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ANALYSIS OF $C_{19}O_3$ STEROIDS BY THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

The separation of twenty-six saturated and two unsaturated $C_{19}O_3$ steroids has been studied by thin-layer chromatography on silica gel F_{254}, with seven mobile phases, and by gas–liquid chromatography on packed columns with four stationary phases; combination of both techniques permitted separation of all the test compounds. The mass spectra of the steroids were obtained by gas chromatography–mass spectrometry and are presented. Fragmentation processes have been studied, and characteristic ions that may be used for multiple ion detection or lead to identification of biologically produced $C_{19}O_3$ steroids are discussed.

INTRODUCTION

Enzymic hydroxylation leading to 5α-reduced $C_{19}O_3$ steroids has been studied in numerous human, murine and canine tissues, including liver[1,2] and prostate[3,4] and perianal glands[5]. Hydroxylation has been reported to occur at the 2ξ-position[1–3], the 6ξ-position[3,4,6], the 7ξ-position[2,4,5], the 15α-position[2] and the 16α-position[1,6], the naturally occurring $C_{19}O_2$ substrates being mainly 3-oxo-, 17β-hydroxy- or 3ξ,17β-dihydroxy-compounds[1,6]. This study of $C_{19}O_3$ steroids has therefore been limited to those having oxygen functions at the positions cited above. Most of these compounds are not commercially available, and their reported identification is based on comparison of their chromatographic mobilities[1–3] and mass spectra[1,2,4–6] with those of custom-made reference compounds. Few studies of their behaviour in thin-layer chromatography (TLC) and gas–liquid chromatography (GLC), or of their fragmentation during mass spectrometry (MS), have been systematically reported[1,7]. Accordingly, we have obtained reference compounds, established conditions for the resolution of mixtures and sought criteria for identification. In this paper we report the mobilities of some $C_{19}O_3$ steroids in TLC and their retention indices in GLC, and present their mass spectra.

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EXPERIMENTAL

Unsaturated steroids

Testosterone and androst-5-ene-3β,16α,17β-triol were purchased from Sigma (St. Louis, Mo., U.S.A.). Androst-5-ene-3β,16β,17β-triol and 5α-androst-2-en-17-one were obtained from Steraloids (Pawling, N.Y., U.S.A.). The androst-5-ene-3β,17β-diol was obtained from Merck (Darmstadt, G.F.R.).

Saturated $C_{19}O_3$ steroids

3β,11β-Dihydroxy-5α-androstan-17-one was a commercial sample from Ikapharm (Ramat Gan, Israel), and 17β-hydroxy-5α-androstane-3,6-dione and 3β-hydroxy-5α-androstane-6,17-dione were obtained from Dr. P. Ofner (Boston, Mass., U.S.A.). 3β-Hydroxy-5α-androstane-7,17-dione was a gift from Sir Ewart R. H. Jones (Oxford University, Oxford, Great Britain) and was also obtained by incubating isoandrosterone (Merck) with *Rhizopus nigricans*[8]. 5α-Androstane-3,6,17-trione and 5α-androstane-3,7,17-trione were obtained by oxidation of 17β-hydroxy-5α-androstane-3,6-dione and 3β-hydroxy-5α-androstane-7,17-dione, respectively, with chromium trioxide. 6α,17β-Dihydroxy-5α-androstan-3-one and 3α,17β-dihydroxy-5α-androstan-6-one were from Sir Ewart R. H. Jones, who also kindly provided some 3β,17β-dihydroxy-5α-androstan-6-one. This last-named steroid was also obtained by specific reduction of the 3-oxo-group in 17β-hydroxy-5α-androstane-3,6-dione with lithium tri-*tert*.-butoxyaluminate (Merck) for 15 min at 0°. Further reduction by lithium tetrahydroaluminate of the three dihydroxy-steroids mentioned above yielded 5α-androstane-3β,6α,17β-triol, 5α-androstane-3α,6β,17β-triol and its 3β-epimer, respectively. 3β,17β-Dihydroxy-5α-androstan-7-one was prepared by a modification of the method of Valcavi *et al.*[9]. The diacetoxy-derivative of androst-5-ene-3β,17β-diol was prepared and specifically oxidized at the 7-position with sodium chromate (Merck). Hydrogenation on platinum dioxide of the resulting 3β,17β-diacetoxy-androst-5-en-7-one yielded a mixture of 3β,17β-diacetoxy-5α-androstan-7α-ol and the epimeric 7β-ol. Before separation of these two epimers, 3β,17β-diacetoxy-5α-androstan-7-one was prepared by oxidation of a portion of the mixture with chromium trioxide. Hydrolysis carried out with methanolic sodium hydroxide produced the expected 3β,17β-dihydroxy-5α-androstan-7-one. The remaining mixture of 3β,17β-diacetoxy-5α-androstan-7α-ol and the epimeric 7β-ol was resolved by column chromatography on alumina, and each of the isolated and crystallized epimers was hydrolyzed with methanolic sodium hydroxide. Thus, 5α-androstane-3β,7α,17β-triol and its 7β-epimer were obtained; the identity of these steroids was checked by GLC and GLC–MS in comparison with authentic samples provided by Dr. J. C. Orr (St Johns, Newfoundland, Canada).

3β,7β-Dihydroxy-5α-androstan-17-one, its 7α-epimer and 3β,6α-dihydroxy-5α-androstan-17-one were obtained from incubations of isoandrosterone with *Rhizopus nigricans*[8]. Reduction of these compounds with potassium tetrahydroborate yielded major quantities of 5α-androstane-3β,7β,17β-triol, its 7α-epimer and 5α-androstane-3β,6α,17β-triol, respectively.

5α-Androstane-2β,3β,17β-triol and 5α-androstane-2α,3α,17β-triol were kindly provided by Dr. Y. Collet (Collège de France, Paris). The synthesis of 5α-androstane-2β,3α,17β-triol was carried out as follows: 5α-androst-2-en-17-one was reduced with

potassium tetrahydroborate; 2α,3α-epoxy-5α-androstan-17β-ol was then obtained by the action of *m*-chloroperbenzoic acid (overnight in benzene medium), and opening of the epoxy-ring with 2 *N* sulphuric acid (Shopee *et al.*[10]) yielded 5α-androstane-2β,3α,17β-triol.

5α-Androstane-3β,11β,17β-triol and its 3α-epimer were obtained by reduction with potassium tetrahydroborate of 3β,11β-dihydroxy-5α-androstan-17-one (Ikapharm), and its 3α-epimer (Sigma), respectively.

5α-Androstane-3β,15α,17β-triol was obtained from Dr. E. M. Chambaz (Grenoble, France), and 5α-androstane-3β,16α,17β-triol and its 3α-epimer were purchased from Steraloids.

Thin-layer chromatography

Thin-layer plates pre-coated with silica gel 60 F_{254} (Merck) were used in this study. Steroids were applied in 20–30 μg amounts and the plates were developed in unsaturated tanks with different solvent systems (see Table I). Visualization of the steroids on the chromatograms was achieved by spraying with sulphuric acid–methanol (7:3, v/v) and heating at 120° for 15 min.

Derivative formation

All derivatives were prepared from 50 μg of free steroid. The trimethylsilyl (TMS) derivatives were prepared (in 30 min at 60° or overnight at room temperature) after addition to the dry steroid of 100 μl of NO-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 10 μl of pyridine. In some instances, 10 μl of trimethylchlorosilane (TMCS) were added to the reaction mixture; TMCS behaves as a catalyst[11] in the formation of TMS derivatives, and thus residual free hydroxyl-groups in the steroid molecules were eliminated.

Gas–liquid chromatography

Carlo Erba (Milan, Italy) GI-452 and Pye Unicam (Cambridge, Great Britain) 104-84 chromatographs, each equipped with flame ionization detectors, were used, with nitrogen as carrier gas (40 ml/min). The operating conditions and stationary phases are given in Table II.

Gas–liquid chromatography–mass spectrometry

A double-beam MS-30 mass spectrometer (AEI, Manchester, Great Britain) coupled by a silicone-membrane separator to a Pye Unicam 104 gas chromatograph was used. As previously described[12], the chromatograph was fitted with a glass capillary column (60 m × 0.3 mm) coated with OV-101 and operated at 265°. Helium was used as carrier gas and added at the column exit before the molecular separator at a make-up flow of 20–25 ml/min[12]. The steroid derivatives were injected through an all-glass solid injector[13]; the temperatures of the molecular separator, introduction line and ion source were 215°, 250° and 200°, respectively. In the mass spectrometer, the energy of the bombarding electrons was 24 eV and the ionizing current was 100 μA; mass spectra were taken at a scan speed of 3 sec per decade at a resolution of 1000.

RESULTS

Thin-layer chromatography

All TLC was carried out in the presence of testosterone, and the R_F values of the steroids (relative to that of testosterone) are presented in Table I. From visualization of the steroids on the chromatograms, we estimated that sufficient separation between steroid pairs was achieved when these values differed by at least 0.05. In all the systems tested, resolution between 3α,17β-dihydroxy-5α-androstan-6-one and 3β,17β-dihydroxy-5α-androstan-7-one was unsatisfactory; the same was true for 5α-androstane-3α,16α,17β-triol and its 3β-epimer and for 5α-androstane-3β,6α,17β-triol and the analogous 3β,7α,17β- and 3β,15α,17β-triols.

TABLE I

TLC OF TESTOSTERONE AND $C_{19}O_3$ STEROIDS

The mobile phases used were: A, chloroform–ethyl acetate (4:1, v/v) once; B, chloroform–ethyl acetate (4:1, v/v) twice; C, benzene–ethyl acetate (3:1, v/v) twice; D, cyclohexane–ethyl acetate (3:2, v/v) twice; E, cyclohexane–ethyl acetate–ethanol (45:45:10, v/v/v) once; F, chloroform–ethanol (9:1, v/v) once; G, benzene–ethanol (9:1, v/v) three times.

Steroid	*Mobility (relative to testosterone) in mobile phase*						
	A	*B*	*C*	*D*	*E*	*F*	*G*
Testosterone	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Androst-5-ene-3β,16α,17β-triol	0.03	0.07	0.06	0.25	0.56	0.68	0.35
Androst-5-ene-3β,16β,17β-triol	0.35	0.40	0.42	0.77	0.80	0.80	0.51
5α-Androstane-3,6,17-trione	1.06	1.00	1.10	0.90	0.91	1.08	1.32
5α-Androstane-3,7,17-trione	1.28	1.11	1.08	0.90	0.90	1.12	1.31
3β-Hydroxy-5α-androstane-6,17-dione	0.31	0.30	0.23	0.34	0.69	0.75	0.68
3β-Hydroxy-5α-androstane-7,17-dione	0.48	0.50	0.34	0.51	0.71	0.80	0.71
17β-Hydroxy-5α-androstane-3, 6-dione	0.60	0.60	0.51	0.64	0.79	0.83	0.80
3α,17β-Dihydroxy-5α-androstan- 6-one	0.17	0.19	0.16	0.37	0.72	0.74	0.56
3β, 6α-Dihydroxy-5α-androstan-17-one	0.04	0.06	0.02	0.10	0.45	0.60	0.48
3β, 7α-Dihydroxy-5α-androstan-17-one	0.10	0.12	0.05	0.21	0.61	0.64	0.49
3β, 7β-Dihydroxy-5α-androstan-17-one	0.11	0.13	0.07	0.19	0.54	0.61	0.46
3β,11β-Dihydroxy-5α-androstan-17-one	0.32	0.34	0.43	0.80	0.93	0.74	0.64
3β,17β-Dihydroxy-5α-androstan- 6-one	0.17	0.19	0.13	0.30	0.65	0.65	0.54
3β,17β-Dihydroxy-5α-androstan- 7-one	0.21	0.23	0.20	0.40	0.68	0.69	0.58
6α,17β-Dihydroxy-5α-androstan- 3-one	0.18	0.21	0.17	0.43	0.73	0.63	0.55
5α-Androstane-2α, 3α,17β-triol	0.10	0.12	0.11	0.38	0.65	0.55	0.53
5α-Androstane-2β, 3α,17β-triol	0.06	0.08	0.09	0.33	0.67	0.45	0.48
5α-Androstane-2β, 3β,17β-triol	0.17	0.20	0.19	0.57	0.83	0.63	0.56
5α-Androstane-3α, 6β,17β-triol	0.14	0.13	0.12	0.35	0.69	0.63	0.51
5α-Androstane-3α,11β,17β-triol	0.06	0.08	0.09	0.36	0.76	0.56	0.50
5α-Androstane-3α,16α,17β-triol	0.02	0.03	0.03	0.14	0.50	0.41	0.39
5α-Androstane-3β, 6α,17β-triol	0.04	0.06	0.02	0.13	0.46	0.43	0.42
5α-Androstane-3β, 6β,17β-triol	0.05	0.07	0.04	0.25	0.60	0.50	0.47
5α-Androstane-3β, 7α,17β-triol	0.06	0.05	0.03	0.17	0.48	0.40	0.38
5α-Androstane-3β, 7β,17β-triol	0.06	0.06	0.04	0.21	0.50	0.46	0.42
5α-Androstane-3β,11β,17β-triol	0.05	0.06	0.07	0.35	0.70	0.47	0.53
5α-Androstane-3β,15α,17β-triol	0.09	0.06	0.04	0.15	0.46	0.43	0.39
5α-Androstane-3β,16α,17β-triol	0.05	0.07	0.07	0.21	0.57	0.54	0.42

Gas–liquid chromatography

Retention indices on four stationary phases were calculated and expressed according to Kovàts[14] for each steroid derivative and the free triketones. The operating conditions and data are shown in Table II.

TABLE II

RETENTION INDICES OF TMS DERIVATIVES FROM TWENTY-SIX $C_{19}O_3$ STEROIDS AND TWO TRIONES IN GLC

The GLC systems used were as follows: I, 2.1-m × 4-mm column with 3.08% of OV-101 at 263°; II, 2.1-m × 4-mm column with 1.82% of DEXSIL at 263°; III, 3.1-m × 3-mm column with 1.57% of OV-7 at 270°; IV, 2.1-m × 4-mm column with 3.08% of SP-2250 at 280°. In each systen the inert support was Gas-Chrom Q (100–120 mesh).

Steroid	*Retention index in system*			
	I	*II*	*III*	*IV*
Androst-5-ene-3β,16α,17β-triol	2889	2886	2899	2941
Androst-5-ene-3β,16β,17β-triol	2914	2923	2922	2972
5α-Androstane-3,6,17-trione	2789	3127	3080	3346
5α-Androstane-3,7,17-trione	2584	2740	2808	2797
3β-Hydroxy-5α-androstane-6,17-dione	2826	3063	3022	3230
3β-Hydroxy-5α-androstane-7,17-dione	2790	2987	2962	3137
17β-Hydroxy-5α-androstane-3, 6-dione	2920	3093	3026	3210
3α,17β-Dihydroxy-5α-androstan- 6-one	2793	2893	2872	2970
3β, 6α-Dihydroxy-5α-androstan-17-one	2755	2861	2861	2972
3β, 7α-Dihydroxy-5α-androstan-17-one	2685	2777	2767	2862
3β, 7β-Dihydroxy-5α-androstan-17-one	2798	2910	2883	2998
3β,11β-Dihydroxy-5α-androstan-17-one	2849	2968	2932	3043
3β,17β-Dihydroxy-5α-androstan- 6-one	2899	3024	2984	3100
3β,17β-Dihydroxy-5α-androstan- 7-one	2860	2956	2948	—
6α,17β-Dihydroxy-5α-androstan- 3-one	2856	2990	2938	3037
5α-Androstane-2α, 3α,17β-triol	2755	2749	2741	2760
5α-Androstane-2β, 3α,17β-triol	2737	2714	2713	2730
5α-Androstane-2β, 3β,17β-triol	2869	2881	2854	2882
5α-Androstane-3α, 6β,17β-triol	2697	2675	2672	2701
5α-Androstane-3α,11β,17β-triol	2743	2740	2739	2771
5α-Androstane-3α,16α,17β-triol	2802	2802	2773	2807
5α-Androstane-3β, 6α,17β-triol	2854	2817	2823	2856
5α-Androstane-3β, 6β,17β-triol	2775	2748	2775	2810
5α-Androstane-3β, 7α,17β-triol	2692	2654	2688	2716
5α-Androstane-3β, 7β,17β-triol	2836	2844	2833	2868
5α-Androstane-3β,11β,17β-triol	2854	2864	2851	2887
5α-Androstane-3β,15α,17β-triol	2846	2811	2833	2849
5α-Androstane-3β,16α,17β-triol	2898	2896	2907	2945

Problems in the formation of tri-TMS derivatives were encountered with steroids bearing a 6β-hydroxyl-group (5α-androstane-3α,6β,17β-triol and its 3β-epimer). Thus, di-TMS derivatives with a free 6β-hydroxyl-group, as confirmed by MS, were obtained when TMCS was not added to the BSTFA–pyridine reaction mixture. Retention indices on OV-101 were 2784 and 2876 for 3α,17β-di-TMS-5α-androstan-6β-ol and the 3β,17β-di-TMS-5α-androstan-6β-ol, respectively. Derivatization of all hydroxyl-groups was obtained when TMCS was present in the reaction

mixture. No enol-trimethylsilyl ethers were formed when 3-oxo- or 17-oxo-steroids were subjected to silylation with BSTFA–TMCS (10:1) at room temperature.

In contrast to TLC, the separation of 3α,17β-dihydroxy-5α-androstan-6-one from 3β,17β-dihydroxy-5α-androstan-7-one, and of 5α-androstane-3β,7α,17β-triol from 5α-androstane-3β,6α,17β-triol and 5α-androstane-3β,15α,17β-triol, (as TMS derivatives), was achieved on all the stationary phases tested; nevertheless, separation of androst-5-ene-3β,6α,17β-triol from 5α-androstane-3β,15α,17β-triol was incomplete with each phase.

In all systems, steroids oxygenated in the axial configuration (2β-, 3α-, 6β- and 7 α-positions) yielded lower retention times than the respective 2α-, 3β-, 6α- and 7β-epimers with an equatorial configuration. The greater flatness of the latter molecules may favour interactions with the stationary phase and so explain the difference in mobilities. In addition, 16α-oxygenated steroids had lower retention times than their 16β-epimers. Such a phenomenon may be explained by co-participation of the 16β- and 17β-radicals in interaction with the stationary phase.

Resolution of 3β-hydroxy-5-ene from 3β-hydroxy-5α-reduced steroids was difficult on packed columns of limited efficiency. In contrast, molecules differing by only 9 index units were completely resolved on glass capillary columns. In the course of this work, capillary columns coated with OV-101 were used for different separations and identifications in synthesis-reaction mixtures.

Mass spectra of $C_{19}O_3$ steroids

All mass spectra of hydroxylated steroids were those of TMS derivatives. Only fragments with a relative intensity of 5% or more are shown in the figures.

5α-Androstane-3,6,17-trione and 5α-androstane-3.7,17-trione (Fig. 1). As expected, both molecules show a molecular ion at $m/e = 302$, but the fragmentation patterns are quite different. Thus, fragments at $m/e = 255$ and 137 are characteristic of 5α-androstane-3,7,17-trione, and those at $m/e = 273$ and 123 only occur with 5α-androstane-3,6,17-trione.

The mass spectra from these two triones were discussed by Obermann *et al.*[15] and by Hammerschmidt *et al.*[16]. Our results agree with the characteristic frag-

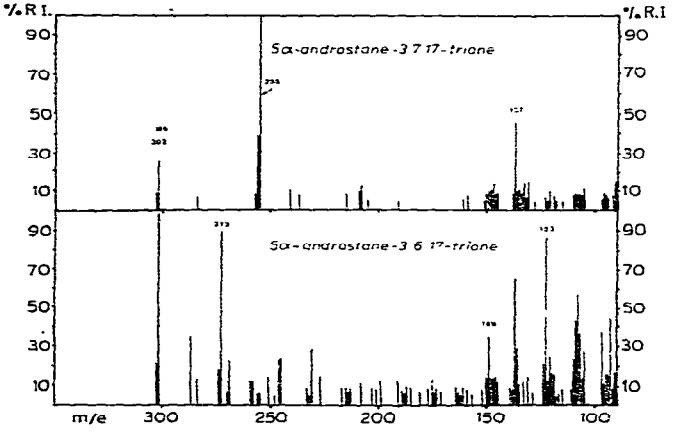


Fig. 1. Mass spectra of 5α-androstane-3,7,17-trione and 5α-androstane-3,6,17-trione.

mentation patterns of the two molecules, but there are differences as regards the relative intensities of low-mass ions. The cited authors explained formation of the ion at $m/e = 273$ by loss of H_2O and CHO[15]. Our finding of another ion at $m/e = 274$ with a relative intensity higher than expected for an isotopic ion suggests the loss of C_2H_5 (M−29) from carbons 1 and 2 of 5α-androstane-3,6,17-trione and loss of CO (M−28) from the 6-oxo-function[16]. Such fragmentation would be characteristic of a trione incorporating a 6-oxo-group. On the other hand, the fragmentation mechanism described by Djerassi *et al.*[17] for 6-oxosteroids may be involved in the formation of fragments at $m/e = 137$ and 109.

3β-Hydroxy-5α-androstane-7,17-dione, 3β-hydroxy-5α-androstane-6,17-dione and 17β-hydroxy-5α-androstane-3,6-dione (Fig. 2). The molecular ion for the TMS derivatives of these three molecules is at $m/e = 376$, but the fragmentation patterns and relative intensities of the fragments are different. The base peaks are at $m/e = 329$ (M−47), 361 (M−15) and 129 for the respective compounds.

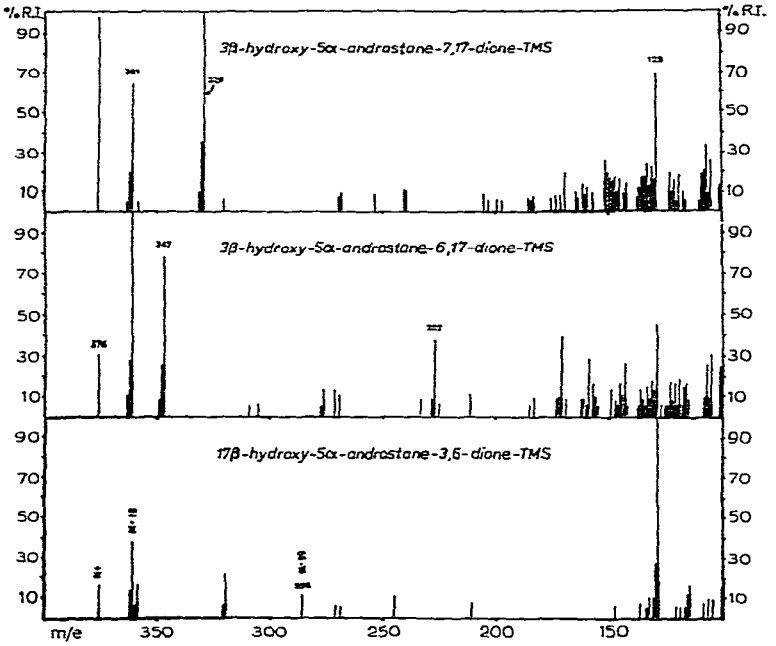
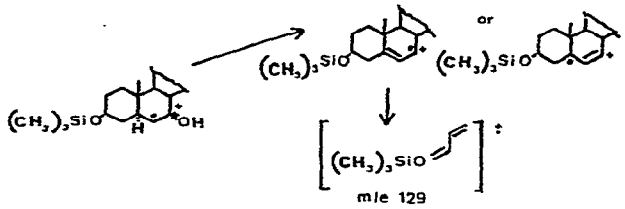


Fig. 2. Mass spectra of TMS derivatives of 3β-hydroxy-5α-androstane-7,17-dione, 3β-hydroxy-5α-androstane-6,17-dione and 17β-hydroxy-5α-androstane-3,6-dione.

Comparison between the spectra of 3β-hydroxy-5α-androstane-7,17-dione and that of 5α-androstane-3,7,17-trione shows that the same fragment (at 47 a.m.u.) is lost by both molecules; this implies that such loss is characteristic of the 7,17-dione function. A one-step mechanism for the loss of H_2O and CHO· is suggested by the metastable ion found at 287.8 a.m.u., which corresponds to the transition $m/e = 376$ to $m/e = 329$ for the dioxosteroid. Formation of an ion at $m/e = 129$ in the spectrum of 3β-hydroxy-5α-androstane-7,17-dione may arise through 7-enol formation and elimination of water[18] as follows:



Such structures agree with elimination of an ion of 129 a.m.u. according to the classical mechanism[19]. Further, it was noted that loss of silanol, which corresponds to the M—90 ion ($m/e = 286$) is very low when compared with that of the TMS derivatives of 5α-androstanediols.

The spectrum of 3β-hydroxy-5α-androstane-6,17-dione differs from that of the analogous 7,17-dione through elimination of an ion of 29 a.m.u. ($m/e = 347$) and an ion at $m/e = 227$. It was also noted that the ion at $m/e = 139$ described by Hammerschmidt and Spiteller[16] as characteristic of 3β-hydroxy-5α-androstane-6,17-dione was not present. The base peak at $m/e = 129$ in the spectrum of 17β-hydroxy-5α-androstane-3,6-dione may result from ring-D fragmentation when an -O-TMS group is in the 17β-position[19]; other fragments can be related with loss of that -O-TMS. Some previously described fragments[19] were not observed.

3β,6α-Dihydroxy-5α-androstan-17-one, 3α,17β-dihydroxy-5α-androstan-6-one and its 3β-epimer and 6α,17β-dihydroxy-5α-androstan-3-one. These four molecules bear oxygen functions in the 3-, 6- and 17-positions of the androstane skeleton and differ only by the positions of the two hydroxyl-groups and that of the ketone (Fig. 3). When analyzed as TMS derivatives, they all exhibit a molecular ion at $m/e = 450$ and have similar fragmentation patterns, but different relative intensities. Thus, the relative intensities of the ions at $m/e = 345$ [M—(90+15)] and $m/e = 270$ [M—(2×90)] differ significantly for 3α,17β-dihydroxy-5α-androstan-6-one, its 3β-epimer and 6α,17β-dihydroxy-5α-androstan-3-one, even though these three molecules have the same reference fragment at $m/e = 129$.

In contrast, 3β,6α-dihydroxy-5α-androstan-17-one has its base peak at $m/e = 271$. Fragmentation in this steroid is induced by both the 17-oxo- and the 3β- and 6α-O-TMS groups. The base peak at $m/e = 271$ [M—(90+89)] is unusual for a di-TMS derivative of the androstane series. In this instance, elimination of silanol seems to follow a 1–3 mechanism[20]: elimination from the 6α-position involves the H atom on carbon 8, which is a β-position; the distance is then too large for silanol liberation and results in elimination of -O-TMS as a free radical (89 a.m.u.). Such a mechanism creates an unsaturated site in ring B and favours the formation of the ion at $m/e = 129$, positive charges being located either on the M—129 fragment (231 a.m.u.) or on the "lost" ion at $m/e = 129$.

The 17-ketone is characterized by some fragments involving loss of water, *viz.*, 271 → 253, and 231 → 213 (metastable at $m/e = 196.9$), or ethylene elimination, *viz.*, 271 → 243 (ref. 21).

The spectra of the three other steroids are similar. As expected, the base peak is at $m/e = 129$ (see ref. 19). We cannot explain the M—56 ion that also appears in spectra of TMS derivatives of 5α-androstanediols.

3β,7α-Dihydroxy-5α-androstan-17-one, its 7β-epimer and 3β,17β-dihydroxy-5α-

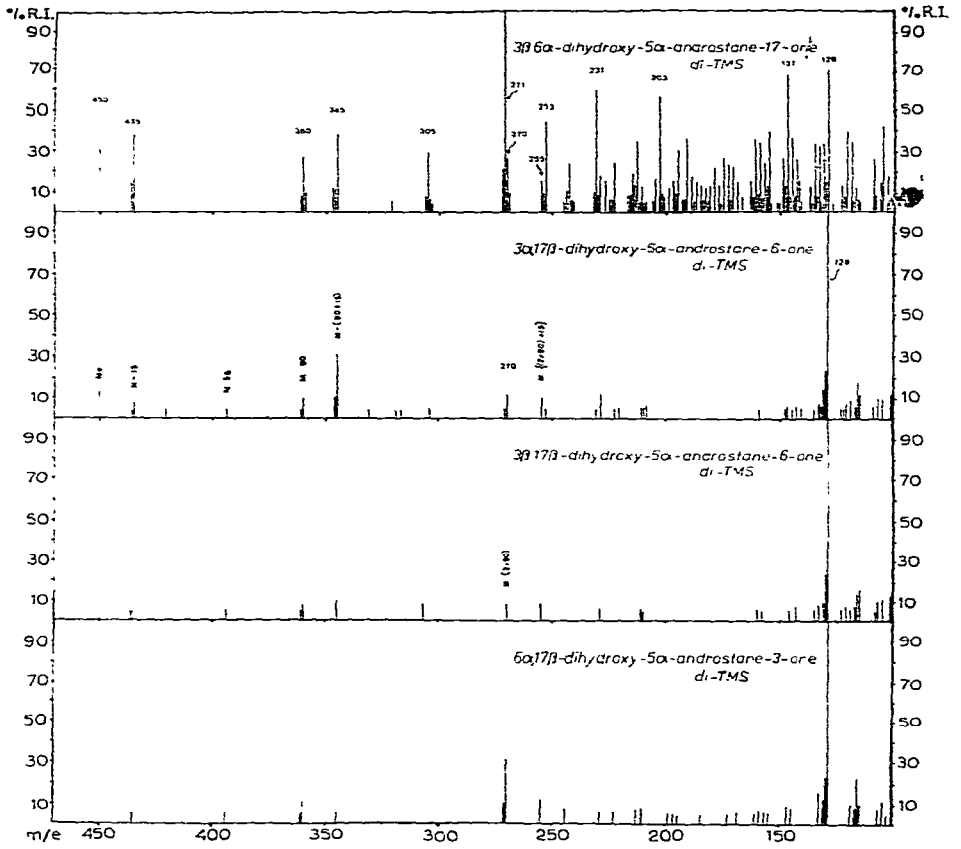


Fig. 3. Mass spectra of TMS derivatives of 3β,6α-dihydroxy-5α-androstan-17-one, 3α,17β-dihydroxy-5α-androstan-6-one, its 3β-epimer and 6α,17β-dihydroxy-5α-androstan-3-one.

androstan-7-one. These three molecules bear oxygen functions in positions 3, 7 and 17 of the androstane skeleton and differ only in the positions of the two hydroxyl-groups and that of the ketone (Fig. 4). When analyzed as TMS derivatives, each exhibits a molecular ion at $m/e = 450$ and similar fragmentation patterns, but the relative intensities of the fragments differ. Thus, the molecular ion at $m/e = 450$, the fragment at $m/e = 435$ (M−15) and that at $m/e = 129$ are the most abundant for 3β,17β-dihydroxy-5α-androstan-7-one, 3β,7β-dihydroxy-5α-androstan-17-one and its 7α-epimer, respectively. With these last two steroids, the ion at $m/e = 129$ can be explained by loss of silanol from position 7, involving the H atom on carbon 5. The resulting unsaturation on carbon 5 favours elimination of $m/e = 231$ and $m/e = 129$ fragments from the ion at $m/e = 360$ (M−90). Loss of water (231 → 213) and of ethylene (360 → 332) involves the 17-oxo-group and agrees with the similar losses described by Djerassi for the TMS derivative of 3β-hydroxyandrost-5-en-17-one[19]. No satisfactory explanation can be given for the ion at $m/e = 243$.

Loss of silanol is more extensive for the 7α-epimer and may be explained by the distance between the -O-TMS and the 5α- and/or the 9α-H being shorter than for the 7β-epimer.

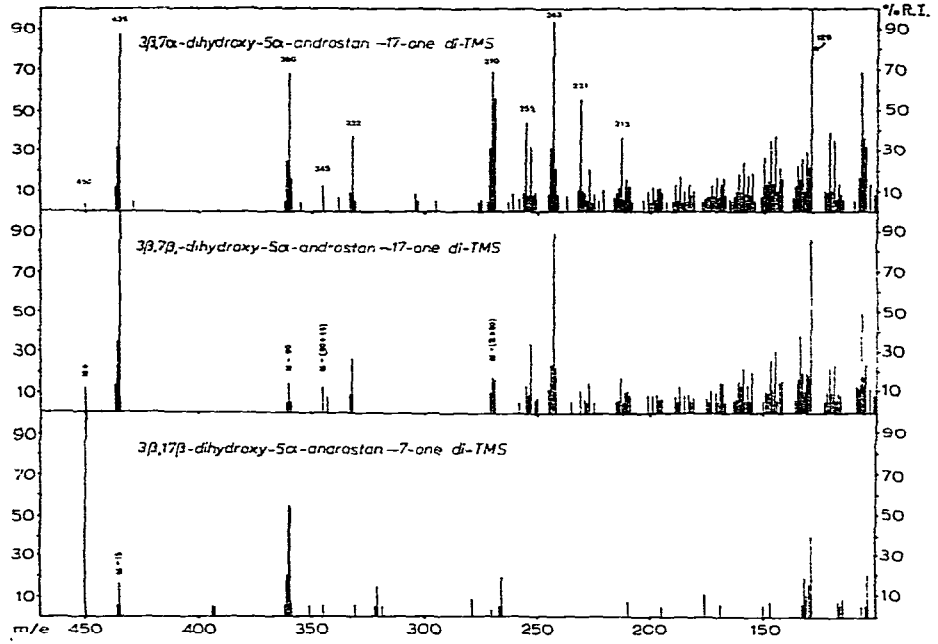


Fig. 4. Mass spectra of TMS derivatives of 3β,7α-dihydroxy-5α-androstan-17-one, its 7β-epimer and 3β,17β-dihydroxy-5α-androstan-7-one.

The spectrum of the di-TMS derivative of 3β,17β-dihydroxy-5α-androstan-7-one shows the base peak to be the molecular ion. Comparison of this spectrum with that of the analogous derivative of 5α-androstane-3ξ,17ξ-diol[22] suggests that a 7-oxo-function results in stabilization of the molecule.

3β,11β-Dihydroxy-5α-androstan-17-one. This compound, when analyzed as a TMS derivative (Fig. 5), differs from those giving the spectra shown in Figs. 3 and 4 by the 11β-hydroxyl-group. Fragmentation agrees with previously published data[23] and resembles that of the steroids mentioned above, but the most abundant fragment is at $m/e = 156$ and cannot be satisfactorily explained. Most of the fragmentation results from loss of silanol (90 a.m.u.) or ring D (56 a.m.u.) according to Zaretskii[21] and loss of a methyl group (15 a.m.u.) or from a combination of these losses.

Androst-5-ene-3β,16α,17β-triol and its 16β-epimer. When these compounds are analyzed as TMS derivatives, they both yield base peaks at $m/e = 129$ and have

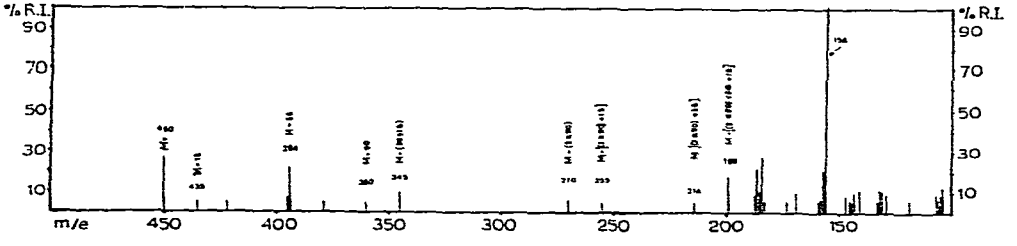


Fig. 5. Mass spectrum of TMS derivative of 3β,11β-dihydroxy-5α-androstan-17-one.

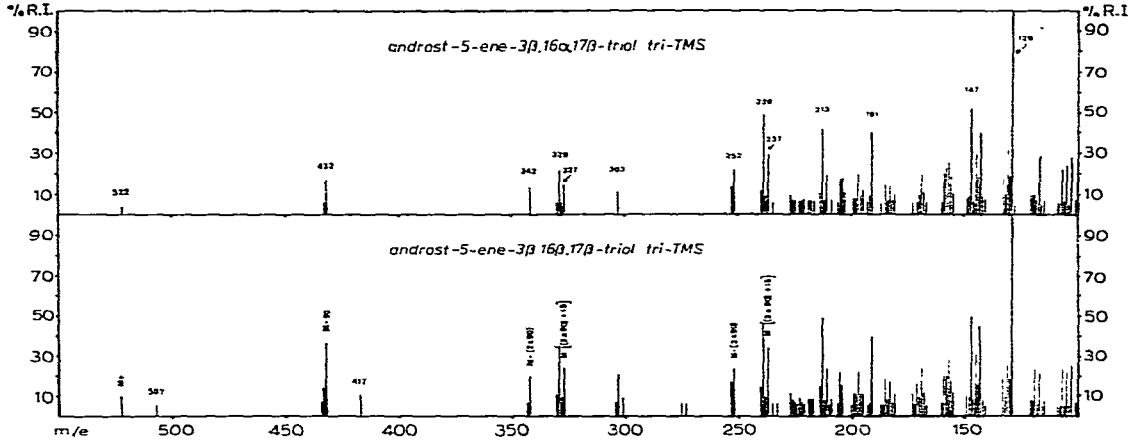


Fig. 6. Mass spectra of TMS derivatives of androst-5-ene-3β,16α,17β-triol and its 16β-epimer.

identical fragmentation patterns; the relative intensities of the fragments are not significantly different (see Fig. 6). The ion at $m/e = 147$ may originate from ring D according to Sloan *et al.*[24], and the other fragments are those expected from TMS derivatives of 5(6)-unsaturated androstenediols.

5α-Androstane-2α,3α,17β-triol, its 2β-epimer and 5α-androstane-2β,3β,17β-triol. The mass spectra of TMS derivatives for these steroids are identical with those presented by Lisboa[7] and show a molecular ion at $m/e = 524$ (Fig. 7); the fragmen-

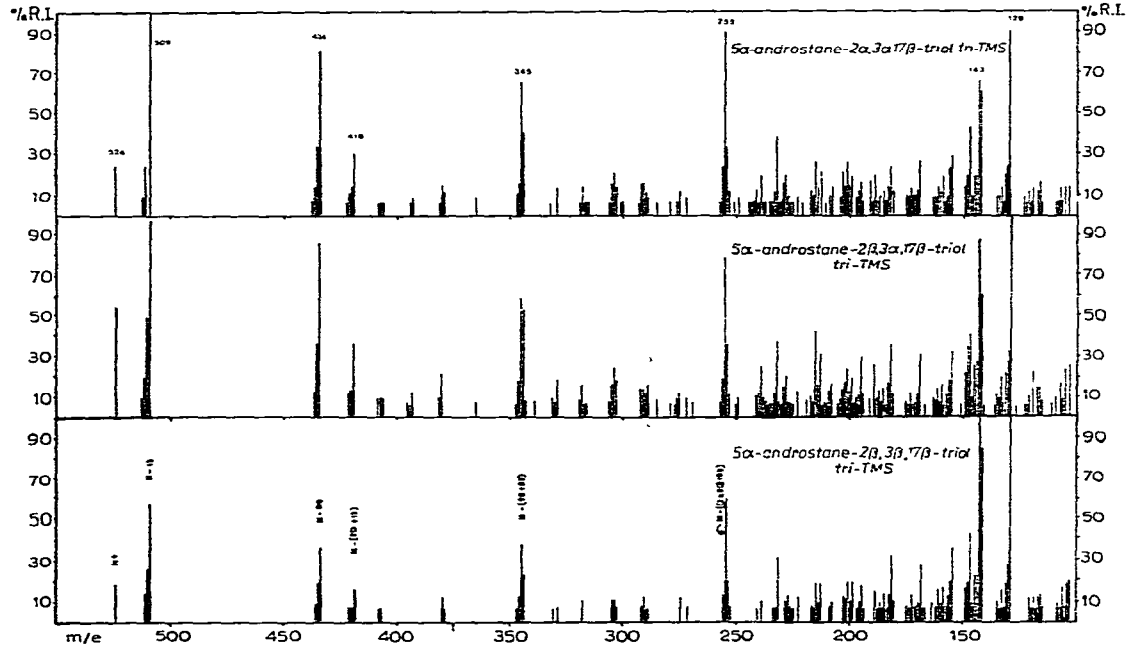


Fig. 7. Mass spectra of TMS derivatives of 5α-androstane-2α,3α,17β-triol, its 2β-epimer and 5α-androstane-2β,3β,17β-triol.

tation patterns are identical, but have different relative intensities. Thus, the base peak is at $m/e = 509$ (M—15) for 5α-androstane-2α,3α,17β-triol whereas the most abundant fragment of the other two steroids is at $m/e = 129$. The patterns show successive losses of fragments of either $m/e = 90$ or $m/e = 89$. This difference may be explained by the proximity of the hydroxyl-groups on carbons 2 and 3 of the androstane skeleton. Such loss of fragments of $m/e = 89$ is characteristic of TMS derivatives of 2ξ,3ξ-dihydroxysteroids. No explanation is as yet available for the ion at 143 a.m.u.

5α-Androstane-3α,6β-17β-triol, its 3β-epimer and 5α-androstane-3β,6α,17β-triol. The mass spectra of the TMS derivatives of these three epimers are closely related. They show the same molecular ion at $m/e = 524$ and a base peak at $m/e = 129$ (see Fig. 8). The fragmentation patterns are identical, with little differences in relative intensities, and are governed by the -O-TMS functions.

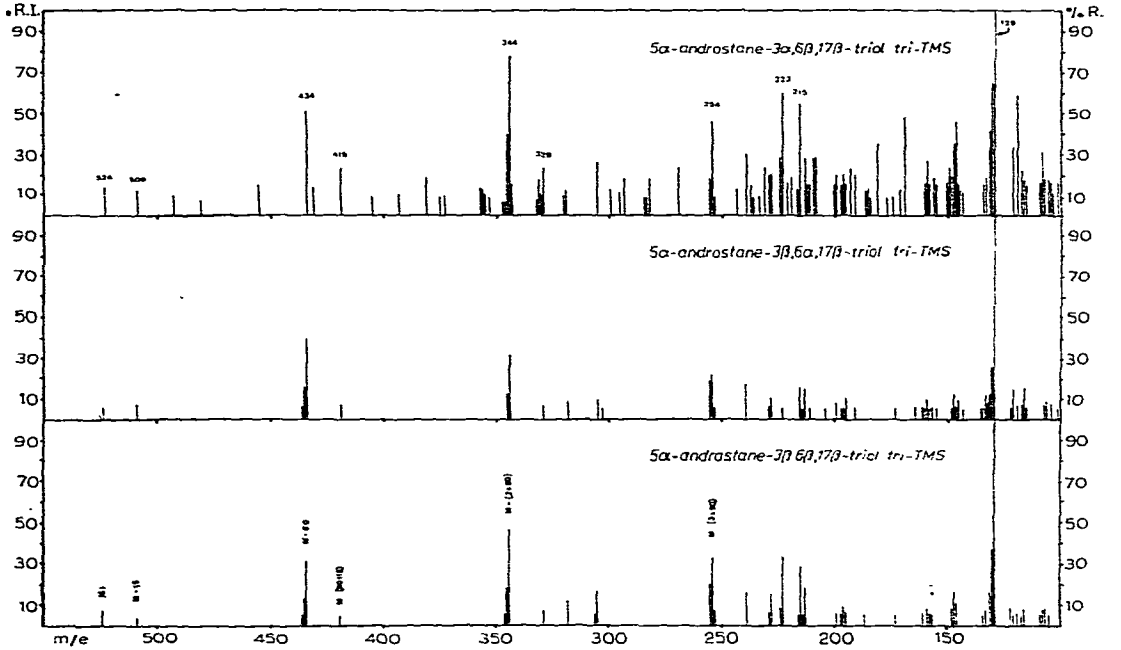


Fig. 8. Mass spectra of TMS derivatives of 5α-androstane-3α,6β,17β-triol, its 6α-epimer and 5α-androstane-3β,6β,17β-triol.

5α-Androstane-3β,7α,17β-triol and its 7β-epimer. The mass spectra of the TMS derivatives of these epimers show the same molecular ion at $m/e = 524$ and identical fragmentation patterns (see Fig. 9). The base peaks are at $m/e = 434$ (M—90) for 5α-androstane-3β,7β,17β-triol and $m/e = 393$ (M—131) for the 7α-epimer, thus permitting differentiation between the compounds. The fragment at 393 a.m.u. corresponds with the loss of ring D and determines the expected diminished intensity of the ion at $m/e = 129$. It is also noteworthy that peaks attributable to fragmentation of rings A, B and C in both steroids (masses lower than 217 a.m.u.) have an unusually low intensity.

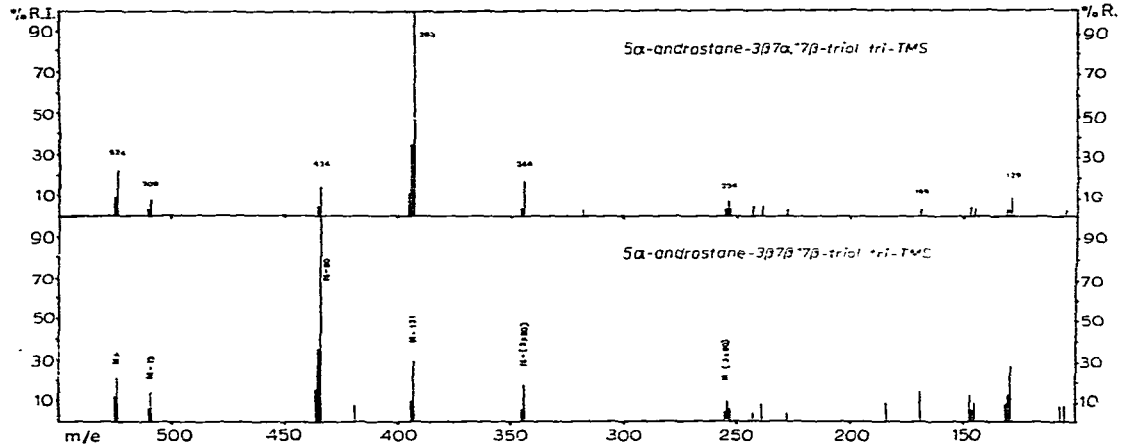


Fig. 9. Mass spectra of TMS derivatives of 5α-androstane-3β,7α,17β-triol and its 7β-epimer.

5α-Androstane-3α,11β,17β-triol and its 3β-epimer. The mass spectra of the TMS derivatives of these epimers show the same molecular ion at $m/e = 524$, the same base peak at $m/e = 169$ and identical fragmentation patterns (see Fig. 10). This last ion may be explained by loss of ring D and carbons 13, 14 and 19. Further loss of a chain containing carbons 19, 13, 12, 11 and the 11-O-TMS may correspond with the ion at $m/e = 143$ (see ref. 25).

5α-Androstane-3β,15α,17β-triol. The TMS derivative of this triol gives a spectrum (see Fig. 11) with a molecular ion at $m/e = 524$ and a base peak at $m/e = 217$ as previously reported[2]. This last ion was attributed by Gustafsson *et al.*[26] to the breaking of C_{13}–C_{17} and C_{14}–C_{15} bonds; the ion at $m/e = 191$ results from complete transfer of the 17-O-TMS group to position 15 (see ref. 26).

5α-Androstane-3α,16α,17β-triol and its 3β-epimer. In agreement with Lisboa[7], the mass spectra of the TMS derivatives of these epimers show identical fragmentation patterns with close relative intensities (see Fig. 12). The molecular ion is at

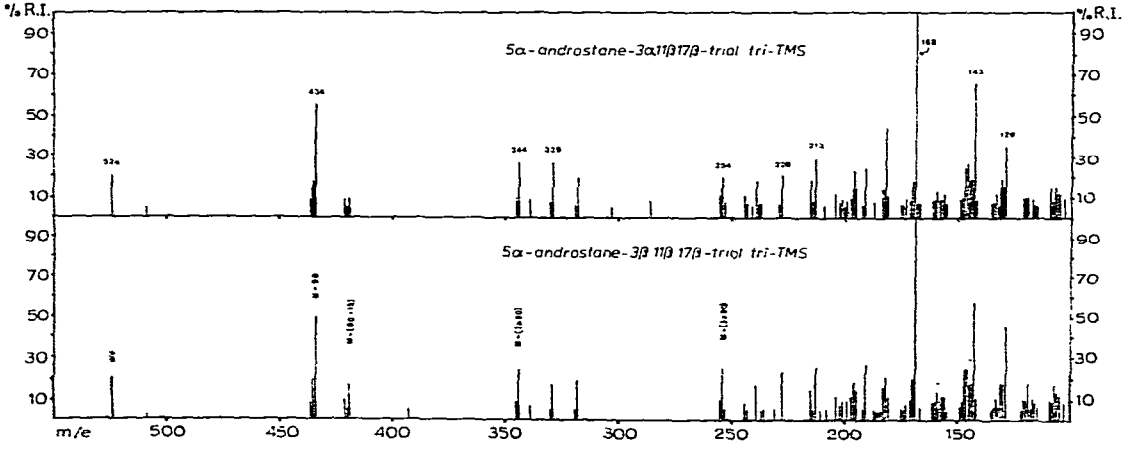


Fig. 10. Mass spectra of TMS derivatives of 5α-androstane-3α,11β,17β-triol and its 3β-epimer.

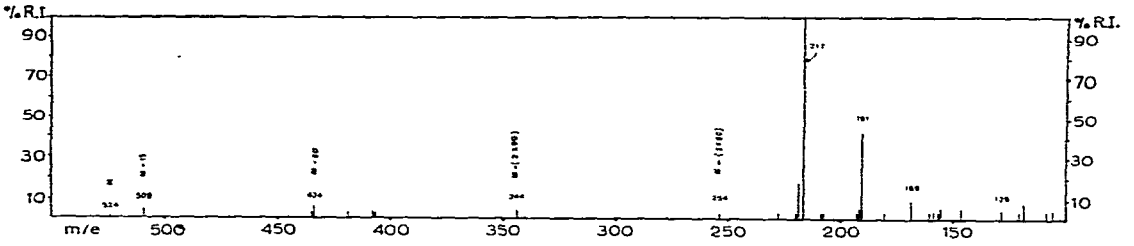


Fig. 11. Mass spectrum of TMS derivative of 5α-androstane-3β,15α,17β-triol.

m/e = 524 and the base peak at *m/e* = 191. This last ion may be explained by a complex transfer of 17-O-TMS to position 16. The fragmentation is also consistent with that observed for the TMS derivatives of androst-5-ene-3β,16ξ,17ξ-triol epimers (see Fig. 6).

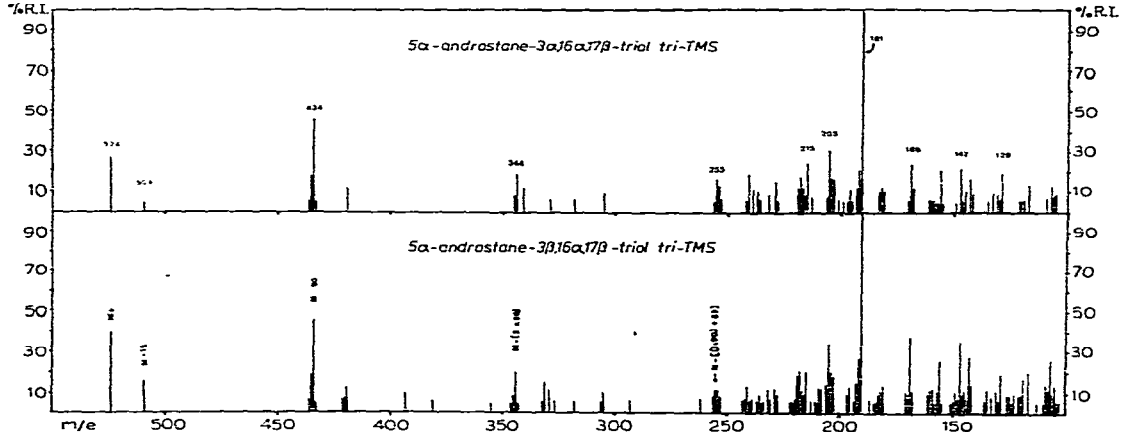


Fig. 12. Mass spectra of TMS derivatives of 5α-androstane-3α,16α,17β-triol and its 3β-epimer.

Characteristic fragmentation of TMS derivatives of dihydroxy-5α-androstanones

The *m/e* values for characteristic fragments obtained from these derivatives are shown in Table III; fragmentation differences between steroids may be used for multiple ion detection or for confirmation of identity.

Characteristic fragmentation of TMS derivatives of androstanetriols

The *m/e* values of characteristic fragments obtained from these derivatives are shown in Table IV; the differences may prove useful for confirming the identity of a steroid or in multiple ion detection.

DISCUSSION

The chromatographic data and mass spectra presented in this paper are limited to twenty-eight $C_{19}O_3$ steroids. Many more such steroids may be chemically available, but their number was limited both by their availability and by our choice of molecules that could be naturally occurring[1–6]. TLC, GLC and GLC–MS are the most common

TABLE III

VALUES OF *m/e* FOR FRAGMENTS OF TMS DERIVATIVES OF DIHYDROXYANDROSTANONES

Positions of hydroxyl-groups	*Position of oxo-group*	*Characteristic m/e values*
3β, 6α	17	271, 253, 231, 203, 137
3α,17β	6	129
3β,17β	6	129
6α,17β	3	129
3β, 7α	17	435, 360, 332, 270, 255, 243, 231, 213, 129
3β, 7β	17	435, 253, 243, 129
3β,17β	7	450, 360
3β,11β	17	394, 214, 199, 156

TABLE IV

VALUES OF *m/e* FOR FRAGMENTS OF TMS DERIVATIVES OF ANDROSTANETRIOLS

Positions of hydroxyl-groups	*Characteristic m/e values*
3β,16α,17β(5-ene)	329, 327, 239, 237, 191, 147, 129
3β,16β,17β(5-ene)	329, 327, 239, 237, 191, 147, 129
3α,16α,17β	524, 434, 205, 191, 169, 147
3β,16α,17β	524, 434, 205, 191, 169, 147
2α, 3α,17β	509, 345, 255, 143
2β, 3α,17β	509, 345, 255, 143
2β, 3β,17β	509, 345, 255, 143
3α, 6β,17β	434, 344, 233, 215, 129
3β, 6α,17β	434, 344, 129
3β, 6β,17β	434, 344, 129
3β, 7α,17β	393
3β, 7β,17β	434
3α,11β,17β	228, 213, 169, 143, 129
3β,11β,17β	228, 213, 169, 143, 129

techniques currently used in steroid-metabolism research for the separation and identification of transformation products; in such instances, the structure of the initial compound being known, only a limited number of new hydroxylated steroids may be formed[1,4].

We tested our $C_{19}O_3$ steroids by TLC in seven different solvent mixtures, all but one of which differed from those used by Gustafsson *et al.*[1]. Our purpose was to extend their study and to avoid both multiple development and the use of acetic acid in the mobile phases.

In GLC, the TMS derivatives of hydroxylated steroids give clear symmetrical peaks. Calculated retention indices generally differ between molecules and between stationary phases; nevertheless, effective resolution of two steroids also depends on the efficiency of the column. Thus, a column with 2000 theoretical plates will separate (with a resolution factor greater than 1) two steroids differing by only 20 index units.

Use of capillary columns with 100,000 theoretical plates[27] may permit the separation of molecules differing by 5 index units with a resolution factor of 1.40. The separation of molecules by GLC must be achieved before the GLC can be coupled with MS.

Few papers report mass spectra of $C_{19}O_3$ steroids as their TMS derivatives. Those reported by Gustafsson *et al.*[1] and by Lisboa[7] differ only slightly from those discussed here. Thus, a base peak at $m/e = 344$ was reported for 5β-androstane-3β,6β,17β-triol instead of at $m/e = 129$, but our fragment at $m/e = 344$ had an intensity 80% of that of the peak at m/e 129. Such a difference may be explained by the fact that the published spectrum[1] was given by a micro-amount of steroid obtained after double enzymic transformation.

Our investigation of the fragmentation of the studied molecules during MS may be used as a criterion for multiple ion detection or to ascertain the identity of $C_{19}O_3$ steroids of biological origin; identification may not be achieved, but useful leads for confirmatory experiments may be obtained.

ACKNOWLEDGEMENTS

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REFERENCES

1 J. Å. Gustafsson, B. P. Lisboa and J. Sjövall, *Eur. J. Biochem.*, 6 (1968) 317.
2 J. Å. Gustafsson and M. Ingelman-Sundberg, *Febs Lett.*, 31 (1973) 292.
3 H. F. Acevedo and J. W. Goldzieher, *Biochim. Biophys. Acta*, 82 (1964) 118.
4 P. Ofner, R. L. Vena and R. F. Morfin, *Steroids*, 24 (1974) 261.
5 R. F. Morfin, I. Leav, P. Ofner and J. C. Orr, *Fed. Proc.*, 29 (1970) 247.
6 J. Å. Gustafsson and B. P. Lisboa, *Acta Endocrinol. (Copenhagen)*, 65 (1970) 84.
7 B. P. Lisboa, *J. Chromatogr.*, 48 (1970) 364.
8 V. E. M. Chambers, W. A. Denny, J. M. Evans, Sir E. R. H. Jones, A. Kasal, D. Meakins and J. Pragnell, *J. Chem. Soc., Perkin Trans. I*, (1973) 1500.
9 U. Valcavi, P. Martelli, U. C. Sironi and S. Tedeschi, *Farmaco, Ed. Sci.*, 30 (1975) 464.
10 C. W. Shoppee, D. N. Jones and G. H. R. Summers, *J. Chem. Soc.*, (1957) 3100.
11 E. M. Chambaz and E. C. Horning, *Anal. Biochem.*, 30 (1967) 7.
12 F. L. Berthou, D. Picart, L. G. Bardou and H. H. Floch, *J. Chromatogr. Sci.*, 12 (1974) 662.
13 A. Ros, *Monit. Ostet. Ginecol. Endocrinol Metab.*, 39 (1968) 777.
14 E. Kovàts, *Helv. Chim. Acta, 41* (1958) 1915.
15 H. Obermann, M. Spiteller-Friedmann and G. Spiteller, *Chem. Ber.*, 103 (1970) 1497.
16 F. Hammerschmidt and G. Spiteller, *Tetrahedron*, 29 (1973) 3995.
17 C. Djerassi, R. H. Shapiro and M. van Dewalle, *J. Amer. Chem. Soc.*, 87 (1965) 4892.
18 C. G. Mac Donald, J. S. Shannon and G. Sugowdz, *Tetrahedron Lett.*, 13 (1963) 807.
19 H. Budzikiewicz, C. Djerassi and D. H. Williams (Editors), *Mass Spectrometry of Organic Compounds*, Holden Day Inc., San Francisco, Cambridge, London, Amsterdam, 1967, p. 475.
20 D. Picart, *Thèse 3e cycle*, Univ. of Brest, France, 1975.
21 Z. V. Zaretskii, *Mass Spectrometry of Steroids*, Wiley, New York, Toronto, and Israel University Press, Jerusalem, 1976, p. 23.
22 N. Vikho, *Acta Endocrinol (Copenhagen) Suppl.* 109, (1966).
23 J. Å. Gustafsson and J. Sjövall, *Eur. J. Biochem.*, 6 (1968) 227.
24 S. Sloan, D. J. Harvey and P. Vouros, *Org. Mass Spectrom.*, 5 (1971) 789.
25 P. Vouros and D. J. Harvey, *J. Chem. Soc., Perkin Trans I*, (1973) 728.
26 J. Å. Gustafsson, R. Ryhage, J. Sjövall and R. M. Moriarty, *J. Amer. Chem. Soc.*, 91 (1969) 1234.
27 F. Berthou, *Spectra 2000*, 30 (1976) 16.