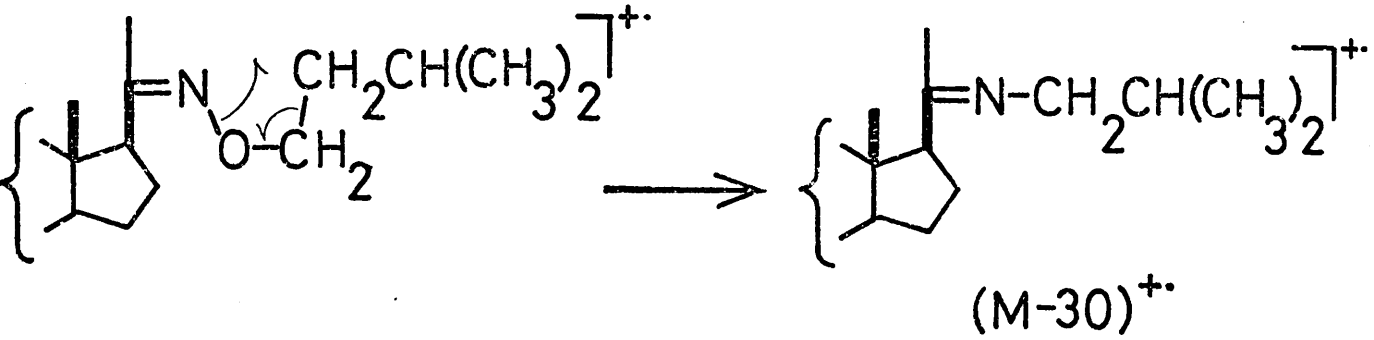


Fig. 38: Synthesis of deuterated isopentoxyamine hydrochlorides.

(A)



(B)

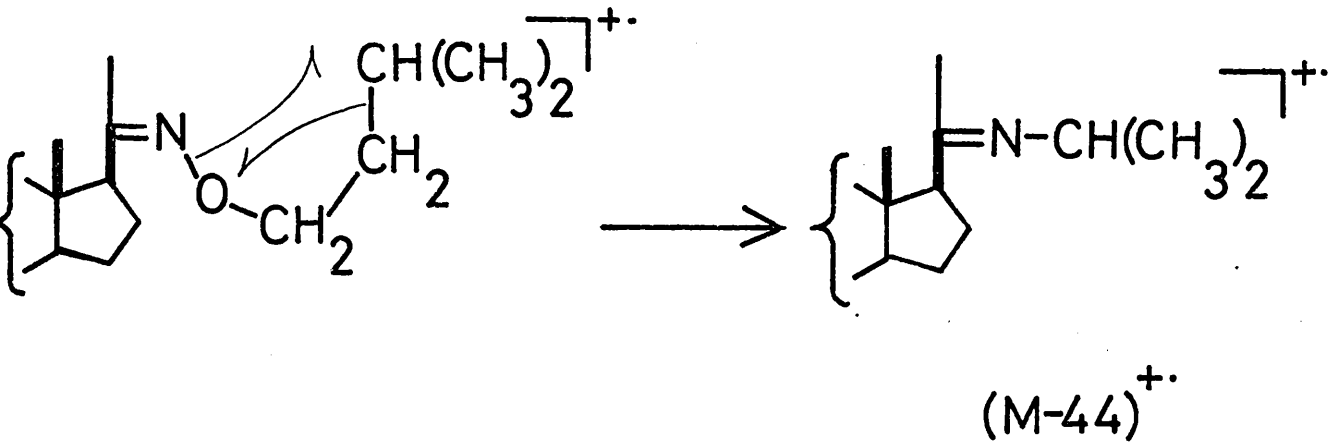


Fig.39: Origin of the $(M-30)^{+\cdot}$ and $(M-44)^{+\cdot}$ ions in the spectrum of pregnenolone O-isopentyloxime;
(A) mechanism confirmed by deuterium labelling.
(B) postulated mechanism.

(molecular weight: 401) as 371.3189. This indicates a molecular formula of $C_{25}H_{41}NO$ (calculated mass: 371.3188), corresponding to the loss of CH_2O from the molecular ion. It appeared probable that the CH_2O unit originated from the alkoxy group, and this was confirmed by data recorded for derivatives labelled by the route indicated (fig.38) with one and two deuterium atoms, respectively: ions at $(M-31)^{+\cdot}$ and $(M-32)^{+\cdot}$ were observed (see Appendix II). The simplest mechanism for the production of these ions would appear to be as in fig.39: their absence in the case of 16- and 17-trimethylsilyloxy-derivatives is presumably due to steric inhibition of the *cisoid* conformation of the N-O-CH_2-R grouping. From models, this is clearly very likely with 17α-substituents, but the effect of a 16α-trimethylsilyloxy group would be expected to be less marked.

The identity of the ions at *m/e* (M-44) and (M-45), which occur in the spectra of many *O*-pentyloximes, particularly those of 17-ketones (Table 17), has not yet been established. However, they appear to be associated with a fragmentation of the alkyloxime group, similar to that described above leading to the $(M-30)^{+\cdot}$ species. Thus, in the labelled pregnenolone-(1-*d*$_1$)*i*PO-TMS ether and pregnenolone-(1-*d*$_2$)*i*PO-TMS ether derivatives, the $(M-44)^{+\cdot}$ and $(M-45)^{+}$ ions had shifted to $(M-45)^{+\cdot}$ and $(M-46)^{+}$, respectively. In the spectrum of dehydroepiandrosterone *i*PO TMS ether (DHA *i*PO TMS), a trio of ions of moderate intensity, at *m/e* (M-43), (M-44) and (M-45) was noted; in the corresponding (1-*d*$_1$) *i*PO derivative, these ions had likewise shifted to *m/e* (M-44), (M-45) and (M-46), respectively.

The possibility that the observed loss of 45 mass units from the molecular ion might represent a combination of losses/

losses of 15 a.m.u. (CH_3 radical) and 30 a.m.u. (CH_2O) was investigated in the case of DHA iPO TMS ether, by examining the corresponding d_9-TMS derivative. Should the methyl radical involved in such a process originate from the trimethylsilyloxy group, the original loss of 45 a.m.u. would become a loss of 48 mass units in the deuterated derivative. However, no peak at m/e (M-48) was present in the spectrum of DHA iPO d_9-TMS ether. Nevertheless, this spectrum did serve to illustrate the dual origin of the $(M-15)^+$ peak, a common ion in the mass spectra of steroid TMS ethers: although still present for the deuterated TMS ether, the intensity of this ion had dimished, while a new peak at m/e (M-18) had appeared. Therefore, this $(M-15)^+$ion must correspond to loss of a methyl radical from the steroid nucleus, while the $(M-18)^+$ fragment results from elimination of a CD_3 radical from the TMS group. (This phenomenon has previously been described by Diekman and Djerassi.[206]). It was concluded that the above $(M-45)^+$ion could conceivably arise from the combined losses of 30 a.m.u. (CH_2O) and a methyl radical (from the steroid nucleus), although an analogous mechanism to that postulated for the formation of the $(M-44)^{+\cdot}$ ion (fig.39) is favoured.

4.3.3 CONCLUSIONS

The butoxyamine and pentoxyamine hydrochlorides were found to react readily and quantitatively with a series of representative ketosteroids. The resulting derivatives were stable, and had good gas chromatographic properties, displaying retention times conveniently intermediate between those of the corresponding MO and BO derivatives. They were also suitable for study by gas chromatography-mass spectrometry, giving molecular ions and/

and informative fragmentation patterns.

These reagents therefore appeared very promising for the study of mono- and diketosteroids by gas phase methods, and their application to the study of urinary steroid profiles is discussed in section 5.

SECTION 5

COMPARISON OF ISOPENTYLOXIME AND BENZYLOXIME TRIMETHYLSILYL ETHERS IN THE CHARACTERISATION OF URINARY STEROIDS OF NEWBORN INFANTS.

5.1 INTORDUCTION

5.1.1 THE URINARY STEROID PROFILE

An important application of GLC and GC-MS in clinical chemistry is in the determination of urinary steroid profiles.[202] This technique permits the simultaneous qualitative and quantitative estimation of the various hormone metabolites in urine, and thus affords a means by which endocrine function may be indirectly assessed, e.g. many disorders of the adrenal glands give rise to markedly altered profiles, which may return to a more normal pattern following drug therapy. Basically, three strikingly different types of human urinary steroid profile can be distinguished: adult male, pregnant female and newborn infant.

The principal components in the adult male (and non-pregnant female) profile are reduction products of the adrenocortical hormones (fig.2), e.g. tetrahydrocortisone (THE), tetrahydrocortisol (THF), cortolone and cortol (fig.4), the 17-ketosteroids androsterone and etiocholanolone (together with their 11-oxygenated analogues), and dehydroepiandrosterone (DHA). With the exception of DHA, which is excreted as its 3β-sulphate, these metabolites are rendered water-soluble mainly through conjugation with glucuronic acid (fig.3).

The urinary steroid profile associated with pregnancy differs from that described above in that elevated levels of certain steroids - notably estriol and pregnanediol - are apparent. The excretion of these two compounds rises steadily throughout pregnancy, and their urinary levels attain values of the order of 25mg/24h[207] and 35mg/24h,[208] respectively, at term. The involvement of the fetus in the production of estrogens has long been appreciated, and the urinary estriol level may be taken as an index of fetal viability.[209,210] The other important estrogens, estrone and estradiol, are found in pregnancy urine in much lower concentration.

Pregnanediol is the principal metabolite of progesterone which, in turn, is secreted in large amounts by the placenta. (It has been estimated that some 250mg of progesterone are secreted per 24h at term.[211]) Both pregnanediol and the estrogens are excreted mainly as their glucuronides, although some conjugation with sulphuric acid may also occur.

The pattern of urinary steroids obtained from newborn infants, however, is strikingly different from those described above. Extensive investigations into the nature of steroid excretion in early infancy were carried out mainly by Mitchell and his colleagues[212-219] and by Reynolds.[220-223] The separation methods employed by these authors were largely those of TLC and paper chromatography; colorimetric and fluorometric determinations of isolated fractions were used for the quantitative estimation of the compounds present. The steroids identified in newborn infant urine differed from those normally encountered for adults in several respects:- (i) the major metabolites possessed a Δ^5-3β-ol structure, as opposed to the ring-B saturated compounds which predominate in adult urine. However, additional saturated metabolites, mostly of the 5β-pregnane series, have been more recently described in infant urine by Shackleton et al;[224] (ii) many of the "newborn" steroids possessed an oxygen function at C-16, usually a 16α-hydroxyl group;[56,219,223] (iii) sulphate conjugation was found to be quantitatively more important than conjugation with glucuronic acid[225]; (iv) the proportion of urinary steroids excreted in unconjugated form, particularly polar metabolites of cortisol, was higher for infants than for adults[225,226]; (v) among the 11-deoxy-17-ketosteroids, DHA, etiocholanolone and androsterone (which form most of the group in the adult) were present in infant urine in only trace amounts, if detectable at all[212]; (vi) the urinary levels of THE and THF, excreted in large amounts by adults, were low in infant urine.[214] Quantitatively, the most important steroids excreted in the urine of newborn infants/

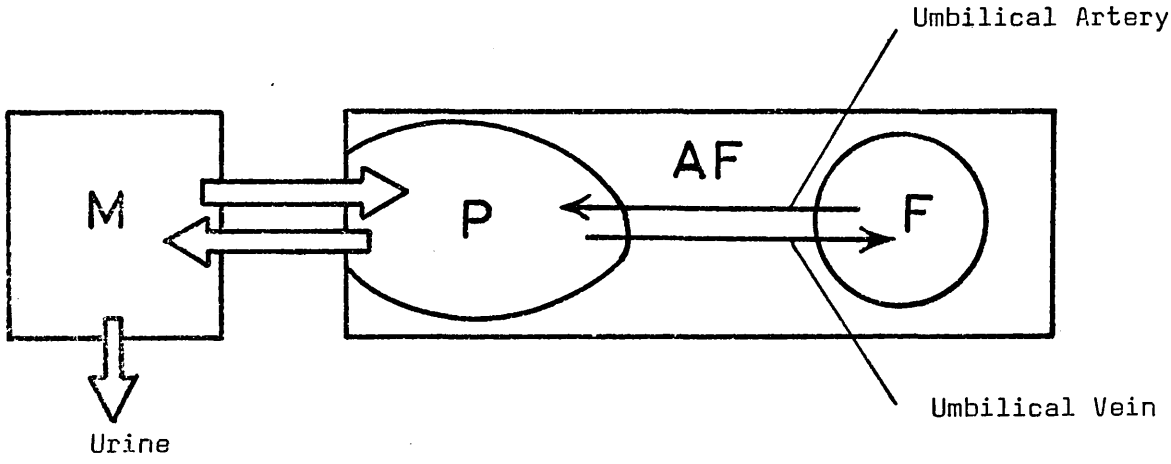


Fig.40: Diagrammatic representation of the feto-placental unit.[9)]

M = maternal compartment

P = placenta

F = fetus

AF = amniotic fluid.

infants were identified as:-

5-androstene-3β,17α (and 17β) -diol,[217,232],

5-androstene-3β,16α,17β -triol,[218,223,228,229],

16α-hydroxy-DHA,[221,227],

16β-hydroxy-DHA,[205],

16-Ketoandrostenediol[221],

16α-hydroxypregnenolone[218,220,222], and

21-hydroxypregnenolone.[56,214-216]

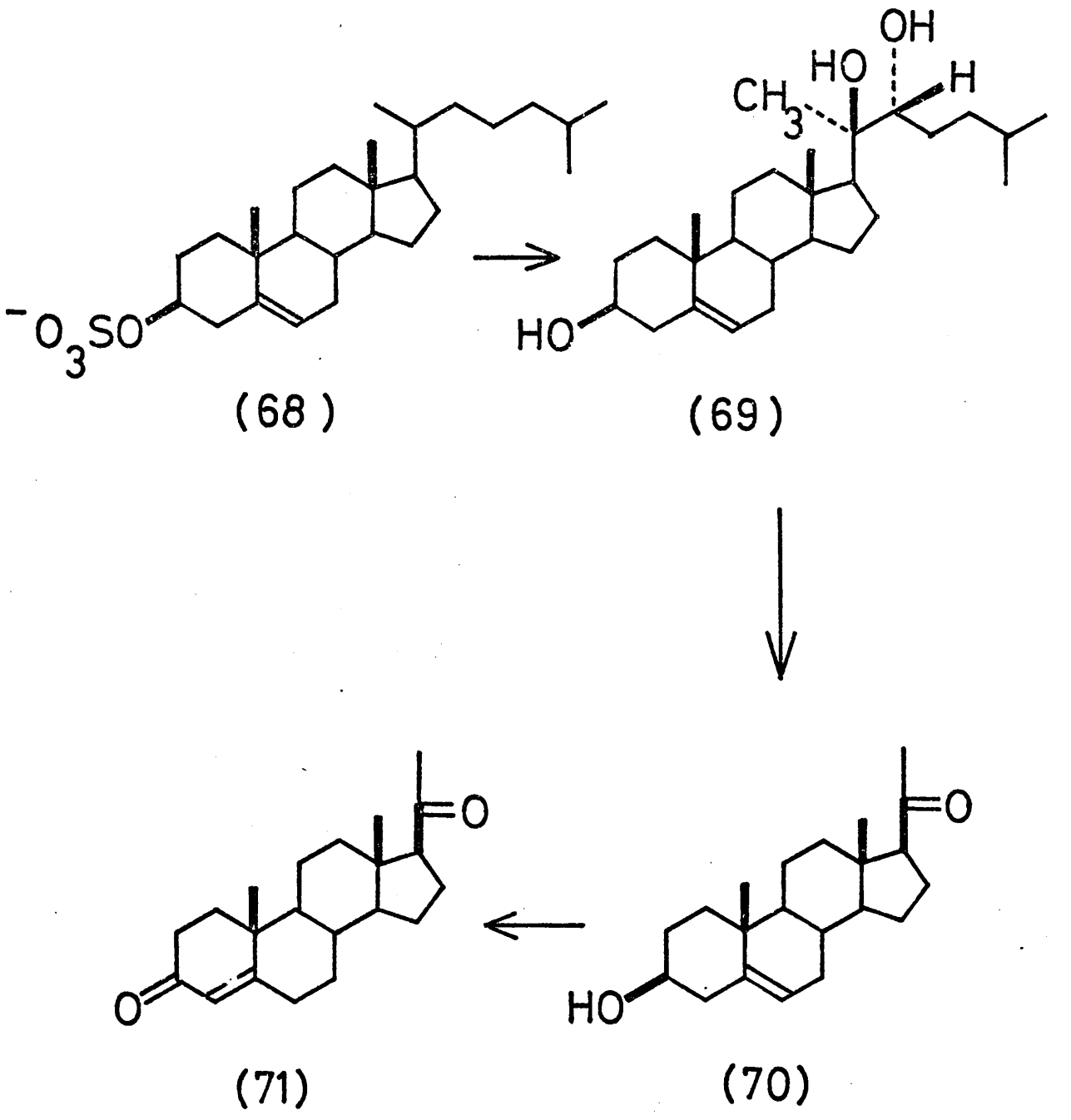
The excretion of these 3β-hydroxy-Δ^5-steroids was observed to persist until the sixth month of life, when a more "adult" steroid pattern began to emerge.[222,230]

Clearly, steroid metabolism in early infancy differs considerably from that obtaining in later life, and the study of urinary metabolites affords an indirect means by which the biosynthetic processes operating *in utero* may be investigated.

5.1.2 STEROID METABOLISM IN THE FETO-PLACENTAL UNIT

The fetus and the placenta are physically distinct, and may be thought of as separate entities. During gestation, the fetus derives its nourishment *via* the placenta, which acts as a "barrier" between fetus and mother. Certain compounds may cross this placental barrier *e.g.* glucose, fats, amino acids, vitamins and certain steroids. However, during intrauterine life, the fetus and the placenta function together, and, particularly with respect to steroid biogenesis, they are advantageously thought of as two specialised compartments of an integrated functional unit. The term "feto-placental unit" is used to emphasise the important inter-relationship, which is illustrated diagrammatically in fig.40.

Steroid metabolism in the feto-placental unit has been investigated extensively by means of both *in vitro* and *in vivo* experiments, and a number of reviews have appeared on the subject.[231-233] Basically, the feto-placental unit is responsible for the synthesis of the large quantities of estrogens and progesterone necessary for the maintenance of pregnancy. While progesterone production is/



(68) (69) (71) (70)

Fig.41: Placental synthesis of progesterone from cholesterol sulphate.

is essentially limited to the placental compartment, the fetus plays an important role in the formation of estrogen precursors. The synthesis of these two types of compound will be dealt with separately:-

Progesterone: Although fetal tissues have been demonstrated to be capable of the de novo synthesis of cholesterol from acetate (cf. fig.1)[234-236], the major precursor of progesterone is believed to be cholesterol from the maternal bloodstream, where it is present as the 3β-sulphate.[237] Placental synthesis of progesterone (71) from cholesterol sulphate (68) (fig.41) has been shown to proceed via removal of the sulphate group (through the action of a sulphatase enzyme), cleavage of the cholesterol side-chain probably via cholesterol 20R, 22R-diol (60)[238,239], and finally conversion of the pregnenolone (70) so produced by the combined action of two enzyme systems, 3β-hydroxysteroid dehydrogenase (3β-HSD) and a Δ^{4-5} isomerase. This last step, the formation of Δ^4-3-ketosteroids from 3β-hydroxy-Δ^5-steroids, is an important reaction in placental tissue. The progesterone formed is distributed between maternal and fetal compartments; in the former, it is mainly reduced to pregnanediol in the maternal liver prior to conjugation and urinary excretion, while hydroxylation at various sites (e.g. 6β,11β,15α,16α,17α and 21) takes place in fetal tissues,[231] along with partial reduction to e.g. the 20α-dihydro derivative.[240]

Estrogens: Whereas the placenta lacks the enzyme system (a 17,20-desmolase) required for complete removal of the cholesterol side-chain (Table 18), the fetus actively converts pregnenolone (70) to dehydroepiandrosterone sulphate (DHAS; 72),[234,241] thus providing a source of estrogen precursors. The first steps in the synthesis of estrogens from C_{19} steroids are the removal of the sulphate ester function and the conversion of the 3β-hydroxy-Δ^5 system to a 4-en-3-one grouping. The fetus, however, has a deficiency in the two enzymes required to bring about these/

Enzyme system	Placenta	Fetus
3β-HSD/ Δ^{4-5} isomerase	High	Low
Aromatisation	High	Low
Sulphatase	High	Low
Sulphokinase	Low	High
16α-Hydroxylase	Low	High
17α-Hydroxylase	Low	High
17,20-Desmolase	Low	High
Steroid synthesis from acetate	Low	High

Table 18: Enzyme activities in the placenta and fetus.[232)]

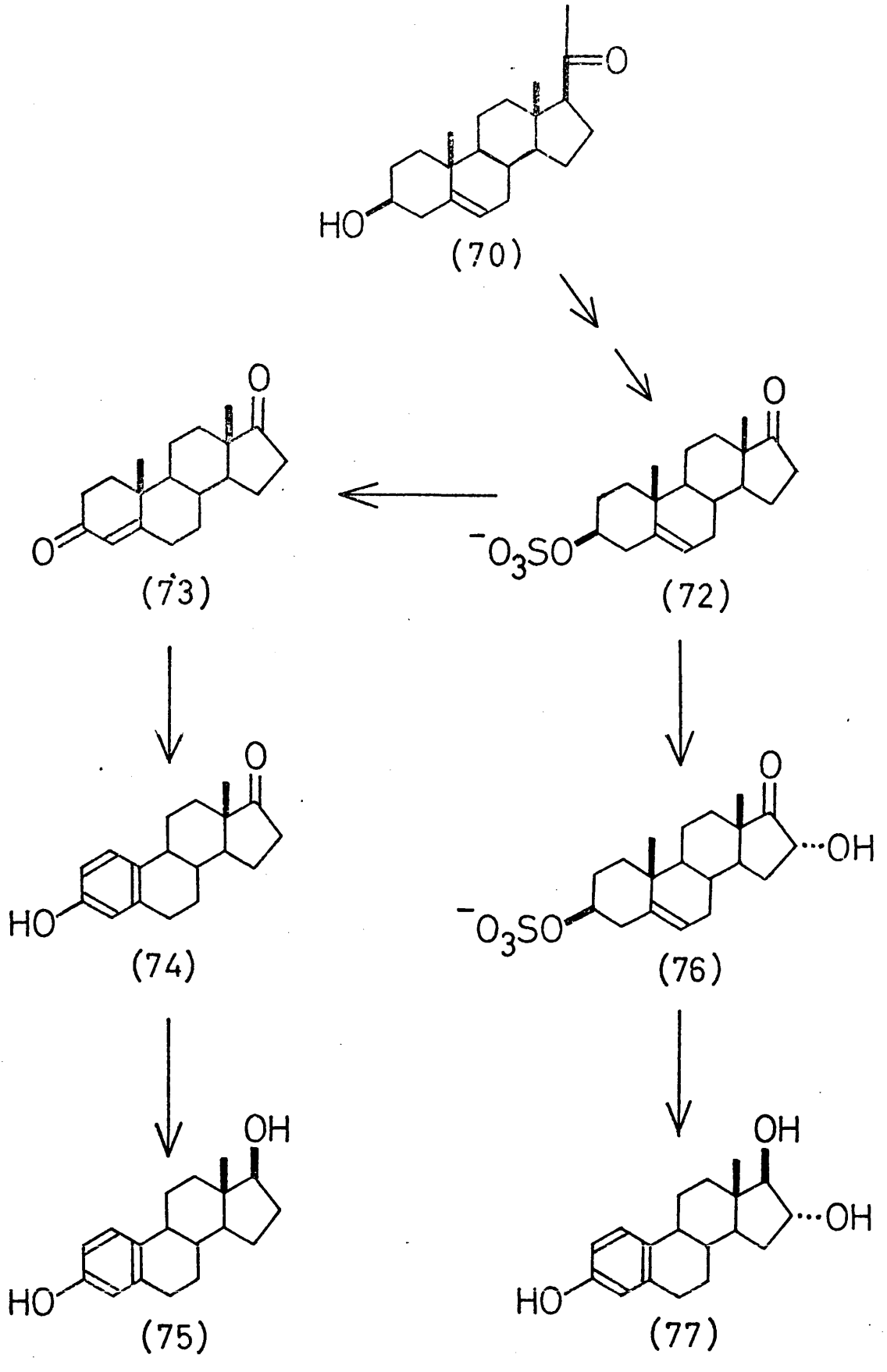


Fig.42: Major pathways for the synthesis of estrogens in the feto-placental unit.

these conversions; the placenta, on the other hand, possesses a high activity of the requisite sulphatase and 3β-HSD/Δ^{4-5} isomerase enzyme systems, and can therefore utilise fetal DHAS, which it converts to androstenedione(73). Aromatisation of ring-A then takes place, again in the placental compartment, via oxidative elimination of C-19,[242-244] and estrone (74) is finally produced (fig.42). Placental reduction of estrone is believed to be the major route to estradiol (75), while estriol (77), quantitatively the most important estrogen in pregnancy, is synthesised in the placenta mainly from 16α-hydroxy-dehydroepiandrosterone sulphate (16α-hydroxy-DHAS; 76).[245] This latter compound is formed in large amounts by the fetus, whose liver tissue possesses a highly active 16α-hydroxylase enzyme (Table 18).

The estrogens produced by the placenta are distributed between both fetal and maternal compartments. In the fetus, sulphurylation rapidly takes place, followed, to some extent, by further hydroxylation (e.g. at C-15α[246-248]); estrogens are then re-cycled to the placenta. Estrogens entering the maternal circulation are eventually conjugated, mainly with glucuronic acid, although sulphate and "mixed" sulphate-glucuronide conjugates are also found in pregnancy urine. The general picture is therefore one of an estrogen/estrogen sulphate cycle between fetus and placenta, which results in estrogens being steadily transferred to the maternal compartment from the pool of estrogen sulphate in the fetus. The above scheme of estrogen biosynthesis illustrates well the complementary nature of steroid production in the feto-placental unit; tissues in fetal and placental compartments act sequentially because the requisite enzymes are present in one compartment, but absent from the other. The relative activities of various enzyme systems in the placenta and fetus are shown in Table 18.

5.1.3 STEROID METABOLISM IN EARLY INFANCY.[249]

At birth, three populations of steroids may be considered to exist in the infant: (1) those arising from the metabolism of the large amounts of estrogen and progesterone received from the placenta; (2) 3β-hydroxy-Δ^5steroids produced *in utero* as precursors of estrogen; and (3) cortisol and other steroids normally found in adulthood. From all three groups, steroids or their metabolites might be expected to be excreted in the urine of the newborn infant. Metabolites of the compounds in group 1 would be excreted after birth in rapidly decreasing quantities, since their source has been eliminated; indeed, the level of urinary estriol has been found to fall to barely detectable levels after the fifth day of life.[250] Excretion of the 3β-hydroxy-Δ^5steroids (group 2), on the other hand, may actually increase rather than decrease during the first month [230], and compounds with this structure predominate over all others in early infancy.[218] This increase in Δ^5-steroid output might be explained by the continued adrenal secretion of these estrogen precursors after birth, and the relative inability of the infant liver to deal with them in the same way as does the placenta *in utero*.[251] However, it would be reasonable to assume that the Δ^5-steroids fulfil some particular biological role, although the nature of this function remains unclear at the present time.

By the sixth month after birth, the excretion of 3β-hydroxy-Δ^5steroids has generally fallen to a negligible level; this decline in Δ^5steroid output has been correlated with physiological changes in the infant adrenal glands, and with the accompanying maturation of the 3β-HSD/Δ^{4-5}isomerase enzyme system which is responsible for the production of Δ^4-3-ketosteroids (*cf.* fig.41). Interestingly enough, of the adult-type steroids considered in group 3, the newborn infant produces adequate supplies of corticosteroid hormones from/

from birth onwards.[252,253] Since these compounds possess the 4-en-3-one grouping, it has been suggested that the 3β-HSD/Δ^{4-5}isomerase system necessary for their formation does exist in certain fetal tissues, but that it is substrate-specific.[254,255] Thus, compounds such as DHAS, 16α-hydroxy-DHAS and pregnenolone sulphate, present in high concentration in the fetal circulation, might remain unaffected, whereas various hydroxylated pregnenolone derivatives might act as corticosteroid precursors in certain fetal tissues. This theory was initially favoured by Mitchell and co-workers, who proposed that, in the newborn, 3β-HSD/Δ^{4-5}isomerase acted subsequent to 17α-,21- and 11β-hydroxylase[218]; such a pathway might explain the presence of 21-hydroxypregnenolone in infant urine. However, a later investigation of polar steroids in plasma from human umbilical cords and in human infant urine failed to detect any of the 3β-hydroxy-Δ^5analogue of cortisol, i.e. 3β,11β, 17α, 21-tetrahydroxy-5-pregnen-20-one.[256]

DHA, a 17-ketosteroid commonly encountered in adult urine, is not normally detected in that of newborn infants,[212] probably due to the high activity of 16α-hydroxylase in fetal and infant liver (Table 18); 16α-hydroxy-DHA is, as previously mentioned, a major infant urinary steroid. The high activity of 16α-hydroxylase is apparent in the first few months of life, and could be a remnant of its necessary function in utero concerned with the production of estriol (cf. fig.42). It may, however, be involved with the important problem of electrolyte balance at birth, and in early independent life, since natriuretic properties have been attributed to 16α-hydroxylated C_{21}steroids.[257]

Steroid metabolism and, consequently, the pattern of urinary steroid excretion in early infancy is thus highly complex, and undergoes a period of continuous change during the first six months of life. It is against this background of constant change, and of variations in the steroid excretion pattern often observed between individual infants,[202] that any deviation from the/

the "normal" metabolism has to be recognised. Such a deviation arises in cases of congenital adrenal hyperplasia, the effects of which on steroid metabolism are discussed below.

5.1.4 CONGENITAL ADRENAL HYPERPLASIA

Congenital adrenal hyperplasia results from an inborn error in steroid metabolism involving defects in the enzyme systems required for the formation of corticosteroid hormones. The inadequate level of hormone in blood causes corticotrophin to be secreted by the pituitary in increasing amount which, in turn, causes hyperplasia of the adrenal glands with overproduction of those steroids not affected by the enzyme block. The most common deficiency is in 21-hydroxylase, but deficiencies have also been reported in infants and children of 3β-HSD, 11β-hydroxylase and cholesterol 20,22-desmolase.[258,259]

In cases of 21-hydroxylase deficiency in infants, the excretion of 3β-hydroxy-Δ^5-steroids is usually much higher than normal,[260] and large amounts of 16α-hydroxy-DHA and 16α-hydroxy-pregnenolone are normally found in the urine.[261] Furthermore, pregnanetriol and the 11-keto derivatives of androsterone, etiocholanolone and pregnanetriol may be excreted in the urine; these compounds are more commonly encountered in older infants with this defect, however, since their formation requires the action of a ring-A reductase which is deficient in the liver of newborn infants.[262-264] Thus the diagnosis of a defect in 21-hydroxylase in infants through a study of the urinary steroid excretion is generally easier and more reliable when the infant is over six months old. At this age, an "adult" type of steroid metabolism has been assumed, and abnormal compounds e.g. pregnanetriol, are clearly visible in the urinary steroid profile.

5.1. 5 DERIVATIVE FORMATION IN STEROID PROFILE ANALYSIS.

GLC and GC-MS methods have been extensively employed by Horning and co-workers in the study of urinary steroid profiles from newborn infants.[55,56,175,200,265-267] In the first week of life, the excretion of corticosteroids of dihydroxyacetone and 20,17-ketol types is very limited, only tetrahydrocortisone (THE) being frequently observed. Accordingly, the major urinary steroids may be studied after simple trimethylsilylation. Under these conditions the principal components are readily identified by GC-MS, but there is considerable overlap of ketonic and hydroxylic steroid derivatives. The MO-TMS ethers provide a more convenient mass spectrometric distinction between the two groups, but show fortuitous coincidence of retention times on non-selective phases (e.g. SE-30 or OV-1), as for example of 5-androstene-3β,16α,17β-triol triTMS with 3α,6α-dihydroxy-5β-pregnan-20-one MO-diTMS. BO-TMS ethers, on the other hand, give rise to a distinct "group separation" of hydroxy- from ketosteroids, due to the large molecular weight increments accompanying their formation. Thus, a BO-TMS profile will generally contain two distinct regions: hydroxysteroids, or steroids possessing unreactive keto groups (e.g. at C-11) are eluted first, followed by a group of peaks representing steroids with reactive ketone functions. Group separations of this nature are clearly desirable in simplifying the overall steroid profile, although the high temperatures (290-300^{o}) required for the elution of many BO-TMS derivatives lead to high GLC column "bleed", and so to elevated levels of "background" ionisation in the mass spectrometer. Furthermore, the large retention increments accompanying BO formation preclude the analysis of most diketonic steroids. In view of the favourable results reported in section 4 for O-alkyloximes with retention times intermediate between those of the MO-TMS and BO-TMS ethers, it was decided to apply one of these, the iPO-TMS derivative, to the study of infant urinary steroid profiles. It was/

was envisaged that the iPO-TMS ethers would complement existing derivatives, and that their use would minimise the practical difficulties, outlined above, associated with the use of BO-TMS ethers in steroid profile analysis.

5.2 EXPERIMENTAL

5.2.1 EXTRACTION OF STEROIDS FROM URINE

All urinary steroid extracts studied in this work were kindly supplied by Dr. E.M. Chambaz (Laboratoire d'Hormonologie, CHR de Grenoble, France). Urine samples were collected from both male and female newborn infants during the first five days of life. A fraction of each urine sample was used to constitute a pool.

The procedures used by Dr. Chambaz for the extraction of urinary steroids were as follows:-

"Total" steroid extracts were obtained after enzymatic hydrolysis (5 to 50ml of urine) with a mixture of β-glucuronidase and sulphatase for 48 h at 37°C in a shaking bath, after addition of 0.1 volume of acetate buffer (0.2 M, pH 5.2). The hydrolysed urines were extracted with 2 volumes of ether and 2 volumes of ethyl acetate; the pooled organic phases were washed with 0.1 volume of 0.1N sodium hydroxide and with distilled water, filtered through anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator: the residues were suitable for derivative formation.

Steroid extracts corresponding to the sulphate and glucuronide - conjugated metabolites were obtained by a two-step differential hydrolysis:
The urine was first extracted with 1 volume of methylene chloride and the organic phase discarded. The aqueous phase was brought to pH 1 with concentrated sulphuric acid and extracted twice with 1 volume of ethyl acetate. The organic phase was kept at 37° for 24 h, then washed with 2 volumes of 1N sodium hydroxide, 0.1 volume 10% acetic acid, distilled water and finally evaporated to dryness. This afforded the "sulphate" fraction.

The 1N sodium hydroxide washings (above) were quickly diluted by 1 volume of water, neutralised and buffered at pH 5.2/

5.2 for enzymic hydrolysis, as previously described. Extraction and work-up, carried out in the same manner as for the "total" steroids, afforded the "glucuronide" fraction.

5.2.2 PREPARATION OF DERIVATIVES

(a) O-Alkyloximes of reference compounds

O-Isopentyloximes (iPO) were prepared by adaptation of the method previously described (section 3.2.1) for O-methyloximes.[161]

O-Benzyloximes (BO)[186]:- The steroid (400μg) was placed in a screw-capped "Reacti-Vial" and benzylhydroxylamine hydrochloride (8mg) added. The mixture was dissolved in pyridine (200μl) and the vial placed in an oven at 60-70° overnight. The solvent was evaporated with the aid of a stream of nitrogen and the residue extracted and worked up in a similar fashion to that previously described for O-methyloximes (section 3.2.1).

(b) Trimethylsilyl (TMS) ethers of reference compounds

Unhindered hydroxyl groups were converted to TMS derivatives by the non-catalysed reaction[162] with BSA or BSTFA outlined in section 3.2.1.

Hindered hydroxyl groups were silylated with TSIM, either by the non-catalysed procedure of Devaux et al.[186] or by the catalysed reaction described by Thenot and Horning[166]:- Non-catalysed reaction: The steroid (500μg) was placed in a "Reacti-Vial" and TSIM (250μl) added. The reaction mixture was then heated at 150° for 4½ h, and examined directly by GLC. Catalysed reaction: This was used in cases where reactive ketone groups had previously been converted to oxime derivatives. Here, the steroid oximes were not extracted from the residue obtained by evaporation of the solvent (pyridine), but the crude oximation product was treated directly with TSIM (1μl reagent per 2μg steroid) and the mixture heated at 100° for 2h. The/

The resulting oxime-TMS derivatives were examined directly by GLC.

(c) BO-TMS and iPO-TMS derivatives of urinary steroid extracts

These were prepared under the conditions described for MO-TMS derivatives by Thenot and Horning[166]:-
The urine extract (approx. 500μg) was placed in a "Reacti-Vial" and the appropriate alkoxyamine hydrochloride (8-10mg) added. The mixture was taken up in dry pyridine (200μl) and heated at 60° for 30 min. The solvent was then removed under a stream of nitrogen and the residue treated with TSIM (100μl). After a period of 2 h at 100°, the reaction mixture was examined directly by GLC.

5.2.3 GLC AND GC-MS CONDITIONS

Gas Chromatography. A Carlo Erba Model GV was used, with W-shaped 12ft (3mm I.D.) glass columns, packed with 1% OV-1 on silanised 100-120 mesh Gas Chrom Q. Flame ionisation detectors were used, with nitrogen (40ml/min) as carrier gas. Separations were carried out with temperature programming from 190° to 275° for iPO-TMS ethers, and from 190° to 300° for BO-TMS derivatives. n-Alkanes (C_{24} to C_{38}) were used to standardise the retention data for the steroids studied. Cholesteryl decylate was added to BO-TMS analyses as an internal standard, while cholesteryl butyrate was used (to some extent) for corresponding iPO-TMS runs.

Gas Chromatography - Mass Spectrometry. This was carried out using an LKB9000 instrument, with the operating conditions previously described (section 1.2.1). A 12ft spiral column was employed, with a packing identical to that used for GLC (above). Urinary steroid samples were injected in silylating reagent (TSIM or BSTFA), and the dual valve was kept closed for approximately/

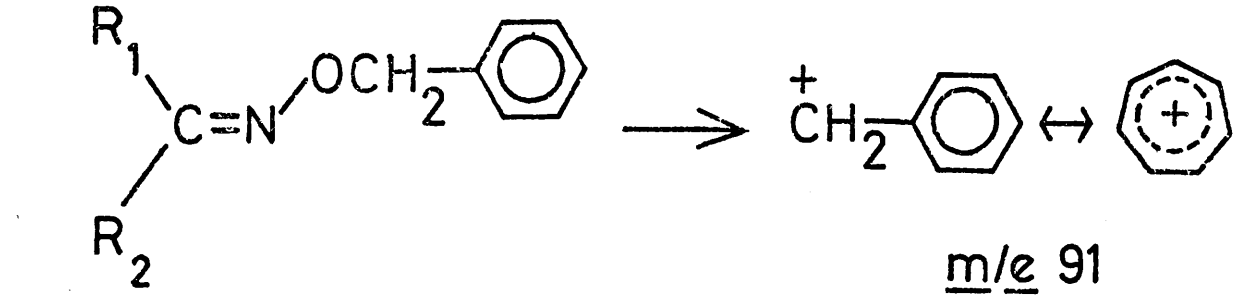
approximately 5 to 10 min following injection. Under these conditions, the solvent front was diverted from the mass spectrometer, resulting in a considerably reduced level of "background" ionisation throughout the run.

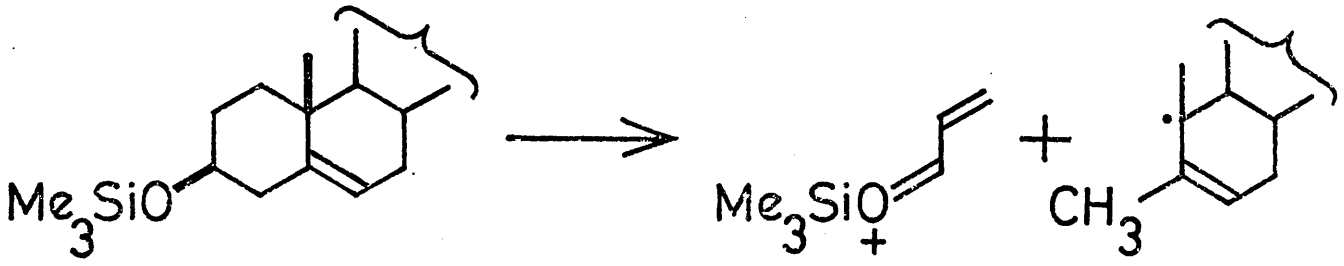
5.3 RESULTS AND DISCUSSION

5.3.1 GAS CHROMATOGRAPHIC CONSIDERATIONS IN URINARY STEROID PROFILE ANALYSIS.

Two modes of operation are routinely employed in the gas chromatography of steroids, namely isothermal analyses and linear temperature programmed (LTP) separations.[132] Under isothermal conditions, members of a simple homologous series e.g. n-alkanes, are eluted with a geometrical progression of retention times. The retention behaviour of a compound may be related to that of a series of n-alkanes using the method of Kováts;[75,268] under this system, which is based on a logarithmic scale, compounds are allocated retention index (I) units. In temperature programmed separations, the temperature of the GLC oven is increased continuously, at a predetermined heating rate, throughout the run. However, the carrier gas flow rate (at constant inlet pressure) decreases with increasing column temperature. Consequently, in temperature programmed analyses, continuous changes in two variables, flow rate and temperature, take place during the separation. During temperature programming, the elution of successive members of a simple homologous series is approximately linear with time, and the retention behaviour of compounds may again be related to that of a series of n-alkanes.

Isothermal procedures are used when relatively few components of a mixture are under study and when the separation can be accomplished in a short time. For the examination of urinary steroid profiles, temperature programmed separations (employing heating rates of 1 to 3^{o}/min) are necessary for the simultaneous determination of estrogen, androgen and corticosteroid metabolites, which have widely differing retention/





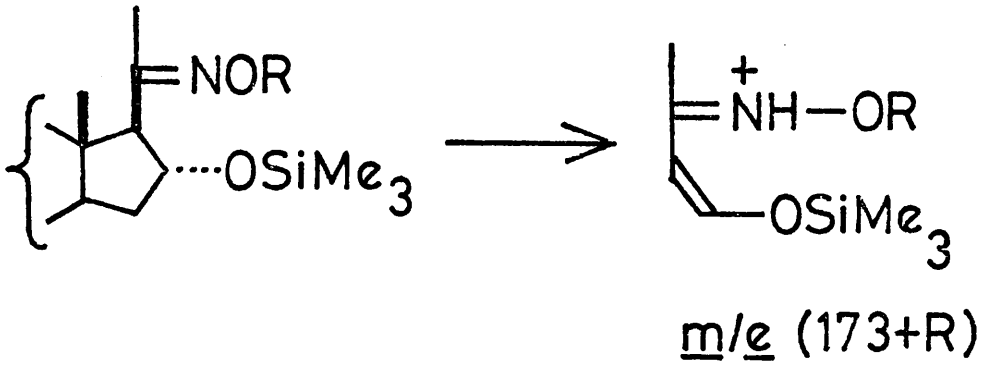


Fig.43: Characteristic ions in the spectra of oxime-TMS ether derivatives of urinary steroids.

retention characteristics. Caution should be used, however, when comparing retention indices of reference steroids, determined under isothermal conditions, with those of components of the urinary steroid profile, since retention indices frequently exhibit some degree of temperature dependence. Thus, I values determined isothermally commonly differ slightly from the corresponding retention indices measured under LTP conditions.[269] In a temperature programmed run, the retention indices of compounds eluted in the earlier stages are generally lower than the corresponding isothermal values; I values of compounds eluted in the later stages, however, agree well with those determined isothermally. This effect, which has also been observed for steroids chromatographed on capillary GLC solumns,[13] is evident from a comparison of the data presented in Appendix I (reference steroids) with those in Tables 19-21 (urinary components). Thus, while direct comparison of a retention index determined from an LTP separation with standard isothermal values is inadequate per se for structural identification, it is nevertheless very useful in narrowing down the range of possible alternatives.

5.3.2 Mass Spectral Fragmentations of iPO-TMS and BO-TMS Derivatives of Infant Urinary Steroids.

Steroidal O-benzyloximes afford characteristic ions in their mass spectra.[185,186] Cleavage of the N-O bond results in the formation of a prominent ion $(M-107)^+$, paralleling the loss of 31 a.m.u. in O-methyloxime, and of 87 a.m.u. in O-isopentyloxime derivatives. In addition, peaks at m/e (M-91) and m/e 91 are observed; the latter fragment, which corresponds to the reasonance - stabilised benzyl or tropylium ion (fig.43), often affords the base peak of the spectrum. The principal fragmentations/

fragmentations of O-isopentyloxime derivatives have been outlined above (section 4.3.2) and the origin of the ions at m/e (M-30), (M-44) and (M-45) in their spectra discussed.

In both BO-TMS and iPO-TMS spectra, prominent fragment ions result from the well-established losses of trimethylsilanol; elimination of this moiety may take place from the molecular ion to give a peak at m/e (M-90), or from suitable fragment ions. TMS ethers of steroids possessing the 3β-hydroxy-Δ^5 grouping, which is the predominant structure in steroids of the newborn infant, afford a characteristic ion at m/e 129.[52,132,175,178,270,271] The genesis of this fragment has been investigated by Diekman and Djerassi[206] and its structure is shown in fig.43. The mass spectra of BO-TMS and iPO-TMS derivatives may provide information about the location of oxime and neighbouring functional groups. Thus, oxime-TMS ethers of 16α-hydroxy-pregnan-20-ones afford an ion comprising the side-chain, together with C-16 and substituent groups[146] (fig.43) - this occurs at m/e 264 for BO-TMS ethers and at m/e 244 for iPO-TMS derivatives. Isomeric 16α, 17α and 21-hydroxy-pregnan-20-ones may therefore be distinguished from one another by examination of the mass spectra of their oxime - TMS ethers; the derivative of the 20,21-ketol gives rise to characteristic peaks at m/e 103 and (M-103) (section 3.3.3), while the 17α-hydroxyl group of the 20,17-ketol does not form a TMS ether under non-catalysed silylation conditions, and may be readily distinguished on this basis.

5.3.3 The Urinary Steroid Profile from a Normal Newborn Infant

By employing the differential hydrolysis procedure outlined above (section 5.2.1), two urinary steroid fractions are obtained, corresponding to metabolites excreted through conjugation with sulphuric or glucuronic acid. Following derivatisation, individual steroid profiles may be recorded from these "sulphate" and/

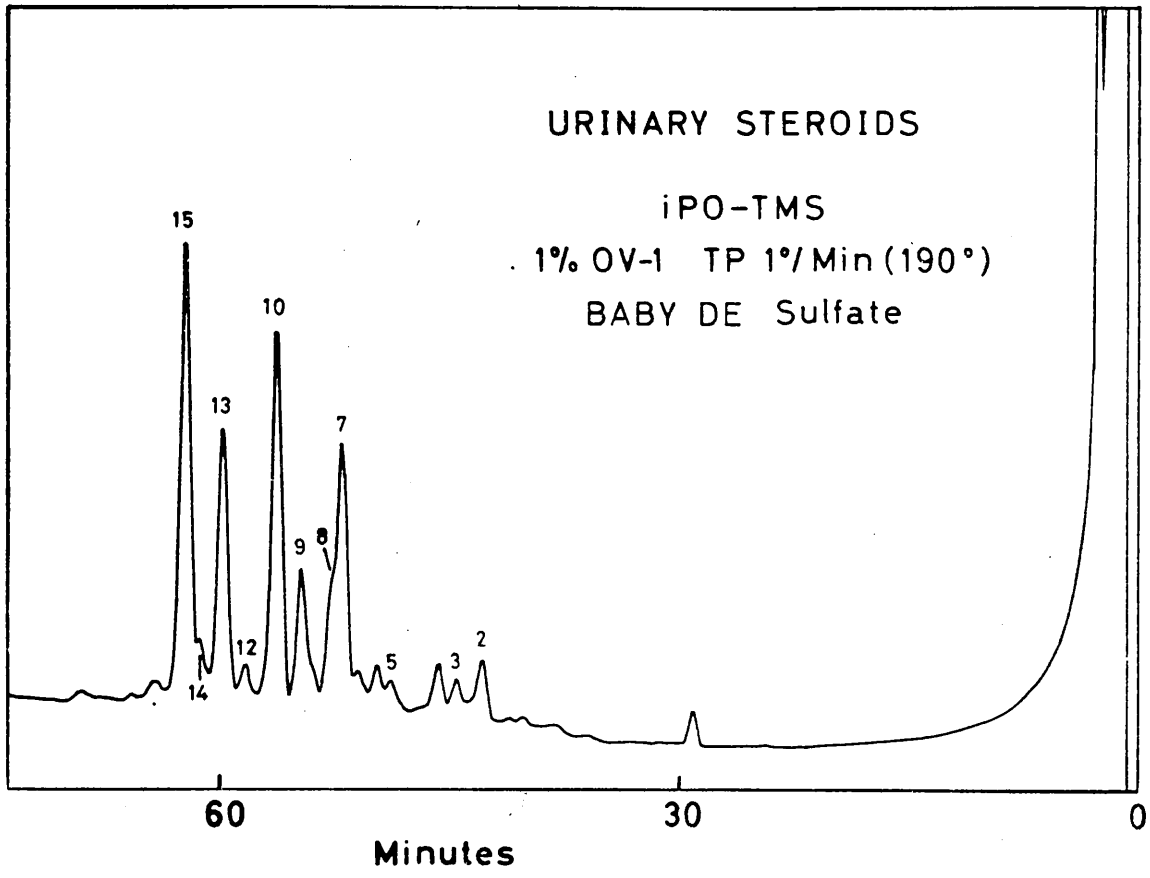


Fig. 44: Separation of urinary steroids from a normal newborn infant; sulphate fraction as iPO-TMS derivatives.

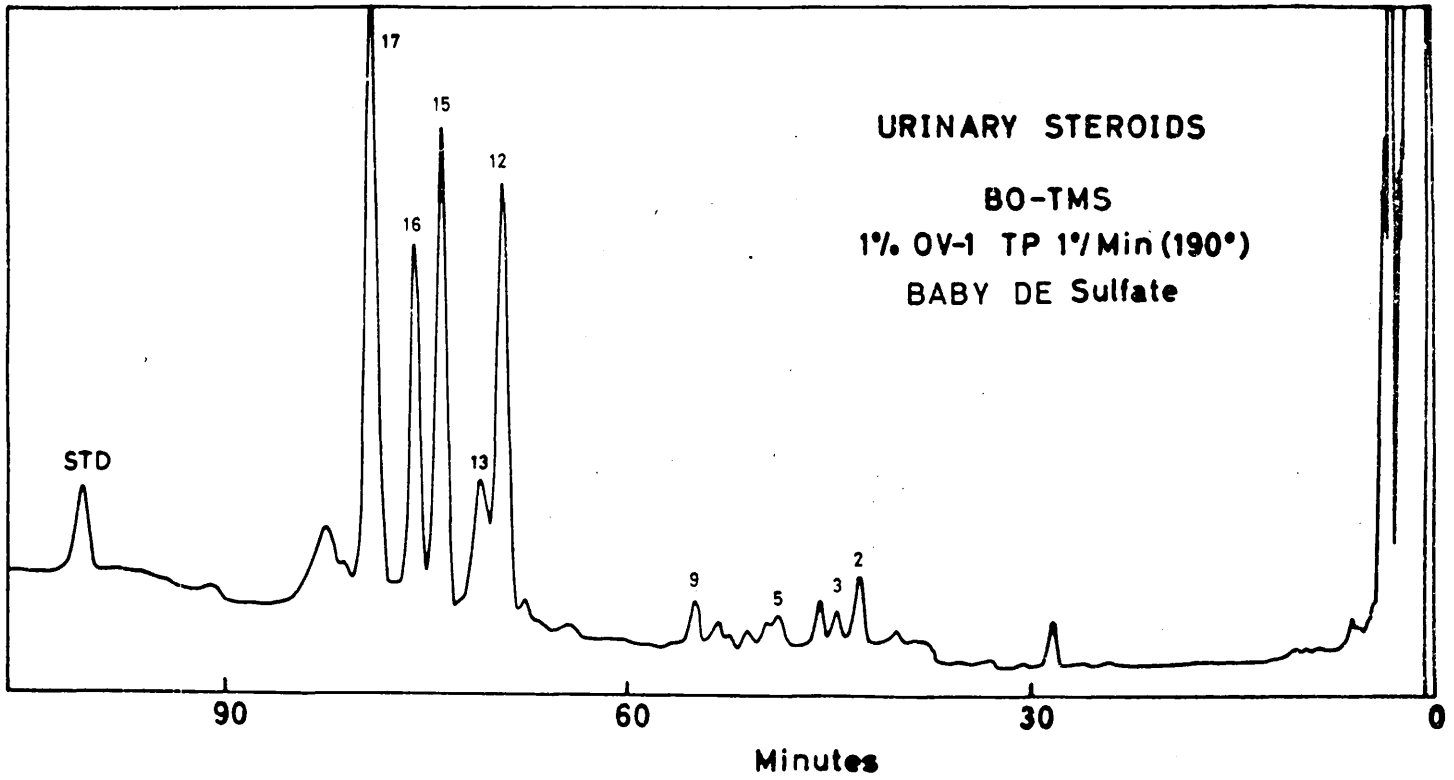


Fig.45: Separation of urinary steroids from a normal newborn infant; sulphate fraction as BO-TMS derivatives.

and "glucuronide" fractions. ("Mixed" conjugates, such as estriol 3-sulphate 16-glucuronide, would afford the free steroid in the "glucuronide" fraction from the above solvolysis/hydrolysis procedure.)

The urinary steroid excretion from a normal newborn infant is discussed below for the example of "Baby DE". A 24 h urine collection afforded, after cleavage of conjugates, 11.8mg of steroid extract; of this total, the sulphate fraction comprised 6.8 mg, while the glucuronide fraction accounted for 5.0mg. Samples of each fraction (0.5-1mg) were converted to iPO-TMS and BO-TMS derivatives, and the gas chromatograms obtained from these derivatised extracts are illustrated in figs. 44-47. Approximate retention parameters were measured for the major components of the mixture, and combined GC-MS yielded mass spectra for each main metabolite. Several GLC peaks appeared as a mixture of metabolites. Identity of retention parameters and mass spectra allowed tentative identification of some of these steroids, but others could only be partially characterised. For the positive identification of steroids in extracts, it is necessary to compare results from more than one type of stationary phase. However, in the exploratory work described in this chapter, urinary profiles were recorded only on a 1% OV-1 column.

Sulphate fraction: The iPO-TMS and BO-TMS urinary profiles are shown in figs.44 and 45, respectively. The pattern of peaks in the two chromatograms is closely similar, although a more pronounced separation between hydroxy - and ketosteroids is evident in the BO-TMS case. The longer analysis time - and, consequently, the higher final temperature - required in the BO-TMS run is also apparent in fig.45. The GC-MS data (Table 19) confirms the preponderance of metabolites with the 3β-hydroxy-Δ^5structure, the major component being 16α-hydroxypregnenolone. The/

iPO-TMS (fig.44)			BO-TMS (fig.45)			Structure Proposed for Parent Steroid
Spectrum No.	I_{OV-1}	$M^{+\cdot}$	Spectrum No.	I_{OV-1}	$M^{+\cdot}$	
2	2835	522	2	2835	522	5-A¹-3β,16α,17β-triol.
3	2870	504	3	2872	504	Estriol.
5	2955	610	5	2963	610	5-A¹-3β,15,16,17-tetrol.
-	-	-	9	3085	566	5β-P-3α,20α,21-triol-11-one.
-	-	-	9	3085	548	P"-triol.
-	-	-	9	3085	458	Cholesterol.
7	3023	533	12	3380	553	16α-Hydroxy-DHA.
8	3033	533	13	3410	553	16β-Hydroxy-DHA.
9	3078	621	13	3410	641	5-A¹-3β,11β,16-triol-17-one.
9	3078	550	-	-	-	5-P¹-3β,20α,21-triol.
10	3113	533	15	3470	553	16-Keto-AD.
12	3153	621	15	3470	641	5-A¹-3β,15,16-triol-17-one.
13	3184	649	16	3515	669	5-P¹-3β,11β,17α-triol-20-one.
14	3212	621	16	3515	641	5-A¹-3β,15, 17-triol-16-one.
14	3212	563	-	-	-	5α-P-3β,6α-diol-20-one.
15	3233	561	17	3580	581	16α-Hydroxypregnenolone.
-	-	-	17	3580	641	5-A¹-triol-one.

Table 19: Urinary steroids - Baby DE; Sulphate fraction.

The co-occurrence of the three isomeric androstenediolones, namely 16α-hydroxy-DHA, 16β-hydroxy-DHA and 16-ketoandrostenediol is regularly observed in newborn infant urine (section 5.1.1). While the role of 16α-hydroxy-DHA in the biosynthesis of estriol is well established, [245] an explanation for the presence of 16β-hydroxy-DHA in quantity in infant urine, and its use by the fetus, is not immediately apparent. Furthermore, 16β-hydroxy-DHA has been shown to undergo spontaneous isomerisation under certain conditions to the more stable 16-ketoandrostenediol.[205] This might therefore indicate that the latter compound is produced from 16β-hydroxy-DHA during extraction and purification, as an artefact.

Comparison of the GC-MS data for iPO-TMS and BO-TMS derivatives in Table 19 demonstrates the complementary nature of these two derivatives. The molecular weights of benzyloximes are 20 a.m.u. higher than those of the corresponding isopentyloximes, while the difference in their retention index values is around 335 units (on OV-1). These regularities assist in the identification of unknown metabolites, particularly where the "unknown" spectrum for one type of oxime derivative is complicated by the presence of a second "overlapping" compound in the same GLC peak. Such interference from accidental coincidences in retention time may often be eliminated by recording the corresponding profile for the other oxime derivative.

Glucuronide fraction: The pattern of compounds excreted as glucuronide conjugates is quite different from that occurring in the sulphate fraction. The major glucuronide components (Table 20) are ring-B saturated metabolites of the pregnane series, mainly pregnanetriols and dihydroxy - and trihydroxy-pregnan-20-ones. These compounds are presumably end-products of the metabolism of placental progesterone received before birth. Pregnanediolones and pregnanetriolones have been detected previously in various/

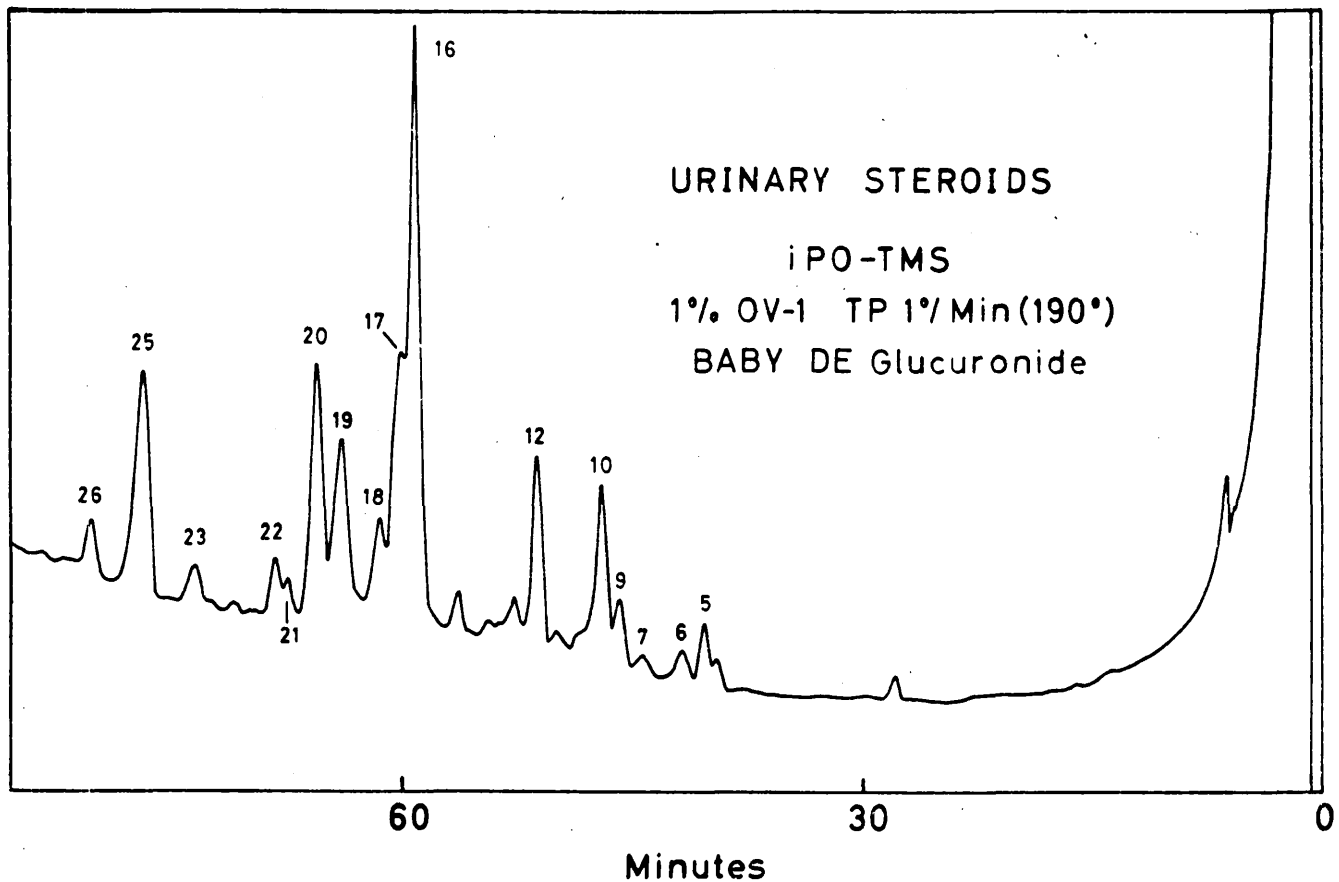


Fig.46: Separation of urinary steroids from a normal newborn infant; glucuronide fraction as iPO-TMS derivatives.

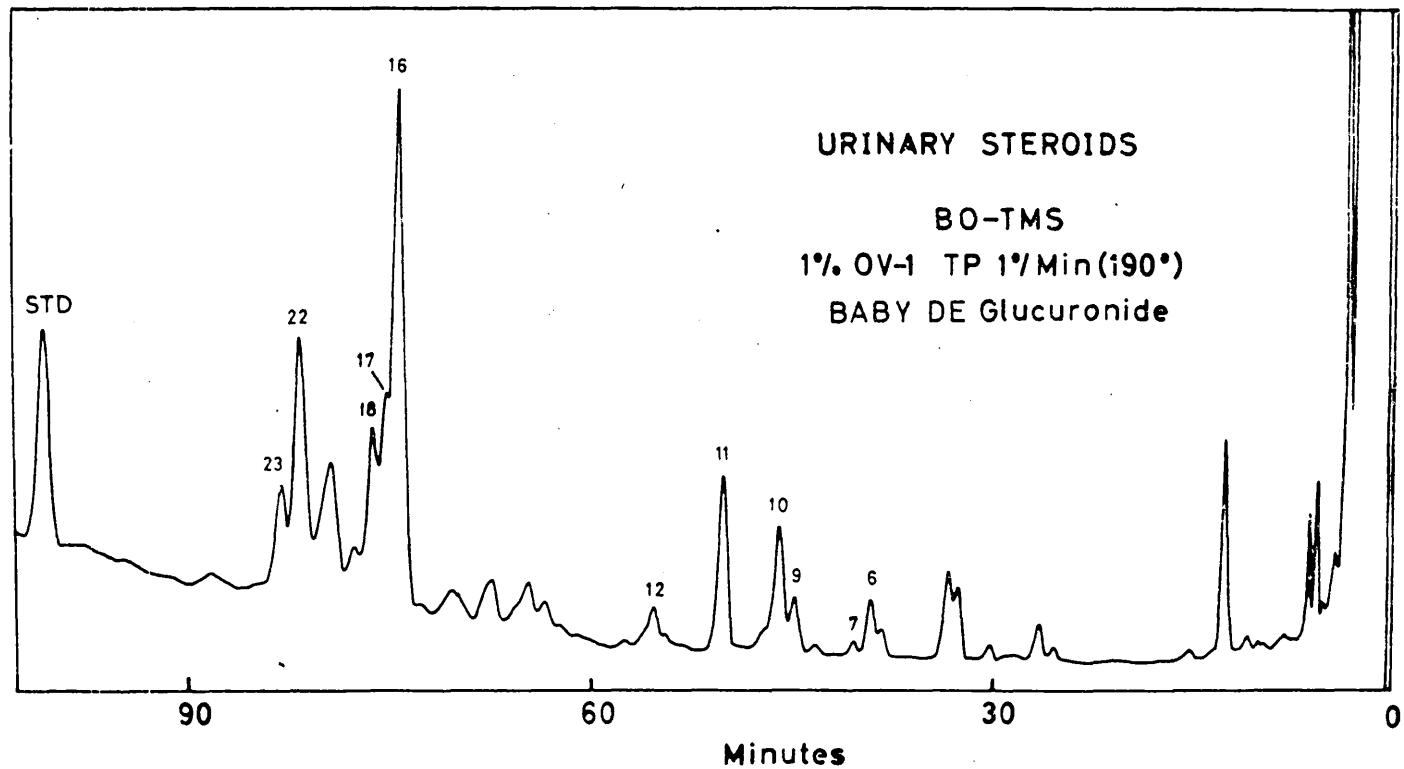


Fig.47: Separation of urinary steroids from a normal newborn infant; glucuronide fraction as BO-TMS derivatives.

iPO-TMS (fig.46)			BO-TMS (fig.47)			Structure Proposed for Parent Steroid
Spectrum No.	I_{OV-1}	$M^{+\cdot}$	Spectrum No.	I_{OV-1}	$M^{+\cdot}$	
5	2760	464	6	2758	464	5β-P-3α,20α-diol.
-	-	-	7	2785	522	5-A¹-3β,11β,17β-triol.
6	2788	552	-	-	-	5β-P-3α,17α,20α-triol.
7	2843	522	-	-	-	5-A¹-3β,16α,17β-triol.
9	2875	504	9	2875	504	Estriol.
10	2898	552	10	2900	552	5β-P-3α,6α,20β-triol.
12	2987	552	11	2987	552	5α-P-3β,16α,20α-triol.
-	-	-	12	3085	550	5-P¹-3β,20α,21-triol.
-	-	-	"	"	458	Cholesterol.
16	3152	563	16	3477	583	5β-P-3α,16α-diol-20-one.
17	3168	563	17	3495	583	5β-P-3α,6-diol-20-one.
18	3199	649	18	3515	669	5-P¹-3β,11β,17α-triol-20-one.
18	3199	563	18	3515	583	5β-P-3α,6-diol-20-one.
19	3252	665	-	-	-	THE
20	3285	563	22	3623	583	5α-P-3β,6-diol-20-one.
21	3318	651	23	3655	671	5β-P-3α,X,16α (or 17α) triol-20-one.
22	3338	651	-	-	-	"
23	3348	662	-	-	-	P-diol-3,20-dione.
25	3513	572	-	-	-	Hydroxyprogesterone.
26	3587	572	-	-	-	"

Table 20: Urinary steroids - Baby DE; Glucuronide fraction.

various biological fluids, e.g. the urine of normal[224] and anencephalic[272] infants, faeces from infants,[273] meconium,[274] fetal intestinal contents[275] and pregnancy urine.[276-279]

An interesting feature in the glucuronide iPO-TMS profile (fig.46) is the presence of three peaks at the upper end of the temperature programme (nos. 23,25, and 26), which afforded mass spectra with even-mass molecular ions ($M^{+\cdot}$ at 662, 572, and 572, respectively). These molecular weights correspond to the di-iPO diTMS ether of a pregnanedioldione (MW=662) and to the di-iPO TMS ether of a monohydroxy-pregnenedione (MW=572). Peaks corresponding to these compounds do not appear in the BO-TMS profile (fig.47) since steroidal di-benzyloximes are not detected owing to their high retention indices (>3800). A possible structure for the derivatives with MW572 would be a hydroxyprogesterone di-iPO TMS ether, since fetal tissues (particularly the adrenals) are known to hydroxylate progesterone at various sites, e.g. 6β[280], 16α[281] and 17α[280,282,283], and the resulting metabolites may undergo urinary excretion during the first few days of life. Furthermore, Δ^4-3-ketosteroid oximes afford two GLC peaks, corresponding to syn/anti isomers, and it appeared feasible that the two urinary components with MW 572 might represent isomers of this type, their mass spectra being very similar. However, the spectra of the "unknowns" differed from those of reference 16α-,17α-and 18-hydroxyprogesterone di-iPO TMS ethers. A further possibility for the $M^{+\cdot}$572 peaks was the di-iPO TMS ether of 16-ketopregnenolone, a derivative which also shows evidence of syn/anti isomers on GLC; this structure was also eliminated on the basis of its mass spectrum. Hence, these "unknown" diketonic steroids still await identification.

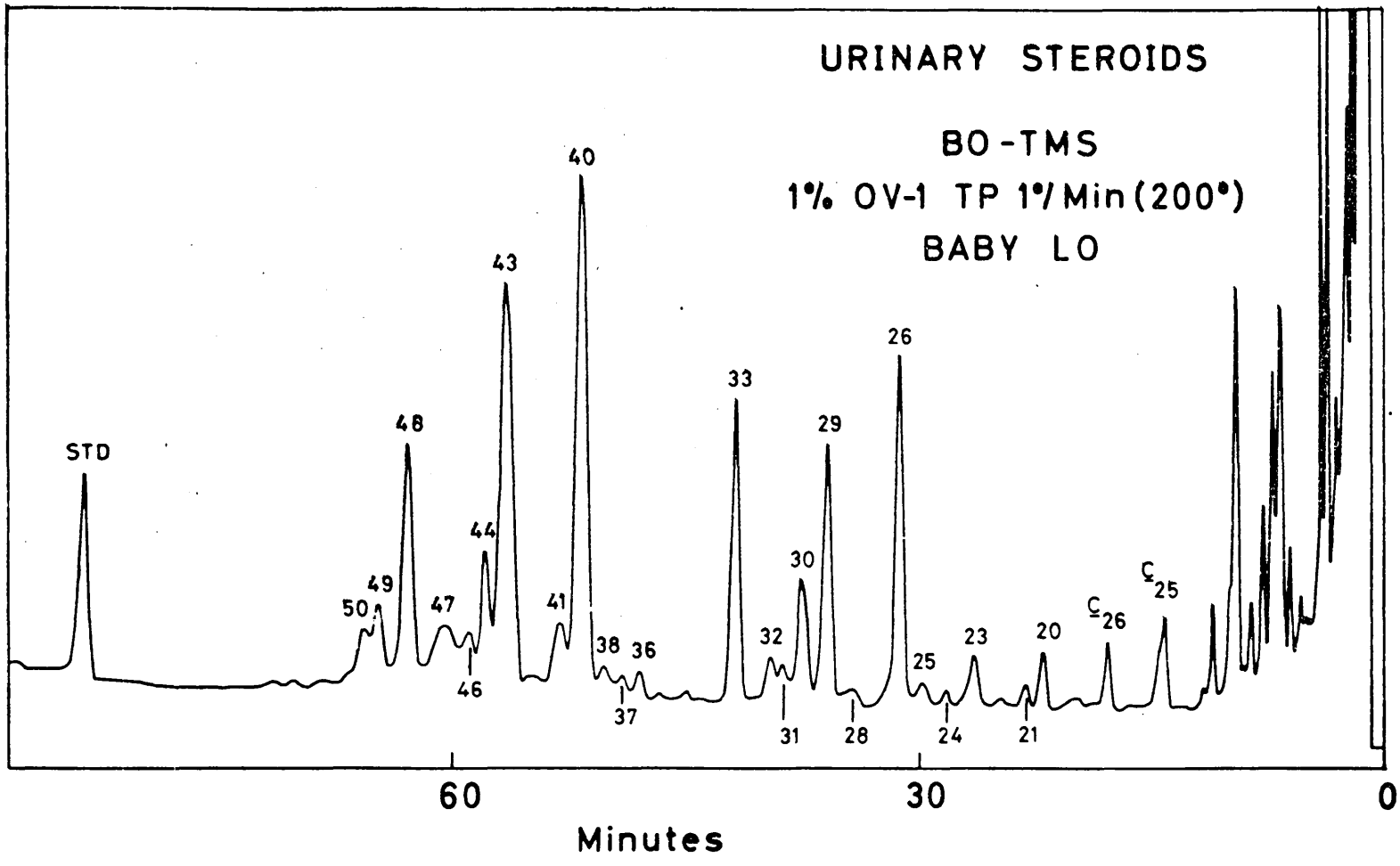


Fig.48: Separation of urinary steroids from an infant with a 21-hydroxylase defect; "total" steroids as BO-TMS derivatives.

Spectrum No.	I_{OV-1}	$M^{+\cdot}$	Proposed Structure
20	2706	522	5-A^1-triol triTMS
21	2730	550	5-P^1-triol triTMS
21	2730	464	5β-P-3α,20β-diol diTMS
23	2803	552	5β-P-3α,17α,20α-triol triTMS
24	2843	522	5-A^1-3β,16α,17β-triol triTMS
24	2843	566	11-Keto-P-triol triTMS
24	2843	478	5β-P-3α,20β-diol-11-one diTMS
25	2877	552	P-3,X,20-triol triTMS
26	2904	552	5β-P-3α,11β,20β-triol triTMS
26	2904	480	5β-P-3α,11β,20β-triol 3,20-di TMS *
28	2968	566	P-3,17α,20-triol-11-one triTMS
28	2968	494	P-3,17α,20-triol-11-one diTMS *
28	2968	552	5β-P-3α,11β,20α-triol triTMS
29	3005	494	5α-P-3, 17α,20-triol-11-one 3,20-diTMS *
30	3042	550	5-P^1-3β,11β,20β-triol triTMS
30	3042	478	5-P^1-3β,11β,20β-triol 3,20-diTMS *
31	3070	550	5-P^1-3β,20α,21-triol triTMS
31	3070	478	5-P^1-3β,11β,20α-triol triTMS
32	3088	458	Cholesterol TMS
33	3130	568	5α-P-3β,15 (or16), 17α,20-tetrol 3,15 (or 16), 20-triTMS*
36	3268	581	5-P^1-diol-one BO-diTMS
37	3290	481	11-Ketoetiocholanolone BO-TMS
40	3343	671	P-triol-one BO-triTMS
40	3343	583	5β-P-3β,X-diol-20-one BO-diTMS
41	3375	671	P-triol-one BO-triTMS
43	3442	671	" "
43	3442	597	5β-P-3β,X-diol-11,20-dione BO-diTMS
44	3476	671	P-3,X,21-triol-20-one BO-triTMS
46	3520	671	P-3,X16α(or 17α)-triol-20-one BO-triTMS
47	3530	671	" " "
47	3530	685	THE BO-triTMS
48	3756	581	16α-Hydroxypregnenolone BO-diTMS
49	3620	581	21-Hydroxypregnenolone BO-diTMS
50	3640	671	P-3, X, 16α (or 17α)-triol-20-one BO-tri TMS

Table 21: Urinary steroids - Baby LO; "Total" steroids as BO-TMS derivatives (cf. fig.48).

* These compounds resulted from incomplete silylation of the sample.

5.3.4 The Urinary Steroid Profile from an Infant with a 21-Hydroxylase Deficiency.

The urinary steroid profile (BO-TMS) of a "total" steroid extract from an infant ("Baby LO") with a defect in 21-hydroxylase is illustrated in fig.48. GC-MS data (Table 21) indicate, somewhat surprisingly, that 3β-hydroxy-Δ^5 steroids are relatively unimportant in this profile, while the principal components are ring-B saturated pregnane derivatives, e.g. pregnanetriols, pregnanediolones and pregnanetriolones. Comparison of the data in Tables 20 and 21, however, indicate that these ring B - reduced metabolites in the pathological profile are different from those encountered in the glucuronide fraction from the normal newborn infant. Numerous 11β-hydroxy - and 11-ketosteroids are also evident in the pathological sample; this feature is commonly observed in cases of 21-hydroxylase deficiency, when the effect of the 11β-hydroxylating enzyme is greatly enhanced. Pregnanetriol, a characteristic urinary steroid in older infants with this disorder, is present as a minor peak in the profile. 16α-Hydroxypregnenolone is the only prominent 3β-hydroxy-Δ^5steroid, although small amounts of androstenetriols and pregnenetriols are present.

Despite the deficiency of 21-hydroxylase, however, the GC-MS data indicate the presence of four steroids bearing a hydroxyl group at C-21 viz. 5-pregnene-3β,20α,21-triol, a pregnane-3, X, 21-triol-20-one, tetrahydrocortisone and 21-hydroxypregnenolone. This latter compound was also identified as a urinary steroid from an infant with a 21-hydroxylase defect by Mitchell et al.[260] Hence, the occurrence of 21-hydroxylated metabolites in the urine cannot, in itself, be taken as evidence that there is no inborn error in 21-hydroxylase.

5.3.5 CONCLUSIONS

Both iPO-TMS and BO-TMS ethers are useful derivatives for the study of urinary steroid profiles. iPO-TMS ethers do not afford the complete group separation of hydroxy - from ketosteroids obtained with the benzyloximes. However, di-isopentyloxime derivatives may be eluted under normal operating conditions, whereas the BO of steroidal diketones have retention times too long to permit their study by gas-phase methods. The two types of oximes give rise to individual fragmentation patterns, while structurally informative mass spectra with clear molecular ions, are usually obtained. The combined application of iPO-TMS and BO-TMS derivatives to the study of urinary steroid profiles thus provides useful complementary data which facilitates the identification of metabolites of unknown structure.

APPENDIX I

GAS CHROMATOGRAPHIC DATA

RETENTION INDICES ON 1% OV-1 (250°) AND 1% DEXSIL-300GC (275°) FOR TMS AND iPO-TMS DERIVATIVES OF REFERENCE STEROIDS

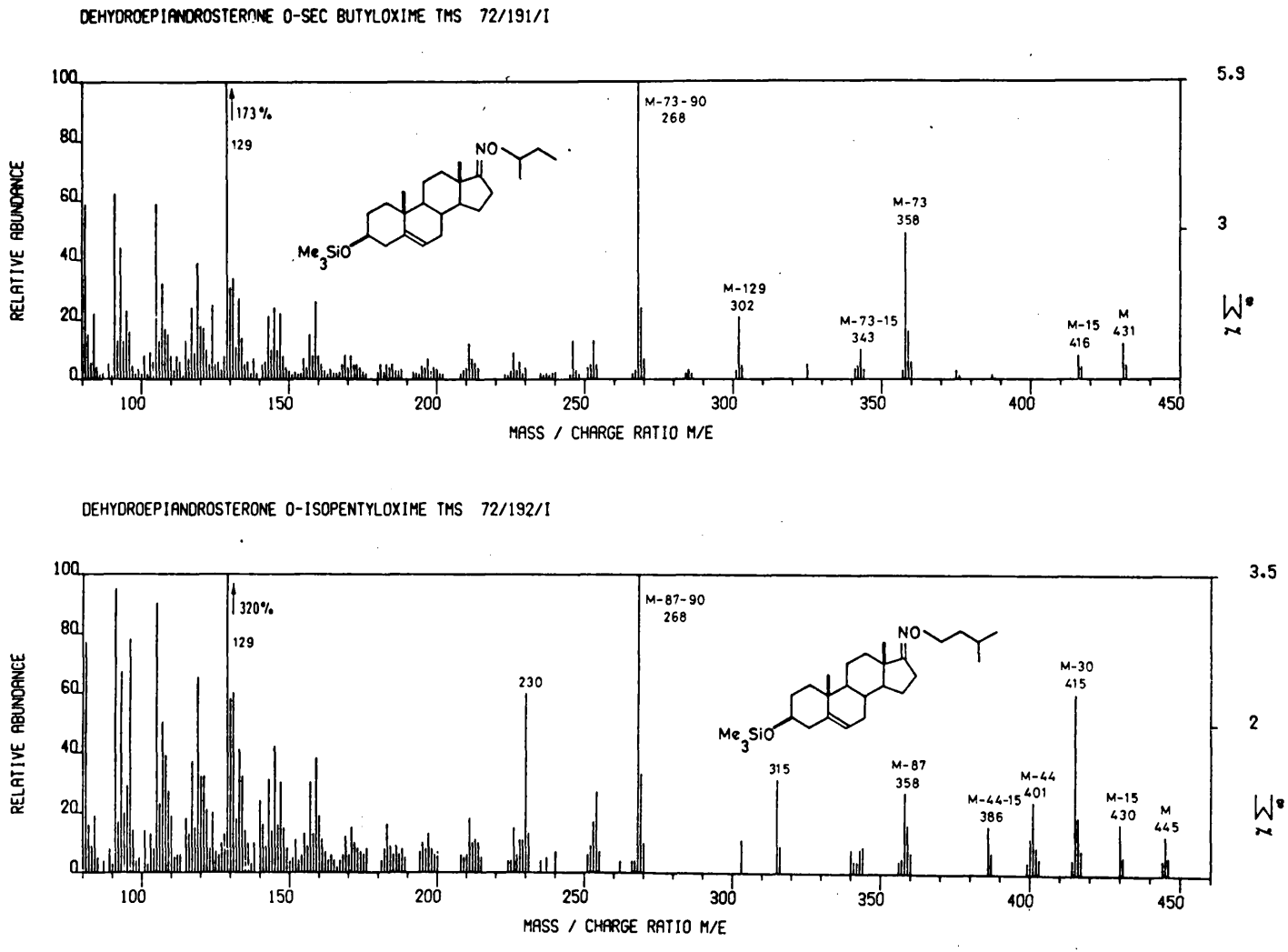
Parent Steroid	TMS or iPO-TMS		MW
	OV-1	Dexsil	
5-A^1-3β,17β-diol	2633	2697	434
5-A^1-3β,11β,17β-triol	2770	2885	522
5-A^1-3β,16α,17α-triol	2847	2872	522
5-A^1-3β,16α,17β-triol	2868	2897	522
5-A^1-3β,16β,17β-triol	2877	2925	522
5-A^1-3β-ol-17-one (DHA)	2948	3031	445
5-A^1-3β,11β-diol-17-one (11β-OH-DHA)	3100	3157	533
5-A^1-3β,16α-diol-17-one (16α-OH-DHA)	3070	3073	533
5-A^1-3β,16β-diol-17-one (16β-OH-DHA)	3104	3138	533
5-A^1-3β,17β-diol-16-one	3125	3178	533
5-A^1-3β,11β,16α-triol-17-one	3187	3162	621
5-P^1-3β,20β-diol	2830	2930	462
5-P^1-3β,16α,20α-triol	3008	3022	550
5-P^1-3β,17α,20α-triol	2989	3008	550
5-P^1-3β,17α,20β-triol	2941	2962	550
5-P^1-3β,20α,21-triol	3044	3117	550
5-P^1-3β-ol-20-one (Pregnenolone)	3132	3224	473
5-P^1-3β,16α-diol-20-one (16α-OH-Pregnenolone)	3253	3270	561
5-P^1-3β,17α-diol-20-one (17α-OH-Pregnenolone)	3200	-	561
5-P^1-3β,21-diol-20-one (21-OH-Pregnenolone)	3303	3308	561
5-P^1-3β,17α,21-triol-20-one	3307	3283	649
5-P^1-3β,11β,17α,21-tetrol-20-one	3452	3388	737
5-P^1-3β,16α,17α,21-tetrol-20-one	3480	3418	737
4-P^1-3,20-dione (Progesterone)	3502/ 3530	3650/ 3677	484
4-P^1-16α-ol-3,20-dione (16α-OH-Progesterone)	3600/ 3626	3688/ 3713	572
4-P^1-17α-ol-3, 20-dione (17α-OH-Progesterone)	3543/ 3587	3613/ 3665	572
4-P^1-18-ol-3,20-dione (18-OH-Progesterone)	3606/ 3640	3702/ 3738	572

Parent Steroid	TMS or iPO-TMS		MW
	OV-1	Dexsil	
5-P[1]-3β-ol-16, 20-dione (16 Keto-pregnenolone)	3560/ 3600	3627/ 3665	572
5,16-P"-3β-ol-20-one	3093	-	471
5α-A-3α-ol-17-one (Androsterone)	2861	2958	447
5α-A-3β-ol-17-one (Epiandrosterone)	2938	3054	447
5β-A-3α-ol-17-one (Etiocholanolone)	2863	2977	447
5α-A-3α,11β-diol-17-one (11β-OH-Androsterone)	3000	3356	535
5β-A-3α,11β-diol-17-one (11β-OH-Etiocholanolone)	3000	3367	535
5α-A-3α-ol-11,17-dione (11-Ketoandrosterone)	2958	3110	461
5β-A-3α-ol-11, 17-dione (11-Ketoetiocholanolone)	2958	2948	461
5β-P-3α,20α-diol (Pregnanediol)	2796	3050	464
5β-P-3α,6α,20β-triol	2887	2892	552
5β-P-3α,17α,20α-triol (Pregnanetriol)	2824	2851	552
5β-P-3β,20β,21-triol	3034	3060	552
5β-P-3α,11β,17α,20α,21-pentol (Cortol)	3144	3063	728
5β-P-3α,11β,17α,20β,21-pentol (β-Cortol)	3100	3010	728
5β-P-3α-ol-20-one (Pregnanolone)	3059	3167	475
5β-P-3α,6α-diol-20-one	3187	3196	563
5α-P-3β,11β-diol-20-one	3304	3378	563
5α-P-3β,16α-diol-20-one	3320	3295	563
5β-P-3β,16α-diol-20-one	3202	3177	563
5α-P-3β,17α-diol-20-one	3217	3244	563
5β-P-3α,17α-diol-20-one	3050	3018	563
5β-P-3α,21-diol-20-one (TH-DOC)	3172	3192	563
5α-P-3α,11β,21-triol-20-one (allo-THB)	3284	3242	651
5α-P-3β,11β,21-triol-20-one	3546	3428	651
5β-P-3α,11β,21-triol-20-one (THB)	3264	3273	651
5β-P-3α,17α,21-triol-20-one (THS)	3146	3096	651
5α-P-3α,11β,17α,21-tetrol-20-one (allo-THF)	3292	3194	739
5β-P-3α,11β,17α,21-tetrol-20-one (THF)	3270	3188	739
5β-P-3α,17α,20α,21-tetrol-11-one (Cortolone)	3083	3060	654

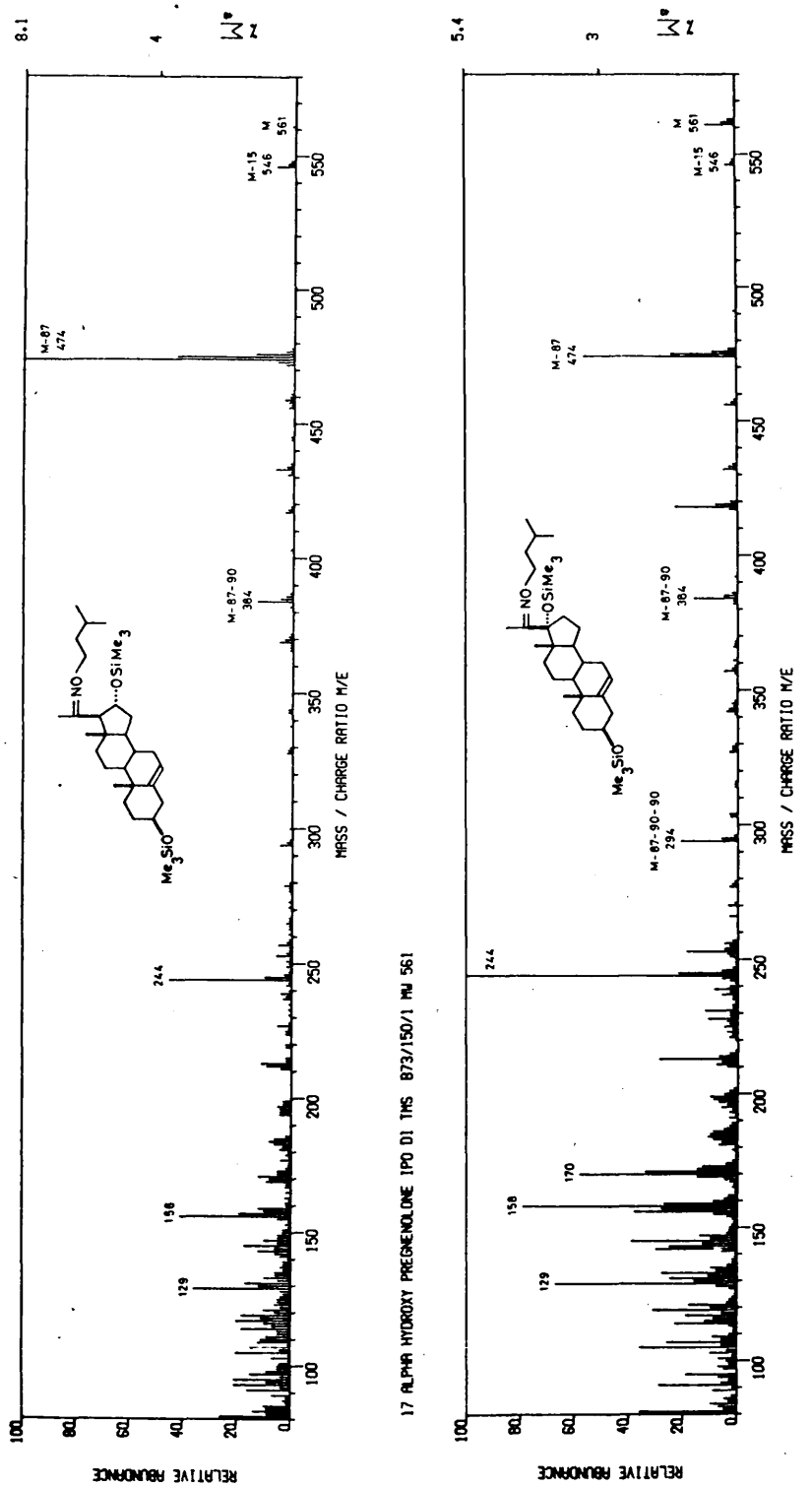
Parent Steroid	TMS or iPO-TMS		MW
	OV-1	Dexsil	
5β-P-3α,17α,20β,21-tetrol-11-one (β-Cortolone)	3104	3104	654
5α-P-3α,21-diol-11,20-dione (allo-THA)	3305	3377	577
5β-P-3α,21-diol-11, 20-dione (THA)	3283	3340	577
5β-P-17α,21-diol-3,20-dione (DHS)	3502/ 3535	3494/ 3538	662
5α-P-3α,17α,21-triol-11,20-dione (allo-THE)	3234	3237	665
5β-P-3α,17α,21-triol-11, 20-dione (THE)	3234	3236	665
Cholesterol	3100	3218	458
1,3,5(10)-Estratriene-3, 16α,17β-triol (Estriol)	2904	2943	504

APPENDIX II

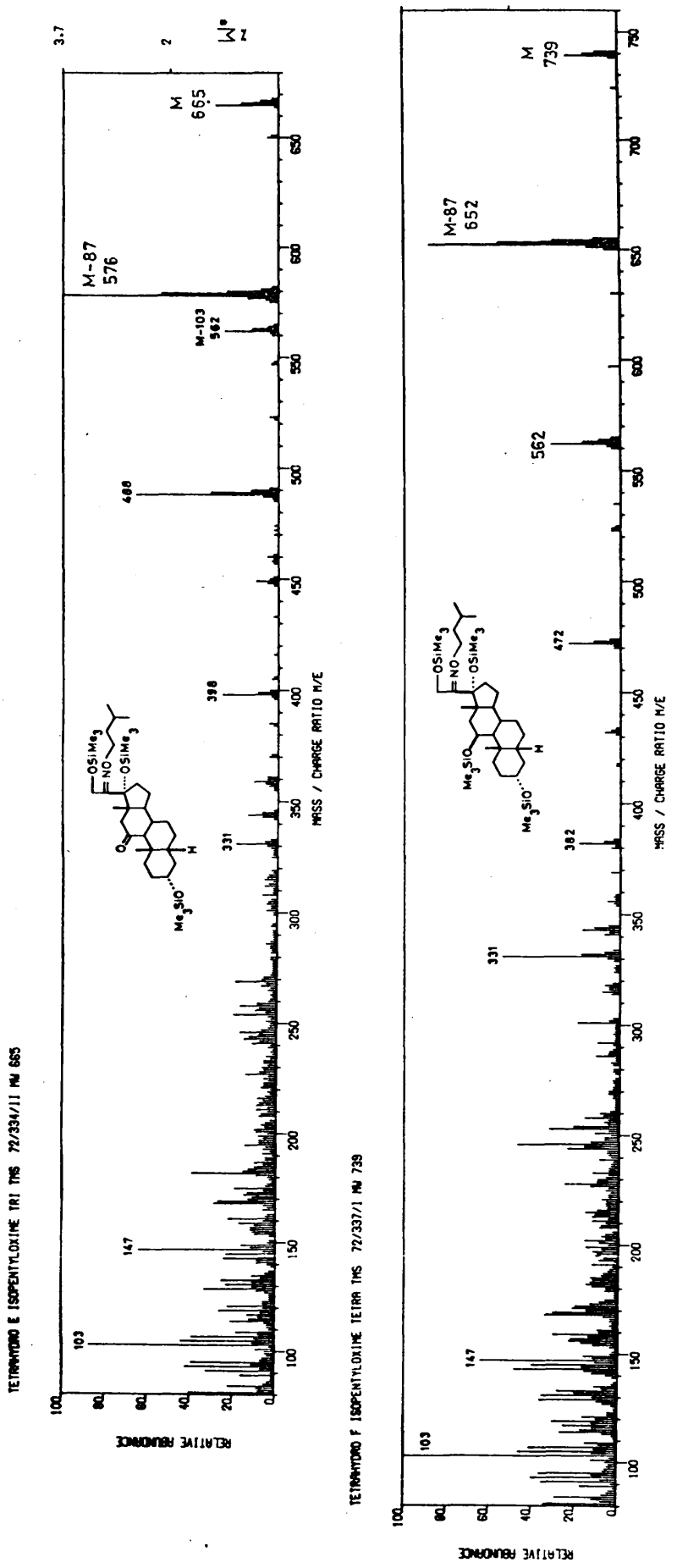
MASS SPECTRA



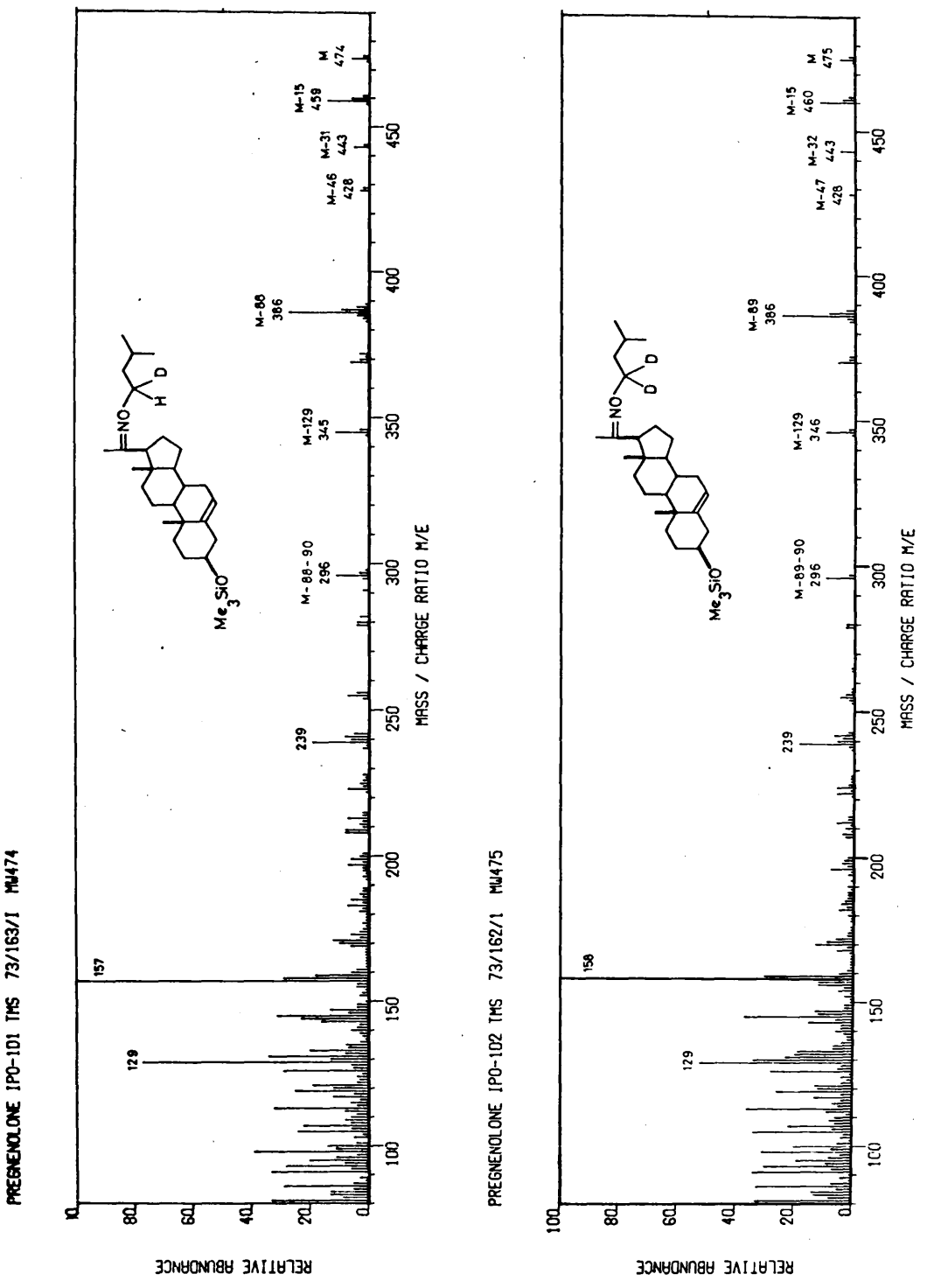
Mass spectra (70eV) of dehydroepiandrosterone O-sec-butyloxime TMS (upper) and dehydroepiandrosterone O-isopentyloxime TMS (lower).



Mass spectra (70eV) of 16α-hydroxypregnenolone iPO-diTMS (upper) and 17α-hydroxypregnenolone iPO-diTMS (lower).



Mass spectra (70eV) of tetrahydrocortisone (THE) iPO-triTMS (upper) and tetrahydrocortisol (THF) iPO-tetraTMS (lower).



Mass spectra (70eV) of pregnenolone iPO (1-d_1)-TMS (upper) and pregnenolone iPO (1-d_2)-TMS (lower).

APPENDIX III

THE CRYSTAL STRUCTURE OF CYCLIC BORONATE ESTERS FROM PREGNANE-17α,20,21-TRIOLS.

INTRODUCTION

Cyclic boronate esters are useful derivatives for the gas-phase characterisation of compounds with various suitably orientated bifunctional groupings.[147] The derivatives may be simply and rapidly prepared, in essentially quantitative fashion, while hydrolysis of the resulting boronate - and regeneration of the parent compound - may often be achieved by the addition of several equivalents of propane-1, 3-diol.[284]

Corticosteroids (with the exception of 20, 17-ketols) may be stabilised for gas chromatography as their boronate esters.[94,95] These derivatives possess good GLC properties, and provide informative mass spectra which usually exhibit molecular ions in moderate abundance. The degree of stability of corticosteroid boronates towards hydrolysis depends primarily on the nature of the steroid side-chain involved.[95] Thus, while boronates of the dihydroxyacetone type of side-chain may be hydrolysed relatively easily, those of corticosteroid 17α,20,21-triols are much more resistant to hydrolysis, and are amenable to study by TLC and to further derivatisation reactions. The structure of these triol boronates may be formulated as either a five-membered (17α,20- or 20,21-boronate) or six-membered (17α,21-boronate) cyclic ester, since examples of each type are encountered in boronates of sugar 1,2,3-triols.[285,286] Evidence which favours a six-membered structure was obtained by Anthony *et al.*,[147] who acetylated the *n*-butylboronate of 17α,20β,21-trihydroxy-4-pregnen-3-one, and then selectively hydrolysed the boronate moiety. The resulting triolone monoacetate differed in its chromatographic behaviour from the 21-acetate, prepared by direct acetylation, and was tentatively regarded as being the 20-monoacetate derivative. Further evidence for a 17α,21-boronate/

boronate structure was provided by Brooks and Harvey[95] who demonstrated that the tert.-butylboronates of both 17α,20α,21- and 17α,20β,21-trihydroxy-4-pregnen-3-one were oxidised with dimethyl sulphoxide - acetic anhydride to the corresponding ester of 17α,21-dihydroxy-4-pregnene-3,20-dione. However, attempts to effect the reverse reaction, by reduction of the steroidal dihydroxyacetone boronate with sodium borohydride, were unsuccessful, due to hydrolysis of the boronate ester. Certain of the mass spectrometric data for triol boronates appear to indicate a five-membered ester (section 3.3.3), although the possibility that this arises during fragmentation must be borne in mind.

In view of the difficulties involved in the study of this problem by chemical and chromatographic methods (notably the ease of hydrolysis of derivatives and of functional group migrations) it was decided to investigate the crystal structure of 17α,20,21-triol boronates by X-ray anaylsis. Introduction of a "heavy atom" was accomplished by the use of p-bromophenylboronic acid, and the crystalline derivatives were prepared as outlined below.

Preparation of p-Bromophenylboronate Esters

17α,20α,21-Trihydroxy-4-pregnen-3-one (50.2mg; 144μmole) was treated with p-bromophenylboronic acid (28.9 mg; 144μmole) and the mixture dissolved in AnalaR ethyl acetate (10ml). The reaction was allowed to proceed at room temperature overnight (16h), and the product examined by analytical TLC (mobile phase: ethyl acetate/ chloroform (1:1 v/v)). This indicated that the triolone (R_f 0.07) had been completely converted to the p-bromophenylboronate ester (R_f 0.60). The solution was then taken to dryness under nitrogen, affording a pale yellow oil. Vacuum sublimation was carried out at 255°/0.02mm Hg, using a "cold finger" apparatus, and yielded the p-bromophenylboronate as a colourless glass (m.p. 126-130°) Elemental analysis (Dr. A. Bernhardt, Mikroanalytisches Laboratorium, Elbach, West Germany) gave:- C: 63.00; H: 6.52; Br: 15.79%.

(Calc. for $C_{27}H_{34}O_4BBr$: C: 63.19; H:6.68; Br: 15.57%.) A portion of this material was crystallised from ethyl acetate, by allowing the solution to evaporate slowly to a small volume over a period of several days. The colourless prisms so obtained had m.p. 204-212°.

17α,20β,21-Trihydroxy-4-pregnen-3-one (52.4mg); 150μmole) was similarly treated with p-bromophenylboronic acid (30.2mg; 150μmole). Analysis of the reaction product by analytical TLC (solvent system as above) again showed quantitative conversion of the triolone (R_f 0.09) to its boronate ester (R_f 0.60). Vacuum sublimation (249°/0.02mm Hg) afforded the p-bromophenylboronate as a amorphous solid, which crystallised slowly from ethyl acetate as colourless prisms, m.p. 245-249°. (Found, C: 63.23; H: 6.56; Br: 15.81%. Calc. for $C_{27}H_{34}O_4BBr$: C: 63.19; H: 6.68; Br: 15.57%.)

RESULTS

Only the crystals obtained from 17α,20β,21-trihydroxy-4-pregnen-3-one were suitable for X-ray analysis purposes. The crystals of this compound were very small, however, and the limited data which were collected led to high standard deviations for the parameters involved. Dr. P.D. Cradwick was responsible for solving the structure by Patterson techniques and by applying tangent formula calculations to the phases of partial structure factors. Dr. P.J. Cox completed the refinement of the atom parameters.

Crystal data

Molecular formula	$C_{27}H_{34}O_4BrB$
Molecular weight	513.3 a.m.u.
Crystal system	orthorhombic
Space group	$P2_12_12_1$
Cell dimensions	$\underline{a}$= 16.847(28)Å
	$\underline{b}$= 13.294(18) Å
	$\underline{c}$= 11.082(10)Å
Cell volume (u)	2482.0 $Å^3$
Density (obs.)	1.42 $g.cm^{-3}$
Density (Calc.)	1.37 $g.cm^{-3}$
Molecules per unit cell (z)	4
No. electrons per unit cell ($F_{(000)}$)	1072
Linear absorption coefficient, μ(MoKα)	17.90 cm^{-1}
R	0.136

The boronate ester ring was found to be six-membered, incorporating the oxygen functions at C-17α and C-21, while the 20β-hydroxyl group remained free. The structure and stereochemistry of the molecule are shown in figs. 49 and 50, respectively; some/

some relevant bond lengths and angles are cited in Tables 22 and 23. Mean plane calculations on the boronate moiety indicated that C(17), C(21), O(2), O(3) and the boron atom were all coplanar, while C(20) lay 0.69Å out of the plane; this corresponds to a "flattened" boat conformation of the boronate ester. The unusually high stability of pregnane-17α, 20, 21-triol boronate derivatives towards hydrolysis might be taken to indicate some degree of interaction between the unfilled p-orbital of the boron and a lone pair on the oxygen attached to C(20) (O(4) in fig.50). However, the distance between these two atoms was found to be 3.05Å, which is very close to the Van der Waals contact distance of 3.07Å. Consequently an interaction of the above type cannot be inferred on the basis of the X-ray data.

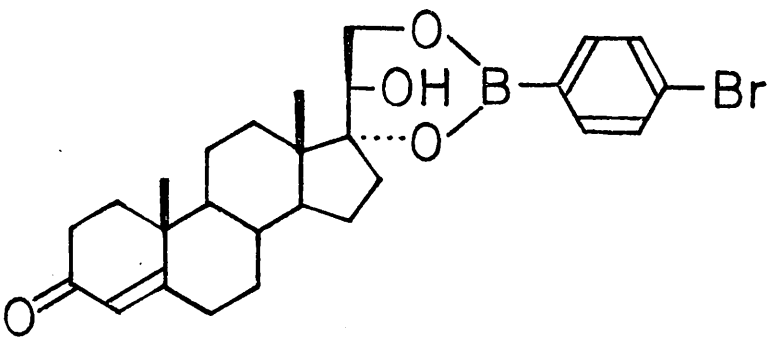


Fig. 49: The structure of the p-bromophenylboronate ester obtained from 17α,20β,21-trihydroxy-4-pregnen-3-one.

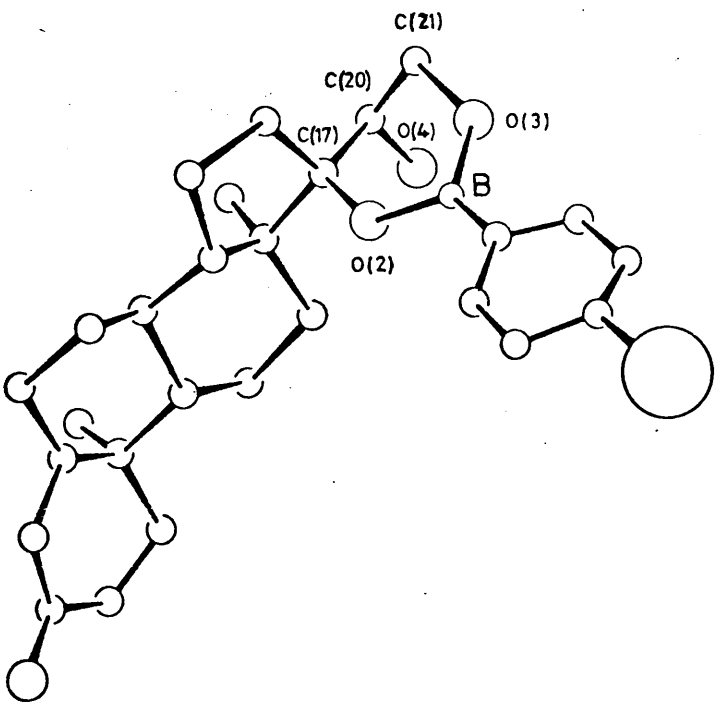


Fig. 50: A general view of the compound in fig.49, indicating three-dimensional structure.

Table 22 Intramolecular bonded distances and estimated standard deviations.[287]

C(17)	-	O(2)	1.46 (5)
O(2)	-	B	1.38 (6)
B	-	O(3)	1.42 (6)
O(3)	-	C(21)	1.59 (5)
C(17)	-	C(20)	1.57 (6)
C(20)	-	C(21)	1.45 (6)
C(20)	-	O(4)	1.47 (6)

Table 23 Valency angles (degrees) and estimated standard deviations.[287]

C(17)	-	O(2) - B	121 (3)
O(2)	-	B - O(3)	121 (2)
B	-	O(3) - C(21)	121 (3)
O(2)	-	C(17)- C(20)	109 (3)
C(17)	-	C(20)- C(21)	114 (3)
C(20)	-	C(21)-O(3)	102 (2)
C(17)	-	C(20)-O(4)	109 (3)
C(21)	-	C(20)-O(4)	113 (3)

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