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PREPARATION OF ENOL-*tert.*-BUTYLDIMETHYLSILYL AND MIXED *tert.*-BUTYLDIMETHYLSILYL-TRIMETHYLSILYL ETHERS FOR GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF STEROIDS AND BILE ACIDS

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SUMMARY

Conditions are described for conversion of testosterone into the 3-enol, 17-bis-*tert.*-butyldimethylsilyl ether derivative without formation of side products. The steroid is treated with *tert.*-butyldimethylsilylimidazole in heptane at 100°C using sodium formate as catalyst. Derivatives are also formed at different rates of 3-keto-5α, 3α/β-hydroxy-, 6α/β-hydroxy-, 7β-hydroxy-, 16α-hydroxy-, 17β-hydroxy(*sec.*)- and 20α/β-hydroxysteroids, whereas hydroxyl groups in 1β, 7α, 12α/β, 15β and 17α(*tert.*) positions do not react to a significant extent. These positions are derivatized by subsequent addition of trimethylsilylimidazole, yielding mixed derivatives which are suitable for gas chromatography–mass spectrometry with selected ion monitoring. Conditions are given for conversion of some biologically important androgens, progestins and bile acids into a single form of derivative. The use of the method is illustrated by an analysis of steroids in a rat testis.

INTRODUCTION

Sample purification procedures, choice of derivative and the technical performance of the equipment are the most important factors determining specificity and sensitivity in gas chromatographic–mass spectrometric (GC–MS) analyses of steroids. Among the derivatives, trimethylsilyl (TMS) and O-methyloxime-TMS ethers[1] are most widely used, but are not best suited for high-sensitivity quantitative work since the fragmentation of TMS ethers is often extensive, and O-methyloximes are lost owing to decomposition in the column or interface to the ion source[2–4]. A variety of other silylation reagents has been studied, some of which give ions carrying a large percentage of the total ionization[5–17]. The *tert.*-butyldimethylsilyl (TBDMS) ethers give intense $[M-57]^+$ ions and have been widely used in analytical applications[18–27]. The important protection of the 3-keto group in 3-keto-Δ^4-steroids can also be achieved by a reaction with TBDMS-imidazole catalysed by potassium acetate[28]. In analogy with the corresponding enol-TMS ethers[29,30], the enol-TBDMS ethers give an intense molecular ion[28]. The high stability of the TBDMS derivatives is an important advantage.

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In the course of studies of the effects of ethanol on the production of testosterone, a method was needed for analysis of testicular steroids and their labelling during metabolism of deuterated ethanol in the rat. The base-catalysed reaction with TBDMS-imidazole appeared to be the most suitable derivatization procedure. However, there are only a few reports on the reaction and its use[28,31], and a more detailed study of conditions for derivatization of different steroids has therefore been carried out. The preparation of mixed TBDMS-TMS ether derivatives of steroids with hindered hydroxyl groups has also been investigated.

EXPERIMENTAL

Solvents, steroids and reagents

Solvents were of reagent grade and were redistilled in an all-glass apparatus.

Unlabelled steroids were from Steraloids (Wilton, NH, U.S.A.) and 17β-hydroxy-5α-[4-^{14}C]androstan-3-one (59 mCi/mmol) and 3β-hydroxy-5-[4-^{14}C]-androsten-17-one (55 mCi/mmol) from the Radiochemical Center (Amersham, U.K.). The bile acids were those used in previous investigations[32].

Trimethylsilylimidazole (TSIM) was distilled *in vacuo* prior to use, and *tert.*-butyldimethylsilylimidazole (TBDMSIM) was synthesized according to Blair and Phillipou[28]. Imidazole was recrystallized from toluene and dried *in vacuo* at 80°C, and *tert.*-butyldimethylsilyl chloride was sublimated before use in the synthesis. Sodium formate was recrystallized from water.

Lipidex 1000 (Packard Instrument Co., Downers Grove, IL, U.S.A.) was washed with hexane.

High-performance liquid chromatography (HPLC)

HPLC was performed on an LDC instrument (Milton Roy, Riviera Beach, FL, U.S.A.) using columns of LiChrosorb DIOL (10 μm, 25 × 0.4 cm I.D.; Merck, Darmstadt, F.R.G.) in hexane–2-propanol (96:4, v/v). A Rheodyne injector (Model 7125, Cotati, CA, U.S.A.) with a 1-ml sample loop was used. The derivatized steroids were injected in 0.1–0.2 ml of the mobile phase and the flow-rate was 1 ml min^{-1}. Biological samples, prior to derivatization, were injected in hexane–2-propanol (3:2, v/v). Radioactivity was determined with a Packard Trace 7140 radioactivity flow monitor (Packard, Downers Grove, IL, U.S.A.) attached to the outlet of the column.

Gas–liquid chromatography (GLC)

GLC was performed on a Pye 104 gas chromatograph with a flame ionization detector, using a 25 m × 0.32 mm I.D. open-tubular fused-silica column coated with 0.15 μm SE-30 (Orion, Espoo, Finland). An all-glass falling-needle injection system was used. Nitrogen was the carrier gas at a pressure of *ca.* 50 kPa and the column temperature was *ca.* 270°C. Retention indices were calculated from the retention times of even-numbered *n*-alkanes.

Gas chromatography–mass spectrometry

A modified LKB 9000 instrument was used with a 25 m × 0.32 mm open-tubular fused-silica column coated with 0.15 μm OV-1 (Skandinaviska GeneTec, Kungsbacka, Sweden) connected to the ion source via a restriction and a single-stage

adjustable jet separator[33]. The column temperature was 250–260°C and the temperatures of the separator and ion source were 290°C and 310°C, respectively. The electron energy was 22.5 eV and the ionizing current 120 μA.

Biological samples

Rat testes weighing *ca.* 1 g were extracted with hexane–2-propanol (3:2, v/v), and a purified steroid fraction was isolated as described previously[34]. The steroids were separated into four groups by HPLC, fractions being collected to include progesterone (fraction I), 17β-hydroxy-5α-androstan-17-one (II), testosterone (III) and 17-hydroxyprogesterone (IV) as determined by previous injections of ^{14}C-labelled steroids. Following evaporation of the solvents the steroids were derivatized using conditions described in Results.

Derivatization procedure

Reactions were carried out in 1-ml micro reaction vessels with Teflon-lined screw-cap seals (Supelco, Bellefonte, PA, U.S.A.). A solution of sodium formate in water (1 mg in 100 μl) was dried under a stream of nitrogen followed by heating at 270°C for 30 min. After cooling, the steroid to be derivatized (usually *ca.* 10 μg) was added in 100 μl of methanol or toluene. The solvent was removed under a stream of nitrogen and 100 μl of heptane and 20 μl of TBDMSIM were added. The vials were purged with nitrogen, closed and placed in an oven at 100°C for different periods of time. After completion of the reaction, excess reagent was destroyed by addition of 20 μl of 2-propanol and continued heating of the closed vial for 10 min at 100°C.

The products were isolated either by extraction or by aspiration into Lipidex gel followed by elution with hexane. In the former case, 0.5 ml of water was added to the reaction mixture which was then extracted three times with 0.5 ml of hexane. In the latter case the reaction mixture was aspirated with a 5-ml syringe through a bed of Lipidex 1000 (10 × 9 mm I.D.) in hexane held in a glass tube between two end pieces of Teflon covered by 10-μm stainless steel gauze. One end piece had a conical fitting to the syringe, the other extended into a tip for aspiration of the sample. The reaction vessel was washed three times with 1 ml of hexane, each wash being aspirated through the Lipidex bed to elute the derivatized steroids.

Steroids containing hindered hydroxyl groups were first treated with TBDMSIM for a suitable time, and 20 μl of TSIM were then added. The vial was purged with nitrogen, closed, and heated for another 2 h. The products were extracted as above.

The HPLC fractions of the biological extract were treated under conditions suitable for steroids that might be present. Following extraction as above, the derivatives were purified by HPLC.

RESULTS AND DISCUSSION

Solvent and catalyst

Initially, progesterone, testosterone, 3β-hydroxy-5-pregnen-20-one and 5-androstene-3β,17β-diol were treated at 100°C for 2 h with 20 μl of TBDMSIM in 100 μl of toluene, using 1 mg of anhydrous potassium acetate as catalyst[28]. While the expected derivatives were the major products, the GLC analyses indicated formation

of several products from testosterone and the 20-ketosteroids. The latter compounds yielded *ca.* 20% of an isomer of the main derivative. The retention times and mass spectra of the respective products suggested that epimerization had occurred at C-17 and that the minor product had a 17α-oriented side-chain. Testosterone gave *ca.* 8% of a product, the mass spectrum of which indicated that it was a 3-enol-TBDMS, 17-acetate. Acetylation has not been described under these conditions, but a trans-acetylation at the tertiary 17α-acetoxy group of medroxyprogesterone acetate has been observed[31].

In attempts to obtain fewer side products, the following compounds were tested as catalysts (or solvents): pyridine, its hydrochloride and hydrobromide, formamide, piperidine, tributylamine, trimethylchlorosilane, sulphuric acid, potassium carbonate and sodium formate. Reactions were carried out at different temperatures for different periods of time. Only sodium formate catalysed a formation of the desired derivatives and did not itself react with hydroxyl groups. However, epimerization at C-17 of 20-ketosteroids occurred also with this catalyst.

The reactions appeared to be slower when sodium formate was used instead of potassium acetate; other solvents were therefore tested. When the reaction was carried out in heptane, the 20α-hydroxyl group of 5α-pregnane-3β,20α-diol was completely derivatized in 6 h, whereas a large percentage remained unchanged when toluene was used. Further experiments were therefore carried out using heptane as solvent and sodium formate as catalyst.

Reaction conditions

Temperature and reaction time were varied with the aim of obtaining single derivatives of steroids containing hydroxyl or keto groups in positions known to be substituted in common androgens, progestins and bile acids. When the reaction with TBDMSIM was very slow, attempts were made to find conditions yielding a mixed TBDMS–TMS derivative. TSIM was added to the reaction mixture at a time when unhindered group(s) had been converted into TBDMS derivative(s). Quilliam and Westmore have shown that TBDMS groups can be exchanged when TSIM is added in the presence of imidazole hydrochloride but not under other conditions[11]. Mixed derivatization with different silylation reagents has been studied by many authors, and conditions can be found when no exchange occurs (see refs. 14, 20, 35, 36). The conditions used by us did not result in significant silyl group exchange in the positions studied (Table I). However, when the reaction was first performed with TSIM and then with TBDMSIM some exchange was observed, *e.g.* at C-3. Also, when the 3,20-bisTBDMS ether of 5-pregnene-3β,17α,20α-triol was further treated with TSIM, the 3,17-bisTBDMS, 20-TMS derivative was formed, probably as the sole product (Fig. 1). This is a larger extent of rearrangement than under conditions used by Quilliam and Westmore[11] and similar to that described by Vouros for the trimethylsilyl group[36].

The conditions used to obtain optimal yields of one derivative of the steroids studied are given in Table I. The yields were estimated from GLC analyses. When no other product was detected (peaks below 2% of the major peak area) the yield was considered to be quantitative for practical analytical purposes. Derivatives were also prepared of ^{14}C-labelled 3β-hydroxy-5-androsten-17-one and 17β-hydroxy-5α-androstan-3-one (*ca.* 20,000 cpm carrier-free steroid) followed by analysis by HPLC

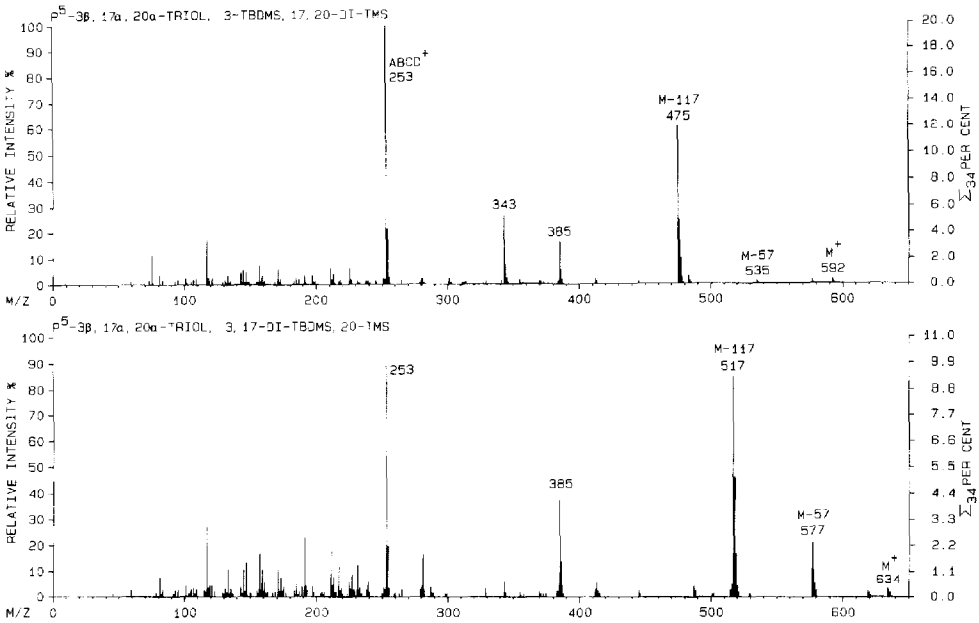

Fig. 1. Mass spectra of the 3-TBDMS, 17,20-bisTMS (upper) and 3,17-bisTBDMS, 20-TMS (lower) ether derivatives of 5-pregnene-3β,17α,20α-triol.

with a radioactivity flow monitor. Only one peak of ^{14}C was obtained having the mobility of the expected derivative. The yield of ^{14}C was *ca.* 90%, not corrected for a possible reduction of efficiency in the counting of the peak of the derivative, which was narrow compared with that of the starting material.

The recovery of steroid derivative in the isolation procedure using Lipidex 1000 was tested with 3β-hydroxy-5-[4-^{14}C]androsten-17-one and found to be 95%.

Selectivity of reaction with TBDMSIM

The reactivities of keto and hydroxyl groups in different positions are indicated by the results shown in Table I. The reaction with 3-keto-Δ^4-steroids[28,31] and absence of reaction with 17-ketosteroids[11] agree with previous studies. Partial formation of an enol-TBDMS derivative of a saturated 3-ketosteroid of the 5α series has been noted in imidazole-catalysed reactions with *tert.*-butyldimethylchlorosilane[9,11]. Complete derivatization occurred under the present conditions. The 20-keto group did not react, but the probable epimerization at C-17 indicates reversible enolization.

As expected, hydroxyl groups at C-3 and C-17 (secondary) were rapidly derivatized[5,7,9–13,28]. The reaction with a 20α-hydroxy group was slow, but faster than that with a 20β-hydroxy group (*cf.* ref. 5). The 11β-hydroxy group did not react (*cf.* refs. 5 and 11). A 16α-hydroxy group in a C_{19} steroid could be completely derivatized, whereas in a C_{21} steroid the reaction was very slow. Furthermore, in the presence of a 20-keto group, elimination of *tert.*-butyldimethylsilanol was favoured, resulting in a molecular ion of low intensity and a base peak at m/z $[M-132]^+$. Thus, preparation of TBDMS ethers of this structure has little practical advantage.

Reactions with TBDMSIM of hydroxyl groups in other positions were studied

TABLE I

GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DATA OF TBDMS AND MIXED TBDMS–TMS DERIVATIVES OF STEROIDS AND BILE ACIDS

Reaction times are given for optimum yield of a single derivative.

*Steroid**	*Retention index*	*Derivative formed*	*Reaction time (h)***	*Yield (%)****	*Major ions above m/z 200*	*Percent of total ions in (m/z)*
C_{19} steroids						
A^5-3β-ol-17-one	2804	3-TBDMS	1	100	345(100), 253(15), 269(7)	29.3(345)
A^5-3β,17β-diol	3164	3,17-bisTBDMS	1	100	461(100), 253(90), 329(63)	11.6(461)
A^4-3,17-dione	2892	3-Enol-TBDMS	4	100	400(100), 342(14), 269(9)	35.1(400)
A^4-17β-ol-3-one	3249	3-Enol, 17-bisTBDMS	4	100	516(100), 459(12), 343(37)	20.0(516)
5αA-17β-ol-3-one	3210	3-Enol,17-bisTBDMS	2	95	461(100), 385(92), 518(35)	16.8(461)
A^4-16α,17β-diol-3-one	3740	3-Enol,16,17-trisTBDMS	6	100	646(100), 245(27), 589(21)	15.0(646)
5βA-3α,11β-diol-17-one	2957	3-TBDMS, 11-TMS	4+2	100	359(100), 435(32), 253(7)	32.5(359)
C_{21} steroids						
P^5-3β-ol-20-one	2967, 3011§	3-TBDMS	1	20+80§	373(100), 297(16), 298(14)	16.0(373)
5αP-3β,20β-diol	3159	3-TBDMS, 20-TMS	2+2	95	449(100), 283(11), 405(6)	20.3(449)
5αP-3β,20α-diol	3365	3,20-bisTBDMS	6	100	491(79), 359(30), 283(12)	13.9(491)
P^4-3,20-dione	3022,3067§	3-Enol-TBDMS	4	20+80§	428(100), 413(10), 312(9)	20.5(428)
P^4-20β-ol-3-one	3460	3-Enol,20-bisTBDMS	21	100	544(100), 487(15), 412(14)	23.8(544)
P^4-16α-ol-3,20-dione	3500	3-Enol, 16-bisTBDMS	6	60	426(27), 558(4), 501(4)	7.4(426)
P^5-3β,17α-diol-20-one	3135	3-TBDMS, 17-TMS	4+2	100	253(100), 475(46), 385(19)	17.2(253)
P^5-3β,17α,20α-triol	3245	3-TBDMS, 17,20-bisTMS	4+2	100	253(100), 475(60), 343(27)	20.0(253)
Bile acids						
5βB-3α,6α-diol	3642	3,6-BisTBDMS	1	100	371(100), 207(19), 280(15)	14.8(371)
5βB-3α,6β-diol	3651	3,6-BisTBDMS	6	100	445(100), 371(40), 339(18)	14.4(445)
5βB-3α,7α-diol	3444	3-TBDMS, 7-TMS	4+2	100	535(100), 255(10), 321(6)	28.6(535)
5βB-3α,7β-diol	3711	3,7-BisTBDMS	5	100	577(59), 369(26), 459(16)	7.1(577)
5βB-3α,12α-diol	3439	3-TBDMS, 12-TMS	4+2	100	535(100), 255(10), 321(5)	28.7(535)
5βB-3α,12β-diol	3385	3-TBDMS, 12-TMS	4+2	100	535(100), 255(17), 503(11)	22.7(535)
5βB-1β,3α,12α-triol	3521	3-TBDMS, 1,12-bisTMS	4+2	100	623(100), 369(9), 259(9)	20.6(623)
5βB-3α,12α,15β-triol	3460	3-TBDMS, 12,15-bisTMS	4+2	100	623(100), 253(10), 369(9)	24.6(623)

* A = androstane, P = pregnane, B = methyl cholanoate; superscript indicates position of double bond.

** The first time is for reaction with TBDMSIM, the second for reaction with TSIM.

*** 100% signifies absence (less than 2%) of peaks of other products in the GLC analysis.

§ Two products probably epimeric at C-17. Ratio between yields is approximate.

using bile acid methyl esters as model compounds. The ester group was stable under the conditions used, as tested with methyl 3-keto-5β-[24-^{14}C]cholanoate and analysis by HPLC. The 6α-hydroxyl group was readily derivatized (*cf.* refs. 10 and 11) while the axial 6β-hydroxyl and equatorial 7β-hydroxyl groups required long reaction times. Hydroxyl groups in the 1β, 7α, 12α/β and 15β positions did not react.

Derivatization for GC–MS analysis

The analytical advantages with TBDMS derivatives are stability and mass spectra suitable for selected ion monitoring[5,7,9–13,17–28,31]. Detection limits (signal-to-noise ratio of 2:1) were determined for some of the derivatized steroids by monitoring of the base peak. They were found to be *ca.* 1 pg for testosterone and 3β-hydroxy-5-pregnen-20-one, *ca.* 5 pg for progesterone and *ca.* 0.5 pg for 3β-hydroxy-5-androsten-17-one. These values were obtained on an LKB 9000 instrument with a single-stage jet separator made of stainless steel, and better sensitivity can be expected on more modern instruments. The higher value for progesterone is partly due to interference by *m/z* 429 from the OV-1 background, and the sensitivity will probably be improved at a higher resolution.

The slow reaction of hydroxyl groups in some positions constitutes a disadvantage. The absence of a reaction is of lesser importance since mixed derivatives can be prepared by subsequent reaction with TSIM. Other disadvantages in quantitative applications, *e.g.* in analyses of corticosteroid metabolites, remain to be investigated.

The slow derivatization of 20-hydroxy groups is a problem in the analysis of

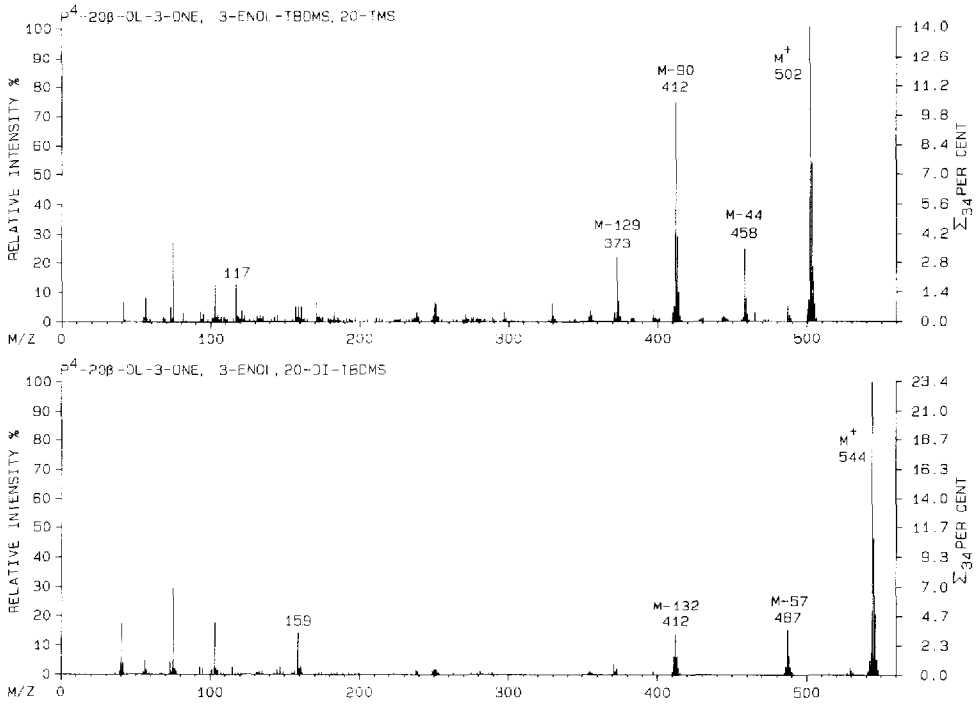

Fig. 2. Mass spectra of the 3-enol-TBDMS, 20-TMS (upper) and 3-enol, 20-bisTBDMS (lower) ether derivatives of 20β-hydroxy-4-pregnen-3-one.

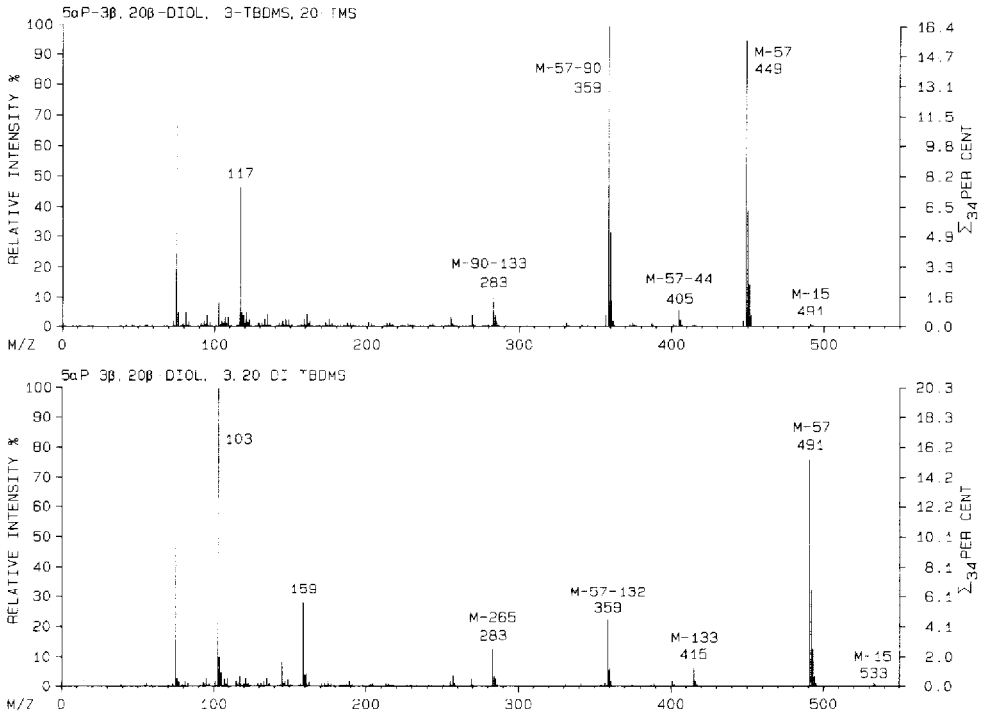

Fig. 3. Mass spectra of the 3-TBDMS, 20-TMS (upper) and 3,20-bisTBDMS (lower) ether derivatives of 5α-pregnane-3β,20β-diol.

progestins. As indicated in Table I, simple steroids with this group may be analysed either as TBDMS derivatives after long reaction times or as mixed TBDMS–TMS derivatives. The choice of method depends on the structure and reactivity of other steroids in the fraction to be analysed. Both types of derivatives give ions suitable for selected ion monitoring (Figs. 2 and 3). The side-chain ion (*m/z* 117) is much less abundant in the 3-TBDMS, 20-TMS derivative than in the 3,20-bisTMS derivative. Spectra with a similar abundance of ions of higher mass are also given by isopropyldimethylsilyl ethers of pregnanediols[16]. An ion formed by loss of a CH_3CHO fragment with migration of the trimethylsilyl group (M − 44 or M − 57 − 44)[37] is seen only with the mixed derivatives having a 20-TMS group (Figs. 2 and 3).

The fragmentation of the mixed TBDMS–TMS derivative of the biologically important 3β,17α-dihydroxy-5-pregnen-20-one is similar to that of the bisTMS derivative, *i.e.* intense ions at *m/z* (M − 43) and 253. The advantages of using the mixed derivative are that the retention time is longer and that $[M-43]^+$ appears at a higher mass. Both factors will help to reduce interference by other compounds.

The mixed derivatives of bile acids methyl esters give mass spectra which are very suitable for selected ion monitoring and for determination of abundance of heavy atoms in metabolic experiments using stable isotopes. The mixed derivatives of di- and trihydroxycholanoates give very intense ions at *m/z* 535 and 623, respectively, corresponding to loss of the *tert.*-butyl group (Figs. 4 and 5). Miyazaki *et al.*[15] have described the use of dimethylethylsilyl ethers to achieve an intense $[M-29]^+$

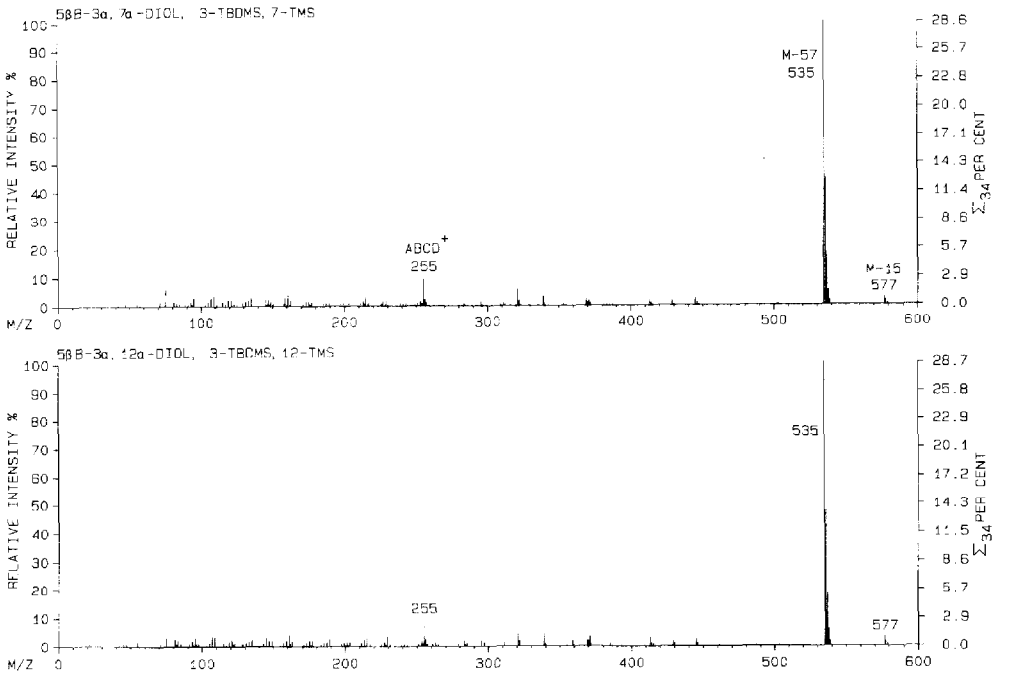

Fig. 4. Mass spectra of the 3-TBDMS, 7-TMS ether derivative of methyl chenodeoxycholate (upper) and the 3-TBDMS, 12-TMS ether derivative of methyl deoxycholate (lower).

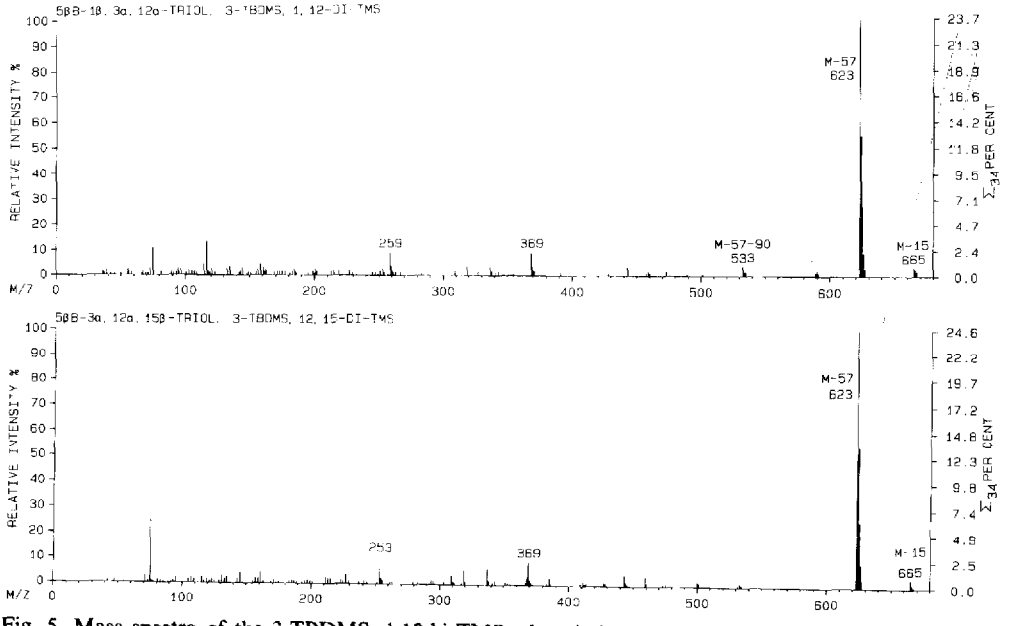

Fig. 5. Mass spectra of the 3-TBDMS, 1,12-bisTMS ether derivative of methyl 1β,3α,12α-trihydroxy-5β-cholanoate (upper) and the 3-TBDMS, 12,15-bisTMS ether derivative of methyl 3α,12α,15β-trihydroxy-5β-cholanoate (lower).

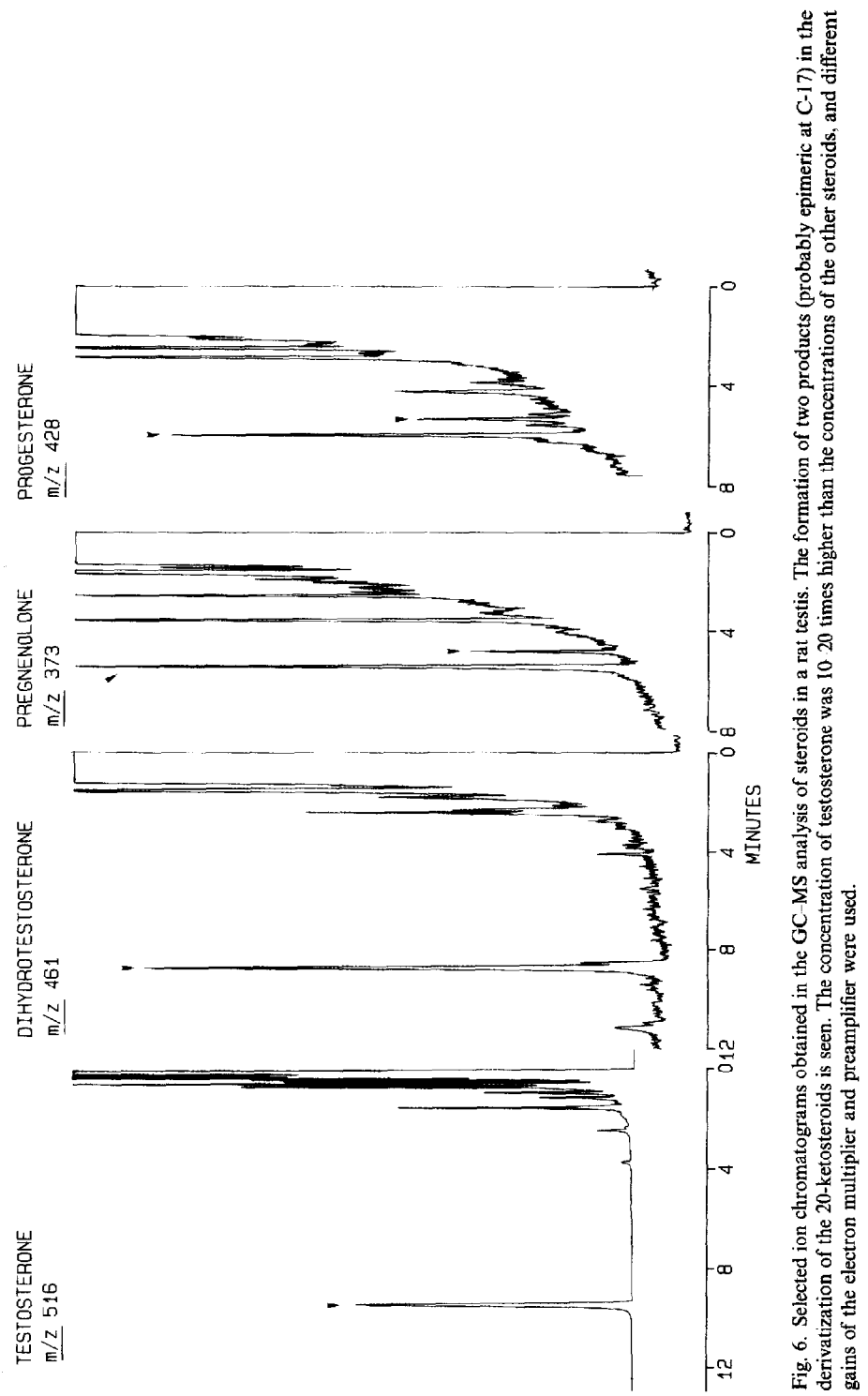

Fig. 6. Selected ion chromatograms obtained in the GC–MS analysis of steroids in a rat testis. The formation of two products (probably epimeric at C-17) in the derivatization of the 20-ketosteroids is seen. The concentration of testosterone was 10–20 times higher than the concentrations of the other steroids, and different gains of the electron multiplier and preamplifier were used.

analogous to $[M-57]^+$ in the mixed TBDMS–TMS derivatives. While the retention times of these derivatives are in the same range as those of the mixed derivatives, a lower percentage of the total ionization is carried by the $[M-29]^+$ than by the $[M-57]^+$ ions. This is particularly true for the derivative of chenodeoxycholic (3α,7α) acid. Use of mixed derivatives may therefore be advantageous for some analytical applications. By choice of appropriate reaction times it may also be possible to obtain information about bile acids with 6α- and 6β-hydroxyl groups. Mass spectra of TMS ethers of 3,6,12- and 3,7,12-trihydroxycholanoates are very similar[38], and the difference in reactivity of the 6-hydroxy group with TBDMSIM may be helpful in structure determinations when only small amounts of bile acid in a mixture are available.

Application to analysis of steroids in testis

A purified extract of testis obtained by a combination of solvent and lipophilic gel extraction[34] was subfractionated by HPLC. Four fractions were collected, the first to contain progesterone and 4-androstene-3,17-dione, the second 17β-hydroxy-5α-androstan-3-one, the third testosterone and the fourth 17-hydroxylated C_{21} steroids. These were derivatized under conditions suitable for steroids expected to be present. The derivatives were purified by HPLC. Each fraction was analysed by GC–MS with monitoring of M^+ for enol-TBDMS ethers of 3-keto-Δ^4-steroids and $[M-57]^+$ for other potentially occurring steroids. Of the C_{19} and C_{21} steroids reported to be present in rat testis[39,40], testosterone, 17β-hydroxy-5α-androstan-3-one, 3β-hydroxy-5-pregnen-20-one and progesterone were readily detected by injection of 1% of the fractions (Fig. 6). The search for 17-hydroxylated C_{21} steroids and 4-androstene-3,17-dione showed that further purification is required to permit specific analysis and injection of larger samples without overloading the capillary column. This is also needed for the more complete characterization of the mixture of steroids in rat testis which is presently being carried out.

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