RESEARCH ARTICLE

Mass spectral fragmentation analyses of isotopically labelled hydroxy steroids using gas chromatography/electron ionization low-resolution mass spectrometry: A practical approach

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Rationale: Gas chromatography coupled to electron ionization mass spectrometry (GC/EI-MS) is used for routine screening of anabolic steroids in many laboratories after the conversion of polar groups into trimethylsilyl (TMS) derivatives. The aim of this work is to elucidate the origin and formation of common and subclass-specific fragments in the mass spectra of TMS-derivatized steroids. Especially in the context of metabolite identification or analysis of designer drugs, isotopic labelling is helpful to better understand fragment ion generation, identify unknown compounds and update established screening methods.

Methods: Stable isotope labelling procedures for the introduction of $[^2H_9]$-TMS or ^{18}O were established to generate perdeuterotrimethylsilylated, mixed deuterated and ^{18}O-labelled derivatives for 13 different hydroxy steroids. Fragmentation proposals were substantiated by comparison of the abundances of isotopically labelled and unlabelled fragment ions in unit mass resolution GC/MS. Specific fragmentations were also investigated by high-resolution MS (GC/quadrupole time-of-flight MS, GC/QTOFMS).

Results: Methyl radical cleavage occurs primarily from the TMS groups in saturated androstanes and from the steroid nucleus in the case of enol-TMS of oxo or α,β-unsaturated steroid ketones. Loss of trimethylsilanol (TMSOH) is dependent on steric factors, degree of saturation of the steroid backbone and the availability of a hydrogen atom and TMSO group in the 1,3-diaxial position. For the formation of the $[M – 105]^+$ fragment ion, methyl radical cleavage predominates from the angular methyl groups in position C-18 or C-19 and is independent of the site of TMSOH loss. The common $[M – 15 – 76]^+$ fragment ion was found in low abundance and identified as $[M – CH_3 – (CH_3)_2SiH – OH]^+$. For the different steroid subclasses further diagnostic fragment ions were discussed and structure proposals postulated.

Conclusions: Stable isotope labelling of oxo groups as well as derivatization with deuterated TMS groups enables the detection of structure-related fragment ion

generation in unit mass resolution GC/EI-MS. This may in turn allow us to propose isomeric assignments that are otherwise almost impossible using MS only.

1 | INTRODUCTION

Analytical challenges for the detection of the misuse of anabolic agents are, for example, the administration of unapproved designer drugs, the use of endogenous substances, and genetic polymorphisms that lead to different metabolic patterns in the tested individual. In addition, nutritional supplements contaminated with endogenous or exogenous steroids are problematic since their intake can cause inadvertent doping.[1–3]

Until the 1980s the fragmentation behavior of mostly underivatized steroids was the subject of extensive and systematic investigation[4–6] but has not been the main focus of research since. ^{18}O-labelling is not usually used for steroid research anymore but for other fields, for example metabolomics or peptide mass spectrometry.[7,8]

In times of MS-library searches for compound identification stable isotopic labelling seems to be old fashioned and laborious. However, when it comes to mass spectral interpretation, isotopic labelling offers a suitable and practical way to trace back and explain diagnostic fragment ions in spectra recorded by gas chromatography/electron ionization mass spectrometry (GC/EI-MS) with the respective mass shifts of +1 for deuterium and +2 m/z units for oxygen-18. Misinterpretation of mass spectral data of closely related substances with similar retention times and identical molecular mass (e.g., the stereoisomers of 5ξ-androst-1-ene-3ξ,17ξ-diol, androst-4-ene-3ξ,17ξ-diol and 17ξ-hydroxy-5ξ-androstan-3-one[9]) may be reduced and characterization of specific structural features facilitated. Especially in the context of designer drugs, isotopic labelling is additionally helpful to better understand fragment ion generation, identify unknown compounds and update established screening methods.

For current GC/MS analyses, steroidal hydroxy and oxo functions are often converted into trimethylsilyl (TMS) ether and enol ether derivatives with trimethyliodosilane (TMIS, formed *in situ* reagent (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)/ ammonium iodide/ethanethiol, 1000:2:3, v/w/v).[10,11] Through silylation, steroid derivatives are more volatile and thermally stable which enhances robust detection by GC/MS and this is the preferred technique for screening of steroids, e.g., in doping control samples. Derivatization greatly affects mass spectral fragmentation[12,13]: TMS derivatives exhibit less fragmentation than their underivatized analogs which increases the abundance of diagnostic fragment ions. Many fragment ions contain one or several TMS groups or are generated through their loss or rearrangement.

Fragment ions explained by losses of TMS groups (e.g., loss of trimethylsilanol (TMSOH) [M − 90]•+) as well as specific fragment ions derived from the steroidal A-, B- or D-ring are described in the literature.[12, 14–18] However, many questions concerning the mass spectra of TMS-derivatized steroids still remain unsolved and need to be addressed.

Thus, the aim of this paper is to contribute possible explanations for allegedly "unspecific" as well as more specific fragment ions in GC/MS for different steroid subclasses. Starting from isotopic labelling techniques for steroids established in the 1960s and 1970s,[6, 19–23] more viable and faster approaches for the introduction of deuterium and [18]O were developed and optimized in the presented work and their suitability shown for mass spectral fragmentation analysis.

2 | EXPERIMENTAL

2.1 | Steroid standards

The reference standards 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone, 5α-DHT, **7**), 3α-hydroxy-5α-androstan-17-one (androsterone, **8**), 3β-hydroxyandrost-5-en-17-one (dehydroepiandrosterone, DHEA, **9**), 17β-hydroxyandrost-4-en-3-one (testosterone, **10**), 16,16,17-[²H₃]-17β-hydroxy-androst-4-en-3-one (16,16,17-[²H₃]- testosterone, **11**), 3α-hydroxy-5β-androstan-17-one (etiochalonolone, **14**), 3β-hydroxy-5β-androstan-17-one (epietiochalonolone, **15**), 3α-hydroxy-5α-estran-17-one (19-norandrosterone, **12**), and 17β-hydroxy-19-norandrost-4-en-3-one (nandrolone, **13**) were purchased from Steraloids (Newport, RI, USA). 1α,2α-[²H₂]-5α-Androstane-3α,17β-diol (**2.4.1**), 2,2,3β,4,4-[²H₅]-5α-androstane-3α,17β-diol (**2.4.2**), 1β,2β-[²H₂]- 5α-DHT (**7.4**), and 2,2,3β,4,4-[²H₅]-androsterone (**8.4**) were previously synthesized in our group. 5α-Androstane-3β,17β-diol (**1**) was synthesized from **7**, 5α-androstane-3α,17β-diol (**2**) from **8**, 5β-androstane-3α,17β-diol (**3**) from **14**, 5β-androstane-3β,17β-diol (**4**) from **15**, androst-5-ene-3β,17β-diol (**5**) from **9**, and androst-4-ene-3β,17β-diol (**6**) from **10** via reduction with NaBH₄ (see below).

2.2 | Reagents and chemicals

Water-[18]O (97 atom% [18]O), ethanethiol (97%), 2-mercaptoethanol (99%), sodium borohydride (NaBH₄) and ammonium iodide (NH₄I, 99.9%) were obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany). Toluene, diethyl ether, isopropylamine (99+%) and titanium tetrachloride (TiCl₄, 99.9%) were from Thermo Fisher Scientific (Dreieich, Germany). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany) and *N,O*-bis(trimethyl-[²H₉]-silyl)acetamide ([²H₁₈]-BSA) from Abcr GmbH (Karlsruhe, Germany).

2.3 | ^{18}O-labelling procedure

Standards **7–10** and **14–15** (1.5 mg) were dissolved in 900 μL toluene each. At a temperature of 0°C, 18 μL TiCl$_4$/toluene (1:2, v/v) and 45 μL isopropylamine were added while stirring. The mixture was incubated at 100°C for 15 min. After cooling, 900 μL water were added to obtain two phases. After centrifugation for 5 min the upper organic phase was used for further experiments. Aliquots of 30 μL (toluene phase) and 60 μL of either H$_2$O (control) or H$_2$^{18}O were evaporated at 100°C in a heating block.

2.4 | Reduction of 3- or 17-oxo groups with NaBH$_4$

For the reduction of 3- or 17-oxo groups (to obtain standards **1–6**), the residue was dissolved in 1 mL diethyl ether. NaBH$_4$ (2 mg) and 200 μL of water were added. After stirring at room temperature for 24 h, 1 mL of water was added. The extraction of the product was performed using 3 × 1 mL diethyl ether. The organic layer was evaporated at reduced pressure.

2.5 | Derivatization

Prior to GC/MS analyses, residues were derivatized with 90 μL MSTFA/TMIS reagent (MSTFA/NH$_4$I/ethanethiol, 1000:2:3, v/w/v) at 60°C for 15 min. Standards **1–13** were additionally derivatized with 80 μL [^{2}H$_{18}$]-BSA/TMIS reagent (5 μL of a saturated NH$_4$I solution in 2-mercaptoethanol added to 100 μL [^{2}H$_{18}$]-BSA) at 90°C for 30 min to achieve perdeuterotrimethylsilylation. Mixed deuterated derivatives were prepared for standards **7–13**. Residues were either first derivatized with 15 μL of [^{2}H$_{18}$]-BSA, evaporated in a gentle stream of nitrogen, and then treated with 65 μL MSTFA/TMIS reagent, or the respective residue was first left to react with 15 μL MSTFA, evaporated in a gentle stream of nitrogen, and subsequently derivatized with 65 μL [^{2}H$_{18}$]-BSA/TMIS reagent.

2.6 | Instrumentation

Unit mass resolution GC/EI-MS analyses were performed on a model 7890 gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a model 5975C single quadrupole mass-selective detector (Agilent Technologies) applying the following parameters: column: Agilent HP-Ultra 1 (17 m × 200 μm × 0.11 μm), carrier gas: helium, constant pressure: 1.14 bar, oven temperature program: 0 min

150°C, +50°C/min, 0 min 240°C, +3°C/min, 0 min 266°C, +50°C/min, 3 min 320°C, injection volume: 2 μL, split 1:20, injection temperature: 300°C, ionization: 70 eV, EI. For additional confirmation of some fragment ions, high-resolution accurate mass spectrometry was performed on an Agilent 7890 gas chromatograph coupled to an Agilent 7200 QTOF mass spectrometer. The same column and the same conditions as described before were used.

3 | RESULTS AND DISCUSSION

3.1 | Isotopic labelling

Investigation of fragmentations in steroid analysis was performed for per-TMS-derivatized analytes. As is well known, the use of TMIS as catalyst in combination with MSTFA results in the derivatization of both hydroxy and oxo groups, the latter after enolization.[24] To achieve labelling of both steroidal hydroxy and oxo groups with [^{2}H$_9$]-TMS, a labelled derivatization agent with enough silylation power was needed. [^{2}H$_9$]-MSTFA was the reagent of choice for generation of the catalyst [^{2}H$_9$]-TMIS and derivatization, but turned out to be unsuitable for in-house synthesis due to very low yields. Vouros and Harvey described a two-step procedure for the preparation of mixed TMS and [^{2}H$_9$]-TMS derivatives of hydroxy steroids in 1973.[25] As reagent they used N,N-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylsilylimidazole (TSIM) or [^{2}H$_9$]-TSIM. While unhindered and hindered hydroxy groups were amenable for derivatization therein, oxo groups were not silylated but separately derivatized to methoximes. In our study, N,O-bis(trimethyl-[^{2}H$_9$]-silyl)acetamide ([^{2}H$_{18}$]-BSA) was found to be an effective alternative although the silylation capacity of BSA is lower than that of MSTFA or BSTFA.[26] For catalyst generation the addition of a saturated solution of ammonium iodide in 2-mercaptoethanol delivered the best results. Using this combination isotopic labelling was performed straightforwardly by introduction of perdeuterotrimethylsilyl ([^{2}H$_9$]-TMS) groups on both the hydroxy and the oxo groups.

Inspired by the observation that 3-oxo steroids form imine derivatives that are easy to hydrolyse with tris(hydroxymethyl) aminomethane during work up of biological samples,[27] a method was developed to use imine intermediates for ^{18}O-labelling. For this purpose, isopropyl amine was used and cleaved off again with H$_2$^{18}O, yielding the original steroid with ^{18}O-oxo groups (Figure 1). This fast and targeted method has also successfully been used in our group for other compounds such as benzophenone derivatives.[28] In contrast to an earlier described method for ^{18}O-labelling of steroids by Lawson et al in 1969[6] as an acid-catalyzed exchange reaction of carbonyl groups with H$_2$^{18}O, this procedure allows fast and reproducible

FIGURE 1 Procedure used for ^{18}O-labelling of steroids exemplified by androsterone (**8**, educt) and 5α-androstane-3α,17β-bis-TMS (**2**, product)
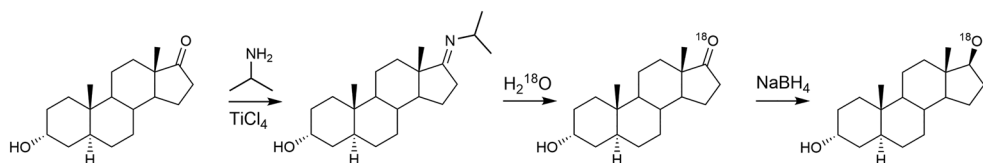
oxygen exchange, while the acid catalysis method showed a strong dependence of exchange rates on the temperature and steric environment of the carbonyl group. Using acid-catalyzed labelling for 4-ene-3-ones such as testosterone, for example, a reaction time of 72 h was needed until the desired equilibrium was achieved, while the exchange procedure described herein only required less than 2 h. To obtain ^{18}O-labelled hydroxy groups, the labelled oxo steroids were reduced with $NaBH_4$ (Figure 1).

3.2 | Mass spectral fragmentation analyses

Methyl radical cleavage ($[M – CH_3]^+$, $[M – 15]^+$), neutral loss of trimethylsilanol ($[M – TMSOH]^{\bullet+}$, $[M – 90]^{\bullet+}$) and their concerted or sequential loss ($[M – TMSOH – CH_3]^+$, $[M – 105]^+$) are common features in GC/MS spectra of trimethylsilylated hydroxy steroids. Generally, these losses are considered to be rather unspecific and an explanation of their exact formation and their origin is rarely given. These three examples together with the $[M – CH_3 – (CH_3)_2SiH – OH]^+$, ($[M – 91]^+$) ion and other diagnostic GC/MS fragment ions used for the comparison of different androgen subclasses are discussed below. Regio- and stereoselectivity were elucidated from the relative abundances of the respective isotopically labelled and unlabelled fragment ions. The percentage of contribution of the individual isotopologous fragment ions was calculated as a proportion of the total abundance of the respective fragment ion (Tables 1 and 2). Based on these findings, explanations for fragment ion generation are proposed.

3.3 | Fragment ion $[M – CH_3]^+$

Methyl radical cleavage from a TMS-derivatized steroid can originate from either one of the TMS groups or one of the angular methyl groups C-18 or C-19 of the steroid nucleus. In the mass spectra of all perdeuterotrimethylsilylated steroids 1–13 both $[M – CH_3]^+$ and $[M – CD_3]^+$ ions are observed with different abundance ratios (fragments #.3B, Tables 1 and 2).

In the mass spectra of the fully saturated androstanes 1–4 methyl radical cleavage primarily takes place from the TMS groups (Figure 2; 2.3B: $[M – CD_3]^+$ = m/z 436.4 and 1.3B, Table 1). In 5α-androstane-3β,17β-diol (1), for example, the $[M – CD_3]^+$ = m/z 436.4 ion contributes 90% and the $[M – CH_3]^+$ = m/z 439.4 ion 10% to the overall abundance (2.3B, Table 1). In case of the enol-TMS steroids 8–13 on the other hand, methyl radical loss originates predominantly from the steroid nucleus. Fragment ion $[M – CD_3]^+$ contributes less than 5% in the 3,17-bis-$[^2H_9]$-TMS and the respective mixed deuterated spectra of androsterone (8.3B, 8.5B, 8.6B, Table 2) and DHEA (9, Figure 3: m/z 432.4 (9.3), m/z 423.3 (9.5, 9.6) and Table 2: 9.3B, 9.5B, 9.6B). In the mass spectra of perdeuterotrimethylsilylated testosterone (10.3B, Table 2) and 16,16,17-$[^2H_3]$-testosterone (11.3B, Table 2) derivatives, less than 15% of the $[M – CD_3]^+$ ion is observed and less than 8% for the mixed deuterated derivatives (10.5B, 10.6B,

11.5B, 11.6B, Table 2). Earlier findings about the position of methyl cleavage in Δ4 steroids from Harvey and Vouros[29] can thus be confirmed.

Compared with its saturated counterparts 1 to 4, the double bond in androst-4-ene-3β,17β-diol (6) directs methyl radical cleavage to the steroid nucleus with the $[M – CD_3]^+$ ion observed with 23% relative abundance only (6.3B, Table 1). In androst-5-ene-3β,17β-diol (5) this cleavage is less dominant as the 5-ene isomer exhibits the ion $[M – CD_3]^+$ with 55%, which indicates that the $[M – CD_3]^+$ and $[M – CH_3]^+$ ions are produced with similar abundances (5.3B, Table 1). Interestingly, in the 5-ene-17-oxo steroid DHEA (9) 95% of the methyl loss originates from the steroid nucleus (observed as $[M – CH_3]^+$), i.e. $[M – CD_3]^+$ represents only 5% (9.3B, Table 2).

In 5α-DHT (7) the mass spectrum of the 3,17-bis-$[^2H_9]$-TMS derivative exhibits both $[M – CD_3]^+$ and $[M – CH_3]^+$ in a ratio of approximately 50:50 (7.3B, Table 2). The ratio is shifted to 84% for $[M – CD_3]^+$ in the mass spectrum of the 3-$[^2H_9]$-TMS,17-TMS derivative (7.5B, Table 2) and to 5% in the spectrum of the 3-TMS,17-$[^2H_9]$-TMS derivative (7.6B, Table 2). Thus, the methyl loss originates from either the steroid nucleus or the TMS-enol group in position C-3.

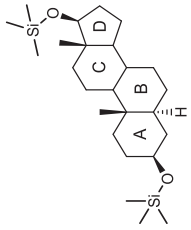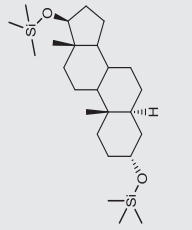## 3.4 | Fragment ion $[M – TMSOH]^{\bullet+}$

In 1972, Vouros and Harvey investigated the site of TMSOH elimination in a number of di- and trihydroxysteroids[21] and found that it is, in fact, not a random process but highly dependent on the steric environment of the respective silylated hydroxy group. Depending on the structure of the precursor for the ^{18}O-labelling procedure, the oxygen of the TMSO group was labelled either in position C-3 (steroids 1, 6–7 and 10, #.2, Table 3) or in position C-17 (steroids 2–5, 8 and 9, #.2, Table 3).

According to the literature, the 5α-androstanediols 1 and 2 preferably cleave off TMSOH from the C-17 position and, if from the A-ring, more easily from the 3α than from the 3β position.[21] ^{18}O-labelling in position C-3 for 1 and position C-17 for 2 shows that 23% (m/z 346.3) of TMSOH loss originates from C-3 in 1 (3β, 1.2C, Table 1) and 39% (m/z 348.3) in 2 (3α, 2.2C, Table 1).

For 5α-androstane-3α,17β-bis-TMS (2.1C, Table 1), the hydrogens in positions 1α and 5α are suitable for elimination together with the TMS oxygen (2.1, Table 3) due to their preferred 1,3-diaxial position.[30] Mass spectra of the 2,2,3β, 4,4-$[^2H_5]$ derivative of 2 (2.4.2C, Table 1) show that almost all the attached deuterium atoms were retained in ring A after TMSOH loss. Fragment ion $[M – 90]^+$ (m/z 351.3) makes up 86% of the signal and $[M – 91]^+$ only 14% (Figure 2, 2.4.2). If position 1α is deuterated, however, the $[M – 91]^+$ ion contributes 41% (2.4.1D, Table 1; Figure 2, 2.4.1). In the case of 1 (the 3β isomer) the 1β hydrogen is the only one available for TMSOH formation and even this is only possible from the unfavored boat conformation (1.1, Table 3). This may explain why the 3α isomer (2) results in higher abundance of TMSOH elimination from the A-ring: compared with the respective base peaks in the ^{18}O-labelled
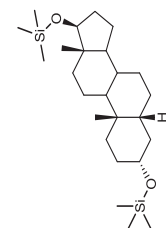
TABLE 1 Experimental data of GC/MS fragment ions of standards 1–6 and stereochemical structures with suitable hydrogens within bonding distance (<3.2 Å) of TMSO groups. Abbreviations: [M – 90]•+ = [M – TMSOH]•+, [M – 99]•+ = [M – [2H9]-TMSOH]•+, [M – 92]•+ = [M – TMS18OH]•+, [M – 92]+ = [M – CH3 – (CH3)2SiH-OH]+, [M – CD3 – 82]+ = [M – CD3 – (CD3)2SiH-OH]+, [M – 90 – CH3]+ = [M – TMSOH – CH3]+, [M – 92 – CH3]+ = [M – TMS18OH – CH3]+, [M – 99 – CH3]+ = [M – [2H9]-TMSOH – CH3]+, [M – 2×90]•+ = [M – 2×TMSOH]•+, [M – 90 – 91]+ = [M – TMSOH – CH3 – (CH3)2SiH-OH]+, [M – 2×90 – CH3]+ = [M – 2×TMSOH – CH3 – (CH3)2SiD-OH]+, [M – CD3 – 83]+ = [M – CD3 – (CD3)2SiD-OH]+, %values given in brackets represent contribution of the individual isotopologous fragment ions to the total abundance of the respective fragment ion

Number	Steroid		Ion	#1: 3,17-bis-TMS	#2: 3/17 18O	#3: 3,17-bis[2H9]TMS	#4: Other (2.4.1: 1α,2α-[2H2])	#4: Other (2.4.2: 2,2,3β,4,4-[2H5])
1	(steroid structure)	A	[M]•+	436.3	3β-18O 438.3	454.5		
		B	[M – CH3]+/[M – CD3]+	421.3	423.3	439.4 (10%) 436.4 (90%)		
		C	[M – 90]•+/[M – 99]•+	346.3 (86%)	348.3 (77%)	355.3 (89%)		
			[M – 92]•+		346.3 (23%)			
		D	[M – CH3 – 76]+ [M – CD3 – 82]+	345.3 (14%)	347.2	354.3 (11%)		
		E	[M – 90 – CH3]+ [M – 92 – CH3]+	331.2	333.2 (56%) 331.2 (44%)			
			[M – 99 – CH3]+ [M – 99 – CD3]+			340.3 (86%) 337.3 (14%)		
		F	[M – 2×90]•+	256.2 (62%)	256.2 (64%)	256.2 (66%)		
		G	[M – 90 – 91]+	255.2 (38%)	255.2 (36%)	255.2 (34%)		
		H	[M – 2×90 – CH3]+	241.2	241.1	241.2		
		I		129.1	131.1 (16%) 129.1 (84%)	138.2		
2	(steroid structure)	A	[M]•+	436.3	17β-18O 438.3	454.5	438.4	441.3
		B	[M – CH3]+/[M – CD3]+	421.3	423.3	439.5 (27%) 436.4 (73%)	423.3	426.3
		C	[M – 90]•+/[M – 99]•+	346.3 (75%)	348.3 (39%)	355.4 (81%)	348.3 (59%)	351.3 (86%)
			[M – 92]•+		346.3 (61%)			
		D	[M – CH3 – 76]+ [M – CD3 – 82]+	345.3 (25%)	347.3	354.4 (19%)	347.3 (41%)	350.3 (14%)
		E	[M – 90 – CH3]+ [M – 92 – CH3]+	331.3	333.3 (72%) 331.3 (28%)		333.2 (67%) 332.2 (33%) ([M – 91 – 15]+)	336.3 (84%) 335.3 (16%) ([M – 91 – 15]+)
			[M – 99 – CH3]+ [M – 99 – CD3]+			340.3 (92%) 337.3 (8%)		
		F	[M – 2×90]•+	256.2 (69%)	256.2 (68%)	256.2 (75%)	258.3 (59%)	261.3 (82%)
		G	[M – 90 – 91]+	255.2 (31%)	255.2 (32%)	255.2 (25%)	257.2 (41%)	260.2 (18%)
		H	[M – 2×90 – CH3]+	241.2	241.2	241.2	243.2 (62%) 242.2 (38%)	246.2 (92%) 245.2 (8%)
		I		129.1	131.1 (87%) 129.1 (13%)	138.1	129.1	129.1

(Continues)

TABLE 1 (Continued)

3 — 17β-^{18}O

	Fragment			
A	[M]•+	436.3	438.4	454.5
B	[M – CH$_3$]+/[M – CD$_3$]+	421.3	423.3	439.4 (16%) 436.4 (84%)
C	[M – 90]•+/[M – 99]•+	346.3 (92%)	348.3 (90%)	355.3 (94%)
	[M – 92]•+		346.3 (10%)	
D	[M – CH$_3$ – 76]+ [M – CD$_3$ – 82]+	345.3 (8%)	347.3	354.3 (6%)
E	[M – 90 – CH$_3$]+ [M – 92 – CH$_3$]+	331.2	333.3 (78%) 331.3 (22%)	
	[M – 99 – CH$_3$]+ [M – 99 – CD$_3$]+			340.3 (59%) 337.3 (41%)
F	[M – 2x90]•+	256.2	256.2	256.2
H	[M – 2x90 – CH$_3$]+	241.2	241.2	241.2
I		129.1	131.1 (85%) 129.1 (15%)	138.1

4 — 17β-^{18}O
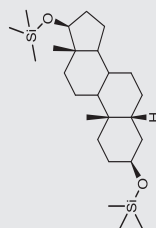
	Fragment			
A	[M]•+	436.3	438.4	454.4
B	[M – CH$_3$]+/[M – CD$_3$]+	421.3	423.3	439.4 (31%) 436.4 (69%)
C	[M – 90]•+/[M – 99]•+	346.3	348.3 (80%)	355.3
	[M – 92]•+		346.3 (20%)	
E	[M – 90 – CH$_3$]+ [M – 92 – CH$_3$]+	331.2	333.3 (83%) 331.3 (17%)	
	[M – 99 – CH$_3$]+ [M – 99 – CD$_3$]+			340.3 (90%) 337.2 (10%)
F	[M – 2x90]•+	256.2	256.2	256.2
H	[M – 2x90 – CH$_3$]+	241.2	241.2	241.2
I		129.1	131.1 (88%) 129.1 (12%)	138.1
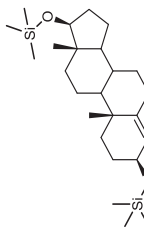
5 — 17β-^{18}O

	Fragment			
A	[M]•+	434.4	436.3	452.4
B	[M – CH$_3$]+/[M – CD$_3$]+	419.3	421.3	437.4 (45%) 434.4 (55%)
C	[M – 90]•+/[M – 99]•+	344.3 (96%)	346.2 (87%)	353.3 (97%)
	[M – 92]•+		344.2 (13%)	
D	[M – CH$_3$ – 76]+ [M – CD$_3$– 82]+	343.2 (4%)	345.3 (10%)*	352.3 (3%)
E	[M – 90 – CH$_3$]+ [M – 92 – CH$_3$]+	329.2	331.2 (87%) 329.2 (13%)	
	[M – 99 – CH$_3$]+ [M – 99 – CD$_3$]+			338.3 (94%) 335.3 (6%)
J	[M – 129]+/[M – 138]+ [M – 131]+	305.2	307.2 (84%) 305.2 (16%)	314.3
F	[M – 2x90]•+	254.2 (85%)	254.2 (80%)	254.2 (90%)
G	[M – 90 – 91]+	253.2 (15%)	253.2 (20%)	253.2 (10%)
H	[M – 2x90 – CH$_3$]+	239.2	239.2	239.2
I		129.1	131.1 (28%) 129.1 (72%)	138.1

TABLE 1 (Continued)

6

			3β-^{18}O	
A	[M]$^{\bullet+}$	434.3	436.3	452.4
B	[M − CH$_3$]$^+$/[M − CD$_3$]$^+$	419.3	421.3	437.4 (77%) 434.4 (23%)
C	[M − 90]$^{\bullet+}$/[M − 99]$^{\bullet+}$	344.3 (82%)	346.2 (16%)	353.3 (86%)
D	[M − 92]$^{\bullet+}$	343.2 (18%)	344.2 (84%)	352.3 (14%)
E	[M − CH$_3$ − 76]$^+$ [M − CD$_3$ − 82]$^+$	329.2	343.2 (25%)* 331.3 (22%) 329.2 (78%)	338.3 (93%) 335.3 (7%)
F	[M − 90 − CH$_3$]$^+$ [M − 92 − CH$_3$]$^+$	254.3 (55%)	254.2 (59%)	254.2 (69%)
G	[M − 99 − CH$_3$]$^+$ [M − 99 − CD$_3$]$^+$	253.2 (45%)	253.2 (41%)	253.2 (31%)
H	[M − 2x90]$^{\bullet+}$	239.2	239.2	239.2
K	[M − 90 − 91]$^+$	143.1	145.1	152.1
I	[M − 2x90 − CH$_3$]$^+$	129.1	129.1	138.1

mass spectra, the m/z 346.3 fragment ion of **1** (**1.2C**) has 3% and the m/z 348.3 ion (**2.2C**, Table 1) 8% relative abundance. If TMSOH elimination occurs in the D-ring, the hydrogen in position 15β and the three hydrogens from the C-18 methyl group are within bonding distance of the OTMS oxygen (**1.1**, Table 3).

The 5β-androstanediols **3** and **4** cleave off TMSOH mainly from the A-ring with the same preference for the 3α position as the 5α-androstanediols. In the mass spectra of the ^{18}O-labelled derivatives [M − 90]$^{\bullet+}$ = m/z 348.3 is observed for 3α (**3.2C**) with 90% and for 3β (**4.2C**, Table 1) with 80%. Suitable hydrogens for the neutral loss of trimethylsilanol are at positions 7α and 9α for 5β-androstane-3α,17β-bis-TMS (**3.1**, Table 3), if the A-ring is in the boat conformation and the TMSO group is located beneath the steroid backbone. In the case of 5β-androstane-3β,17β-bis-TMS (**4**) the hydrogens in positions 1β and 5β are at the right distance for elimination with TMSO from the A-ring (**4.1**, Table 3). Further confirmation will be addressed in future investigations.

If a double bond in position Δ5 is present in the steroid nucleus as in androst-5-ene-3β,17β-diol (**5**), the [M − 90]$^{\bullet+}$ fragment ion originates from the A-ring.[25, 31–33] After labelling of the C-17 oxygen, the mass spectrum shows 87% TMSOH loss from 3β ([M − 90]$^{\bullet+}$) and 13% from 17β ([M − 92]$^{\bullet+}$, **5.2C**, Table 1). This result can be explained by the stability of the resulting [M − 90]$^{\bullet+}$ ion in which a double bond is formed in conjugation to the Δ5 double bond.[21] If the A-ring is in the boat conformation, the bonding distance between the TMS-oxygen and the 1β hydrogen is reduced, making loss of TMSOH more likely (**5.1**, Table 3).

The Δ4 double bond of androst-4-ene-3β,17β-diol (**6**) also tends to direct TMSOH elimination to the A-ring. ^{18}O-labelling substantiates preferred TMSOH loss from position 3β (84% [M − 92]$^{\bullet+}$ and 16% [M − 90]$^{\bullet+}$, **6.2C**, Table 1). Pseudo-boat formation in ring A for bonding of TMSO with the 1β hydrogen is possible (**6.1**, Table 3). According to the literature, cleavage of the C-2/C-3 bond with subsequent TMSO loss is facilitated by the Δ4 double bond.[29] After bond cleavage and free rotation of the C-4/C-5 bond, the 3β-TMSO is also within bonding distance of the C-6 hydrogens.

In general, the TMS enol derivatives **7**–**13** produce [M − 90]$^{\bullet+}$ and [M − 105]$^+$ (see below) fragment ions with a much lower abundance than the diols. Due to enolization, the TMS group is attached to a sp^2 carbon and this together with the neighbouring double bond makes the steroid backbone more rigid (for example, in testosterone, **10.1**, Table 3). Suitable hydrogens for TMSOH loss are out of the required bonding distance of 3.2 Å.[30] Thus, TMSOH elimination predominates from the unlabelled TMS ether function if a TMS ether and a (^{18}O-labelled) TMS enol are present (standards **7**–**10**): In the case of androsterone (**8**), for example, 86% of the TMSOH loss originates from ring A ([M − 90]$^{\bullet+}$ = m/z 346.3) and only 14% from ring D ([M − 92]$^{\bullet+}$ = m/z 344.4, **8.2C**, Table 2). The same applies to fragment ions [M − 105]$^+$ = m/z 331.3 (93%) and [M − CH$_3$ − TMS^{18}OH]$^+$ = [M − 107]$^+$ (7%, **8.2E**, Table 2). In the mass spectrum of DHEA (**9**) the [M − 105]$^+$ = m/z 329.2 ion (**9.2E**, Table 2; 9.2, Figure 3) also mainly originates from the A-ring and in 5α-DHT (**7**, **7.2E**, Table 2) from the D-ring.

TABLE 2 Experimental data of GC/MS fragment ions of standards **7–13** (see Table 1 for abbreviations)
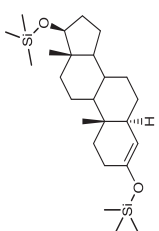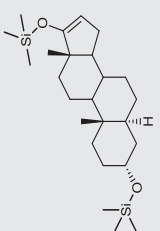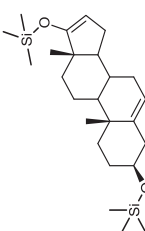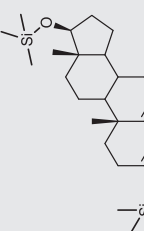
Number	Steroid		Ion	#1: 3,17-bis-TMS	#2: 3/17 18O	#3: 3,17-bis[2H9]TMS	#4: Other	#5: 3[2H9]-TMS, 17-TMS	#6: 3-TMS, 17[2H9]-TMS
7	(steroid structure)				3-18O		1β,2β-[2H2]		
		A	[M]•+	434.3	436.3	452.4	436.3	443.4	443.4
		B	[M − CH3]+ [M − CD3]+	419.3	421.3	437.4 (54%) / 434.4 (46%)	421.3	428.3 (16%) / 425.3 (84%)	428.3 (95%) / 425.3 (5%)
		C	[M − 90]•+ [M − 99]•+	344.3 (44%)	346.3 (63%)	353.3 (64%)	346.3 (48%)	353.3 (69%)	353.3 (37%)
			[M − 92]•+		344.2 (37%)			344.3 (31%)	344.3 (63%)
		D	[M − CH3 − 76]+ [M − CD3 − 82]+	343.2 (56%)	345.2 (69%)*	352.3 (36%)	345.3 (52%)	352.3 (55%)*	343.2 (40%)*
		E	[M − 90 − CH3]+ [M − 92 − CH3]+	329.2	331.2 (64%) / 329.2 (36%)		331.3	338.3 (71%)	338.3 (23%)
			[M − 99 − CH3]+ [M − 99 − CD3]+			338.3 (91%) / 335.3 (9%)		329.2 (29%)	329.2 (77%)
		F	[M − 2x90]•+	254.2 (71%)	254.2 (57%)	254.2 (80%)	256.3 (52%)	254.2 (73%)	254.2 (76%)
		G	[M − 90 − 91]+	253.2 (29%)	253.2 (43%)	253.2 (20%)	255.3 (48%)	253.2 (27%)	253.2 (24%)
		H	[M − 2x90 − CH3]+	239.2	239.1	239.2	241.2	239.2	239.2
		K		143.1	145.1	152.1	145.2 (49%) / 144.2 (51%)	152.1	143.1
		L		127.1	129.1	136.1	129.1 (62%) / 128.1 (38%)	136.1	127.1
8	(steroid structure)				17-18O		2,2,3β,4,4-[2H5]		
		A	[M]•+	434.3	436.4	452.4	439.3	443.4	443.4
		B	[M − CH3]+ [M − CD3]+	419.2	421.3	437.4 (96%) / 434.4 (4%)	424.3	428.4 (97%) / 425.3 (3%)	428.4 (96%) / 425.3 (4%)
		C	[M − 90]•+ [M − 99]•+	344.2 (39%)	346.3 (86%)	353.3 (60%)	349.3 (33%)	353.3 (70%)	353.3 (75%)
			[M − 92]•+		344.4 (14%)			344.2 (30%)	344.2 (25%)
		D	[M − CH3 − 76]+ [M − CD3 − 82]+	343.3 (61%)	345.3 (65%)*	352.3 (40%)	348.3 (67%)	343.2 (49%)*	352.2 (61%)*
		E	[M − 90 − CH3]+ [M − 92 − CH3]+	329.2	331.3 (93%) / 329.2 (7%)		334.2 (77%) / 333.3 (23%) ([M − 91 − 15]+)		338.3
			[M − 99 − CH3]+			338.3		329.2	
		F	[M − 2x90]•+	254.2 (46%)	254.2 (35%)	254.2 (46%)	259.2 (44%)	254.2 (40%)	254.2 (47%)

TABLE 2 (Continued)

Number	Steroid	Ion	#.1: 3,17-bis-TMS	#.2: 3/17 ^{18}O	#.3: 3,17-bis$[^2H_9]$TMS	#.4: Other	#.5: 3$[^2H_9]$-TMS, 17-TMS	#.6: 3-TMS, 17$[^2H_9]$-TMS
		G $[M - 90 - 91]^+$	253.2 (54%)	253.3 (65%)	253.2 (54%)	258.1 (56%)	253.1 (60%)	253.1 (53%)
		H $[M - 2\times90 - CH_3]^+$	239.1	239.2	239.2	244.2 (80%) 243.2 (20%)	239.2	239.2
		M	169.1	171.1	178.2	169.1	169.1	178.2
9				(17-^{18}O)				
		A $[M]^{•+}$	432.3	434.4	450.4		441.4	441.4
		B $[M - CH_3]^+$ $[M - CD_3]^+$	417.2	419.3	435.4 (95%) 432.4 (5%)		426.3 (95%) 423.3 (5%)	426.3 (97%) 423.3 (3%)
		C $[M - 90]^{•+}$ $[M - 99]^{•+}$ $[M - 92]^{•+}$	342.2 (79%)	344.2 (72%) 342.2 (28%)	351.3 (83%)		351.2 (24%) 342.3 (76%)	351.3 (87%) 342.2 (13%)
		D $[M - CH_3 - 76]^+$ $[M - CD_3 - 82]^+$	341.2 (21%)	343.2 (38%)*	350.3 (17%)		341.2 (32%)*	350.3 (35%)*
		E $[M - 90 - CH_3]^+$ $[M - 92 - CH_3]^+$	327.2	329.2 (87%) 327.2 (13%)	336.3		336.3 (4%) 327.2 (96%)	336.3 (96%) 327.2 (4%)
		J $[M - 129]^+$ $[M - 138]^+$	303.1	305.2	312.3		303.2	312.2
		H $[M - 2\times90 - CH_3]^+$	237.1	237.2	237.2		237.2	237.2
		M	169.1	171.1	178.2		169.1	178.2
		I	129.1	129.1	138.1		138.1	129.1
10				(3-^{18}O)				
		A $[M]^{•+}$	432.3	434.3	450.4		441.4	441.3
		B $[M - CH_3]^+$ $[M - CD_3]^+$	417.3	419.3	435.4 (85%) 432.4 (15%)		426.3 (93%) 423.3 (7%)	426.3 (93%) 423.3 (7%)
		C $[M - 90]^{•+}$ $[M - 99]^{•+}$ $[M - 92]^{•+}$	342.2 (61%)	344.3 (82%) 342.2 (18%)	351.3 (62%)		351.3 (94%) 342.2 (6%)	351.3 (8%) 342.2 (92%)
		D $[M - CH_3 - 76]^+$ $[M - CD_3 - 82]^+$	341.0 (39%)	343.3 (46%)*	350.3 (38%)		350.3 (42%)*	341.2 (36%)
		E $[M - 90 - CH_3]^+$ $[M - 92 - CH_3]^+$	327.2	329.2 (80%) 327.2 (20%)			336.3 (92%)	336.3 (10%)
		$[M - 99 - CH_3]^+$ $[M - 99 - CD_3]^+$			336.3 (95%) 333.3 (5%)		327.2 (8%)	327.2 (90%)
		H $[M - 2\times90 - CH_3]^+$	237.2	237.2	237.2		237.2	237.2
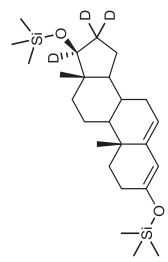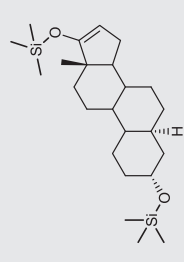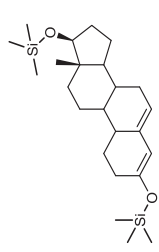
TABLE 2 (Continued)

Number	Steroid		Ion	#1: 3,17-bis-TMS	#2: 3/17 ^{18}O	#3: 3,17-bis[^{2}H$_9$]TMS	#4: Other	#5: 3[^{2}H$_9$]-TMS, 17-TMS	#6: 3-TMS, 17[^{2}H$_9$]-TMS
11		N		301.2	303.2	310.3		310.3	301.2
		O		209.1	211.2	218.2		218.2	209.1
		P		208.1	210.1	217.2		217.2	208.1
		A	[M]•+	435.3		453.4		444.4	444.4
		B	[M − CH₃]+ [M − CD₃]+	420.3		438.4 (86%) 435.4 (14%)		429.3 (92%) 426.3 (8%)	429.3 (92%) 426.3 (8%)
		C	[M − 90]•+ [M − 99]•+	345.2 (55%)		354.3 (54%)		354.3 (93%) 345.3 (7%)	354.3 (8%) 345.3 (92%)
		D	[M − CH₃ − 76]+ [M − CD₃ − 82]+	344.2 (45%)		353.3 (46%)		353.3 (49%)*	344.2 (46%)*
		E	[M − CH₃ − 77]+ [M − CD₃ − 83]+	343.2 (24%)*		352.3 (19%)*		352.3 (23%)*	343.2 (17%)*
			[M − 90 − CH₃]+	330.1		339.3 (82%)		339.3 (91%)	339.3 (9%)
			[M − 99 − CH₃]+ [M − 99 − CD₃]+			336.3 (9%)		330.2 (9%)	330.2 (91%)
		H	[M − 2x90 − CH₃]+	240.1		240.2		240.2	240.2
		N		302.2		311.3		311.3	302.2
		O		301.2		310.3		310.3	301.2
		P		209.1		218.2		218.2	209.1
				208.1		217.2		217.2	208.1
12		A	[M]•+	420.3		438.4		429.3	429.3
		B	[M − CH₃]+ [M − CD₃]+	405.3		423.3 (96%) 420.4 (4%)		414.3 (97%) 411.3 (3%)	414.3 (99%) 411.3 (1%)
		C	[M − 90]•+ [M − 99]•+	330.2 (72%)		339.3 (57%)		339.3 (45%) 330.2 (55%)	339.3 (21%) 330.2 (79%)
		D	[M − CH₃ − 76]+ [M − CD₃ − 82]+	329.0 (28%)		338.3 (43%)		329.2 (47%)	338.3 (55%)
		E	[M − 90 − CH₃]+	315.2		324.3		324.3 (5%) 315.2 (95%)	324.3 (99%) 315.2 (1%)
		F	[M − 2x90]•+	240.1 (41%)		240.2 (52%)		240.2 (54%)	240.2 (48%)
		G	[M − 90 − 91]+	239.1 (59%)		239.2 (48%)		239.2 (46%)	239.2 (52%)
		H	[M − 2x90 − CH₃]+	225.2		225.2		225.2	225.2
		M		169.1		178.2		169.1	178.2

TABLE 2 (Continued)

Number	Steroid		Ion	#1: 3,17-bis-TMS	#2: 3/17 ^{18}O	#3: 3,17-bis[$^2H_9$]TMS	#4: Other	#5: 3[$^2H_9$]-TMS, 17-TMS	#6: 3-TMS, 17[$^2H_9$]-TMS
13		A	$[M]^{\bullet+}$	418.3		436.4		427.3	427.3
		B	$[M - CH_3]^+$ $[M - CD_3]^+$	403.2		421.4 (66%) 418.3 (34%)		412.3 (85%) 409.3 (15%)	412.3 (86%) 409.3 (14%)
		C	$[M - 90]^{\bullet+}$ $[M - 99]^{\bullet+}$	328.2 (54%)		337.3 (58%)		337.3 (53%)	328.2 (56%)
		D	$[M - CH_3 - 76]^+$ $[M - CD_3 - 82]^+$	327.2 (46%)		336.3 (42%)		336.3 (47%)	327.2 (44%)
		E	$[M - 90 - CH_3]^+$	313.2		322.3		322.3	313.2
		H	$[M - 99 - CH_3]^+$ $[M - 99 - CD_3]^+$	223.2		223.1		223.2	223.1
		N	$[M - 2\times90 - CH_3]^+$	287.2		296.2		296.2	287.2
		P		194.1		203.2		203.2	194.1

*Compared with respective mass + 1 m/z unit.

Testosterone (**10**) and 16,16,17-[$^2H_3$]-testosterone (**11**) cleave off TMSOH (m/z 351.3 (94%, **10.5C**) and m/z 354.3 (93%, **11.5C**, Table 2) when the C-3 TMS enol ether is labelled and [$^2H_9$]-TMSOH (m/z 342.2 (92%, **10.6C**) and m/z 345.3 (92%, **11.6C**, Table 2) when the C-17 TMS ether is labelled with deuterium, confirming the dominant elimination from the D-ring. No deuterium from C-16 or C-17 is lost with this elimination in the case of the 16,16,17-[$^2H_3$]-labelled derivative **11** (see below). ^{18}O-labelling of the A-ring of testosterone (**10**) supplies further evidence for the preferred loss of TMSOH from C-17: the $[M - 90]^{\bullet+}$ = m/z 344.3 fragment ion is observed with 82% and $[M - 92]^{\bullet+}$ = m/z 342.3 with 18% in the respective mass spectrum (**10.2C**, Table 2).

3.5 | Fragment ion $[M - CH_3 - TMSOH]^+$

Aberrant from the above mentioned, the $[M - CH_3 - TMSOH]^+ = [M - 105]^+$ ion is primarily shifted to $[M - CH_3 - [^2H_9]\text{-TMSOH}]^+ = [M - 114]^+$ in the perdeutero trimethylsilylated spectra of all standards (**#.3E**, Tables 1 and 2). Even if, for example, in the spectra of the androstanediols **1–4** $[M - CD_3]^+$ (m/z 436.4) is more abundant than $[M - CH_3]^+$ (m/z 439.5), the $[M - CD_3 - [^2H_9]\text{-TMSOH}]^+ = [M - 117]^+$ ion (m/z 337.3, **1.3E**, **2.3E**, **4.3E**, Table 1) is observed with very low abundance (**2**, Figure 2, 2.3); it represents less than 15%, except for **3** (41%, **3.3E**, Table 1). In the course of future studies, androstanediol derivatives doubly labelled with ^{18}O and [$^2H_9$]-TMS may be synthesized. If a $[M - 119]^+$ fragment ion is observed for 5β-androstane-3α-^{18}O,3α,17β-bis-[$^2H_9$]-TMS (**3**), for example, the cleaved [$^2H_9$]-TMSOH group must originate from the A-ring. Overall, the findings shown in Table 1, however, indicate that for the formation of the $[M - 105]^+/[M - 114]^+$ fragment ion the required methyl radical cleavage predominates from one of the angular methyl groups in position C-18 or C-19 and not from the TMS methyl groups.

The mixed 3-[$^2H_9$]-TMS,17-TMS derivatives of 5α-DHT (**7.5**), testosterone (**10.5**) and 16,16,17-[$^2H_3$]-testosterone (**11.5**, Table 3) also produce a $[M - CD_3 - TMSOH]^+ = [M - 108]^+$ fragment ion with relative abundances of 15%, 4% and 5%, respectively, compared with $[M - 105]^+$ (not included in Table 1). In these cases, a CD_3 radical from the [$^2H_9$]-TMS enol group at position C-3 is cleaved off together with the TMSOH group from position C-17. In the mass spectra of the two partially deuterated DHEA derivatives (**9.5E**, **9.6E**, Table 1), neither the $[M - 117]^+$ nor the $[M - 108]^+$ fragment ion is detected, probably due to the low abundance of the $[M - CD_3]^+$ ion (Figure 3, 9.5, 9.6).

The spectra of the mixed deuterated derivatives of 5α-DHT (**7.5**, **7.6**), DHEA (**9.5**, **9.6**), testosterone (**10.5**, **10.6**), and 16,16,17-[$^2H_3$]-testosterone (**11.5**, **11.6**, Table 3) substantiate the cleavage of $^{\bullet}CH_3$-TMSOH ($[M - 105]^+$ or $[M - 114]^+$) from the respective TMS ether and not the enol ether group. For example, if the [$^2H_9$]-TMS group is attached to the A-ring (e.g., in the case for the 3-[$^2H_9$]-TMS,17-TMS derivatives of DHEA (**9**)) the $[M - 114]^+$ ion is detected with 96% relative abundance (m/z 327.2, **9.5E**, Table 2;
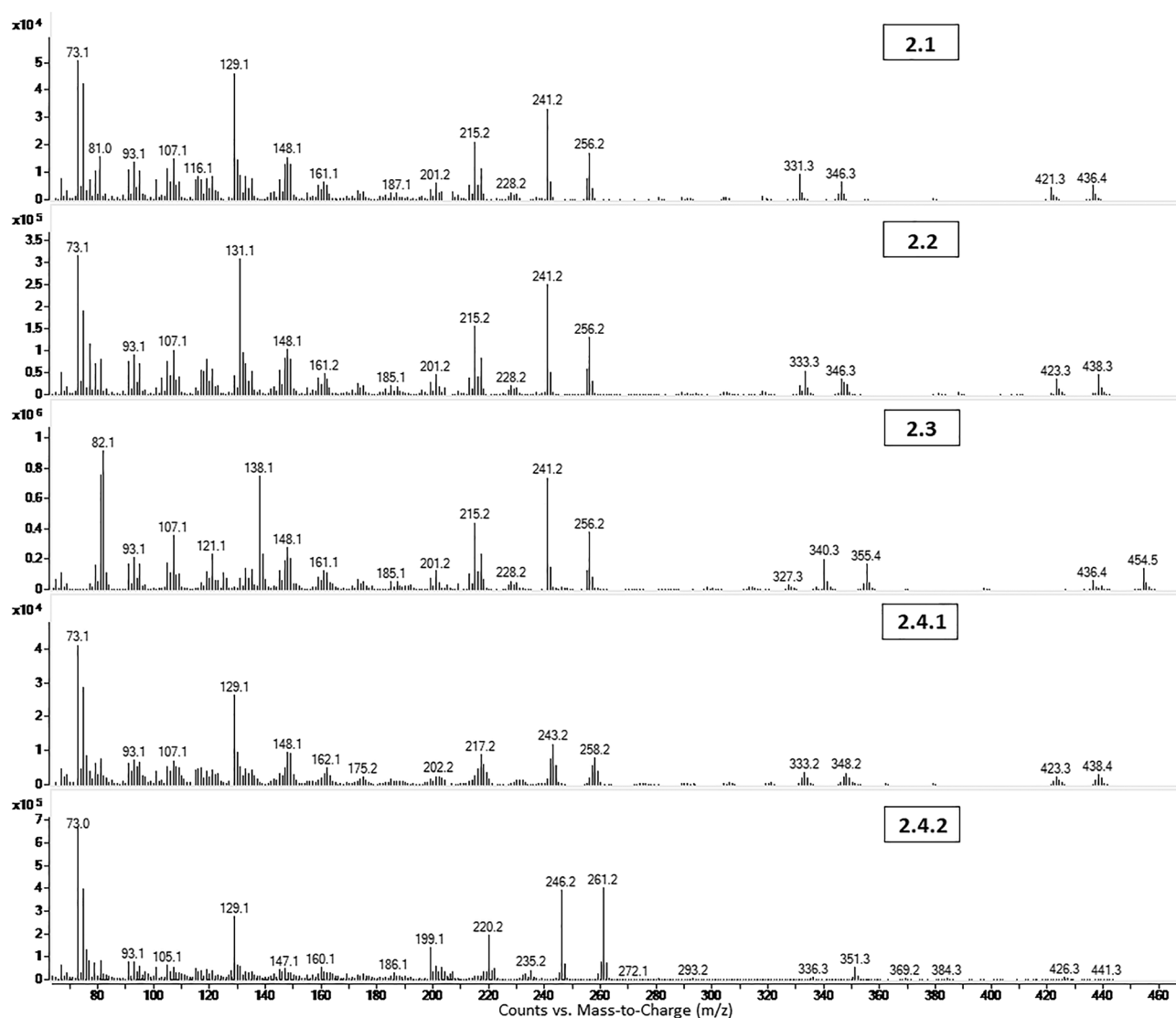
FIGURE 2 GC/MS spectra of 5α-androstane-3α,17β-diol (**2**) as bis-d_0-TMS (2.1), 17β-^{18}O-bis-TMS (2.2), 3,17-bis-[^{2}H$_9$]-TMS (2.3), 1α,2α-[^{2}H$_2$]-bis-TMS (2.4.1) and 2,2,3β,4,4-[^{2}H$_5$]-bis-TMS (2.4.2) derivatives

Figure 3, 9.5). If, however, the TMS group in position C-17 is labelled (3-TMS,17-[^{2}H$_9$]-TMS derivative of DHEA, **9.6E**, Table 2), the [M – 105]$^+$ ion is primarily formed (Figure 3, 9.6: m/z 336.3).

In the mass spectra of the ^{18}O-labelled steroids **1–9** the [M – CH$_3$ – TMS^{18}OH]$^+$ = [M – 107]$^+$ ion can be observed in addition to [M – 105]$^+$, indicating that there are several routes of formation. In the case of steroids **4–7** the [M – 107]$^+$ ion is generated with approximately the same ratio as [M – 92]$^{•+}$ ([M – TMS^{18}OH]$^{•+}$); for example, m/z 329.2 (36%, **7.2E**) and m/z 344.2 (37%, **7.2C**, Table 2) for 5α-DHT (**7**). The ^{18}O-labelled TMS derivatives of androsterone (**8**) and DHEA (**9**) predominantly cleave off TMSOH from the unlabelled A-ring to form the [M – 105]$^+$ ion. The [M – 107]$^+$ fragment ion is detected with 7% relative abundance (m/z 329.2 for androsterone, **8.2E**) and 13% (m/z 327.2 for DHEA, **9.2E**), which is half the relative abundance for the respective [M – 92]$^{•+}$ ions (**8.2C**, **9.2C**, Table 2). For the androstanediols **1–3** no clear correlation between these fragment ions can be made (Figure 2, 2.2). Overall, these findings

show that the [M – 90]$^{•+}$ and [M – 105]$^+$ fragment ions (plus [M – 92]$^{•+}$ and [M – 107]$^+$ for the ^{18}O-labelled derivatives) are generated independently from each other and can originate from different TMS groups within the steroid backbone.

Comparisons of the mass spectra of androsterone (**8**) and testosterone (**10**) with their respective 19-nor analogs (**12**, **13**) show that the [M – 105]$^+$ and [M – 114]$^+$ fragment ions (for the perdeuterotrimethylsilylated derivatives) are generated with similar ratios (for mass spectra, see supporting information). The [M – 15]$^+$ ion (m/z 419.2, **8.1B**) is the base peak in the mass spectrum of androsterone (**8**), and the abundance of the [M – 105]$^+$ (m/z 329.2, **8.1E**, Table 2) fragment ion is 62% of the base peak. In the case of the 19-norandrosterone (**12.1B**) spectrum the abundance of the [M – 105]$^+$ (m/z 315.1, **12.1E**) ion is 51% of the base peak [M – 15]$^+$ (m/z 405.3, **12.1B**, Table 2). The fragment ion [M – 15]$^+$ is also the base peak in the mass spectrum of perdeuterotrimethylsilylated 19-norandrosterone (**12**), where [M – 18]$^+$ is observed with only 5%
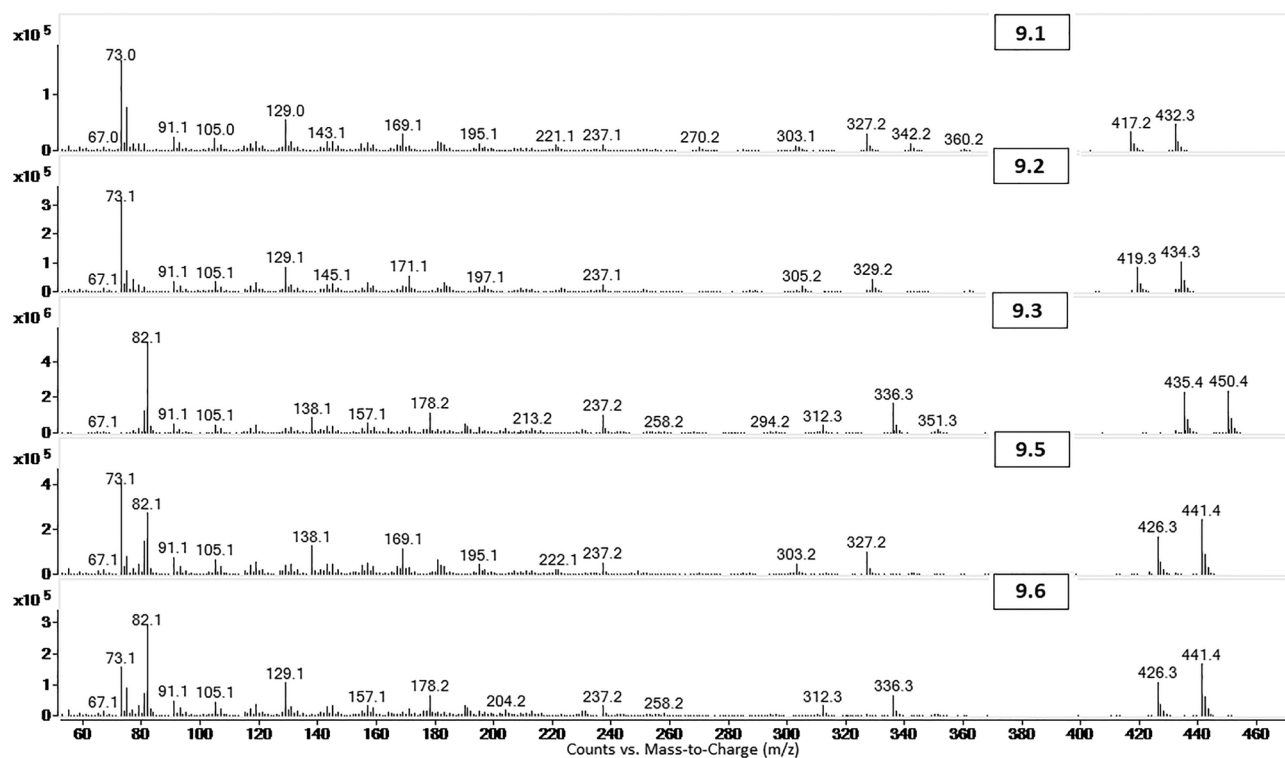
FIGURE 3   GC/MS spectra of DHEA (**9**) as bis-d_0-TMS (9.1), 17β-^{18}O-bis-TMS (9.2), 3,17-bis-[^{2}H$_9$]-TMS (9.3), 3-[^{2}H$_9$]-TMS,17-TMS (9.5), and 3-TMS,17-[^{2}H$_9$]-TMS (9.6) derivatives

(**12.3B**, Table 2) of the overall abundance. These findings suggest that the methyl group cleaved off for the generation of the [M − 15]$^+$ and [M − 105]$^+$/[M − 114]$^+$ ions is derived from the C-18 group in 19-norandrosterone (**12**) and thus probably also in androsterone (**8**). Similarly, the [M − CD$_3$ − [^{2}H$_9$]-TMSOH]$^+$ = [M − 117]$^+$ fragment ion is observed with 5% relative abundance in the spectra of perdeuterotrimethylsilylated testosterone (m/z 333.3, **10.3E**) and 19-nortestosterone (m/z 319.2, **13.3E**, Table 2), suggesting that the [M − CH$_3$ − [^{2}H$_9$]-TMSOH]$^+$ = [M − 114]$^+$ fragment ion is also predominantly formed for both steroids by loss of the C-18 methyl group. However, when comparing the abundances of the [M − CD$_3$]$^+$ fragment ion, the 3,17-bis-[^{2}H$_9$]-TMS derivative of 19-nortestosterone (**13.3**, Table 3) generates approximately twice as much (34%, **13.3B**) as the respective testosterone derivative (15%, **10.3B**, Table 2). Overall, loss of a methyl radical predominates from the steroid nucleus even if only one angular methyl group is present.

3.6 | Fragment ion [M − CH$_3$ − (CH$_3$)$_2$SiH-OH]$^+$

Undervalued as a fragment ion until now, in all the analyzed mass spectra of unlabelled steroids a [M − 91]$^+$ ion is observed next to the [M − 90]$^{+\cdot}$ion. In the mass spectra of steroids **7** and **8**, the [M − 91]$^+$ fragment ion (**7.1D**, **8.1D**) is even more abundant than the [M − 90]$^{+\cdot}$ ion (**7.1C**, **8.1C**, Table 2). This fragment ion may be explained by loss of a methyl radical from a TMS function followed by neutral loss of the

remaining dimethylsilyloxy group plus two additional hydrogen atoms from the steroid backbone ((CH$_3$)$_2$-SiH-OH, exact mass 76.0344). The ion can thus be described as [M − CH$_3$ − (CH$_3$)$_2$SiH-OH]$^+$ = [M − 15 − 76]$^+$. If the respective TMS group is deuterated, the observed loss is shifted to [M − CD$_3$ − (CD$_3$)$_2$SiH-OH]$^+$ = [M − 18 − 82]$^+$ (**7.3D**, **8.3D**, Table 2). GC/QTOF accurate mass measurement of the [^{2}H$_9$]-TMS-labelled testosterone derivatives provides ions at m/z 350.2851 (**10.3D**), 350.2867 (**10.5D**) and 341.2289 (**10.6D**, Tables 2 and 4), which are in accordance with the proposed fragment ion generation pathway (for mass spectra, see supporting information).

In the case of the ^{18}O-labelled derivatives, formation of both [M − 91]$^+$ (**10.2D**) and [M − 93]$^+$ = [M − CH$_3$ − (CH$_3$)$_2$-SiH-^{18}OH]$^+$ ions is observed, showing that there is no preferred cleavage site (from C-3 or C-17). For steroids **1–2**, **5–8** and **12** an additional [M − 181]$^+$ fragment ion is detected, corresponding to losses of TMSOH together with CH$_3$-(CH$_3$)$_2$SiH-OH (**#.1G**, Tables 1 and 2). In the mass spectrum of 16,16,17-[^{2}H$_3$]-testosterone (**11**), there are two possible explanations for the loss of 91 Da: either loss of TMSOD (instead of TMSOH) from the D-ring or cleavage of CH$_3$–(CH$_3$)$_2$SiH-OH. In the QTOF spectrum an ion at m/z 344.2472 is observed (**11.1D**, Tables 2 and 4). If TMSOD is cleaved off, the theoretical exact mass of the resulting fragment ion C$_{22}$H$_{32}$D$_2$OSi$^+$ is m/z 344.2499, with an experimental mass error of 7.8 ppm. With a mass measurement error of only 3.2 ppm, formation of the C$_{22}$H$_{30}$D$_3$OSi$^+$ (**11.1D**, Table 4) ion is thus more likely. The accurate masses of the [^{2}H$_9$]-TMS derivatives also comply with this finding (**11.3D**, **11.5D**,

11.6D, Table 4). Because the mass errors of the differently labelled derivatives are lower for the [M – 15 – 76]⁺ ion than for the [M – TMSOD]⁺ ion and 16,16,17-[²H₃]-testosterone produces in total only 6% more of the [M – 91]⁺ ion than testosterone, the required hydrogen for TMSOH loss from the D-ring probably originates from somewhere other than positions C-16 and C-17. A [M – 15 – 77]⁺ (**11.1D**, Table 2) ion is additionally observed in low abundance in the mass spectrum of 16,16,17-[²H₃]-testosterone and corresponds to [M – CH₃-(CH₃)₂SiD – OH]⁺.

3.7 | Fragment ions representing structural specificity

3.7.1 | m/z 129 and [M – 129]⁺

In all four diastereomeric androstanediols **1–4**, (2.1, Figure 2) m/z 129.1 represents the base peak and is found to originate from the D-ring (84–88% in the ¹⁸O-labelled mass spectra, **#.2I**, Table 1). According to the literature, this fragment ion is indicative of TMS-derivatized 3- or 17-hydroxy functions of steroids from A- or D-ring cleavage, respectively.[16, 19, 22] When the 3β-oxygen in 5α-androstane-3β,17β-diol (**1**) is exchanged with ¹⁸O, almost no mass shift is observed (84% m/z 129.1, **1.2I**, Table 1), while the ion is mainly shifted to m/z 131.1 in the 17β-oxygen-labelled derivatives **2–4** (2.2, Figure 2). In the mass spectrum of the 2,2,3β,4,4-[²H₅] derivative of 5α-androstane-3α,17β-diol (**2**) the m/z 129.1 ion is not shifted (**2.4I**, Table 1; 2.4.2, Figure 2). This substantiates generation of this fragment ion from somewhere other than the A-ring. These findings comply with an earlier study from Middleditch,[34] stating that 85% of the m/z 129 ions from **2** originate from the D-ring. In the case of androst-4-ene-3β,17β (**6**) the m/z 129.1 ion has a low abundance and also originates from the D-ring, as no mass shift is observed when the C-3 oxygen is labelled.

If the D-ring is labelled with ¹⁸O, no mass shift is detectable for the m/z 129.1 fragment ion in the mass spectrum of DHEA (**9.2I**, Table 2; 9.2, Figure 3). In contrast, in the mass spectrum of androst-5-ene-3β,17β-diol (**5.2I**, Table 1) 72% relative abundance m/z 129.1 and 28% relative abundance m/z 131.1 can be observed in the case of ¹⁸O-labelling in the D-ring, indicating that this ion originates predominantly from the A-ring but is also formed from the D-ring. In the mass spectra of 3β-trimethylsilyloxy Δ5-steroids such as DHEA (**9**) and androst-5-ene-3β,17β-diol (**5**) [M – 129]⁺ ions are observed in addition to the m/z 129 ion.[19, 34, 35] In DHEA the [M – 129]⁺ fragment ion is detected at m/z 303.1 (**9.1J**, Table 2; 9.1, Figure 3). It is shifted to m/z 305.2 in the spectrum after ¹⁸O labelling (**9.2J**, Table 2; 9.2 Figure 3), to m/z 312.3 for the 3,17-bis-[²H₉]-TMS (**9.3J**, Table 2, 9.3 Figure 3) and the 3-TMS, 17-[²H₉]-TMS (**9.6J**, Table 2, 9.6, Figure 3) derivatives. These findings comply with the assumption that this fragment ion is formed from the remainder of the molecule including the D-ring after the m/z 129 ion is cleaved off from the A-ring. To a minor extent the [M – 129]⁺ fragment ion can also represent the A-ring as

¹⁸O-labelling data of **5** shows the m/z 307.2 ([M – 129]⁺, D-ring still present) ion with 84% relative abundance, while the m/z 305.2 ([M – 131]⁺, A-ring still present) ion represents 16% of the overall abundance (**5.2J**, Table 1).

3.7.2 | m/z 127

The m/z 127.1 fragment ion is only found in the mass spectrum of 5α-DHT (**7**). Because it is shifted to m/z 129.1 in the spectrum of the ¹⁸O-labelled, to m/z 129.1 (**7.2L**, 62%) and m/z 128.1 (**7.4L**, 38%, loss of one deuterium atom) for the 1β,2β-[²H₂] and to m/z 136.1 for the 3-[²H₉]TMS, 17-TMS (**7.5L**, Table 2) derivatives, it is proposed to originate from the A-ring and might be analogous to the m/z 129 ion with an additional double bond. GC/QTOF data supports the proposed elemental composition of C₆H₁₁OSi⁺ for this ion (**7.1L–7.6L**, Table 4).
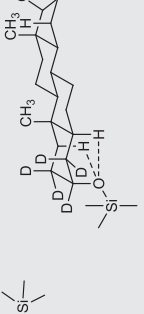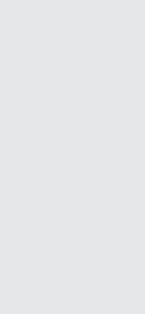
3.7.3 | m/z 143
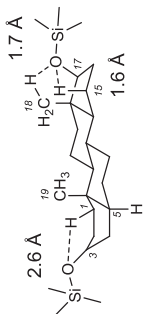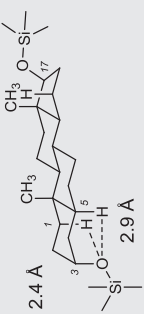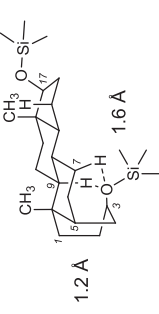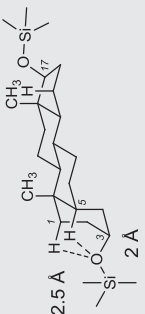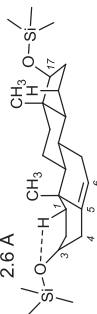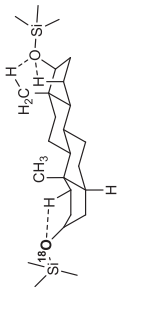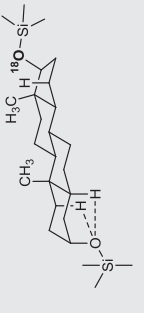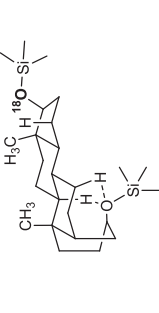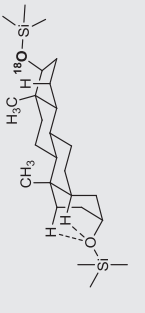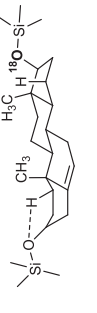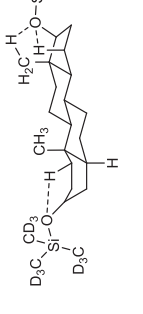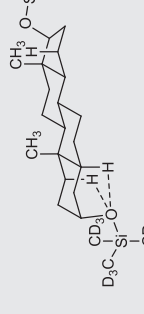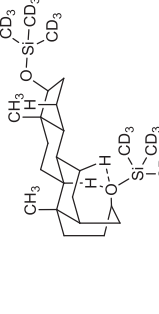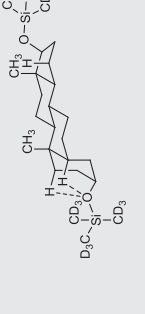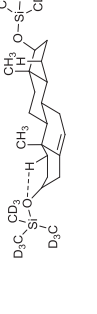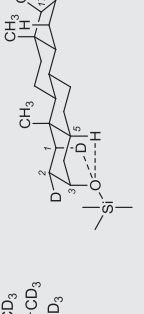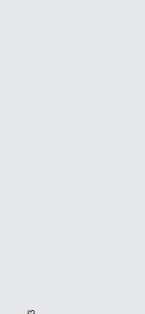
The fragment ion at m/z 143.1 in the mass spectrum of androst-4-ene-3β,17β-diol (**6**) is attributed to the A-ring. It is shifted to m/z 145.1 (**6.2K**) after ¹⁸O-labelling of the oxygen at C-3 and to m/z 152.1 (**6.3K**, Table 1) in the mass spectrum of the perdeuterotrimethylsilylated derivative. This ion is usually described in the literature as a marker for 17α-alkylated synthetic steroids[16] but it also occurs in the mass spectra of 3-keto-enol steroid derivatives such as 5α-DHT or 3-hydroxyandrost-1- or -4-ene steroids.[9] m/z 143 is described as a resonance-stabilized 3-(O-trimethylsilyl)but-1-ene ion with the elemental composition C₇H₁₅OSi⁺.[36] The exact formation of this ion in this case still needs to be determined but the double bond in ring A seems to play an important role.

As mentioned above, m/z 143.1 may also originate from the A-ring in the mass spectrum of 5α-DHT (**7.1K**). It is shifted to m/z 152.1 (**7.5K**) when [²H₉]-TMS is attached to position C-3 but no mass shift is observed when the C-17 TMS group is deuterated (**7.6K**, Tables 2 and 4). In the case of the 1β,2β-[²H₂] derivative (**7.4K**, Table 2), two fragment ions, m/z 144.2 (51%) and 145.2 (49%), are observed, which implies the involvement of either one or two hydrogens from these positions in the A-ring. These results confirm the conclusion of Borges et al[36] that the m/z 143 ion for 5α-DHT must be generated from somewhere other than the D-ring because there was no mass shift in the spectrum of the silylated 16,16,17-[²H₃] derivative.

3.7.4 | m/z 169

A typical fragment ion seen in the mass spectra of androsterone (**8**) and DHEA (**9**) is m/z 169.1 which was previously described as a marker for 17-oxo steroids.[16, 37] It is shifted to m/z 171.1 for the ¹⁸O-labelled derivatives (**8.2M**, **9.2M**) and to m/z 178.2 for the

TABLE 3 Stereochemical structures of unlabelled and isotopically labelled TMS derivatives of steroid standards 1–13 with suitable hydrogens within bonding distance (< 3.2 Å) of TMSO groups. Distances calculated by ChemDraw (version 15.0) displayed as mean values (± 0.5 Å).

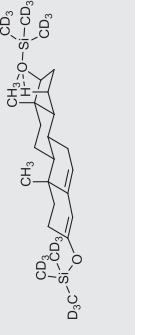| No.	#:1: 3,17-bis-TMS	#:2: 3/17 ^{18}O	#:3: 3,17-bis[^{2}H$_9$]-TMS	#:4.1: Other	#:4.2: Other
1				-	
2					
3				-	
4				-	
5				-	

(Continues)

TABLE 3 (Continued)
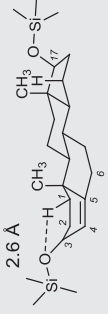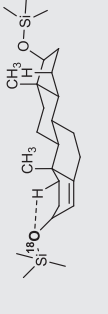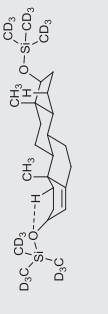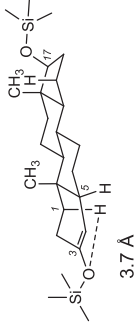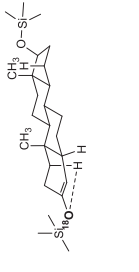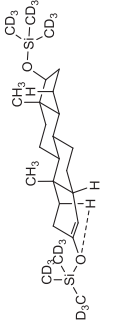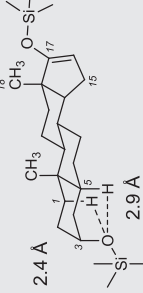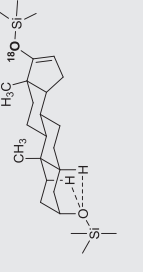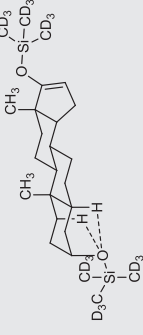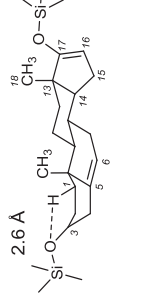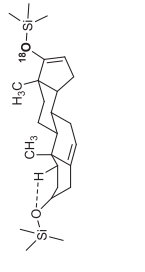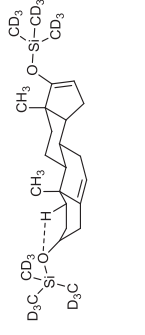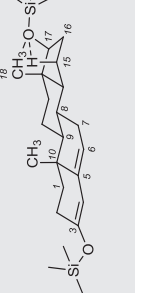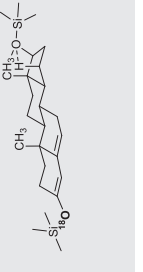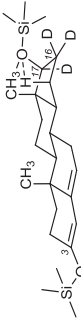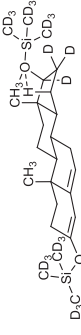
No.	#.1: 3,17-bis-TMS	#.2: 3/17 ¹⁸O	#.3: 3,17-bis[²H₉]-TMS	#.4.1: Other	#.4.2: Other
6				-	

No.	#.1: 3,17-bis-TMS	#.2: 3/17 ¹⁸O	#.3: 3,17-bis[²H₉]-TMS	#.4.1: Other	#.4.2: Other
7					
8					
9					
10					
11		-			
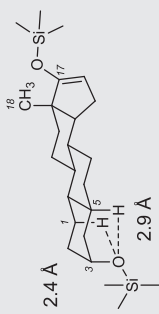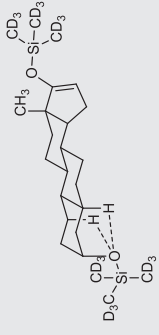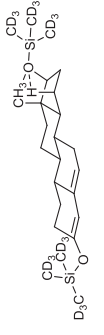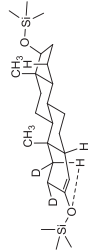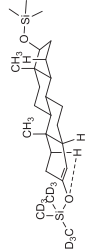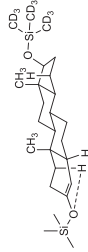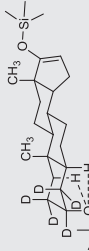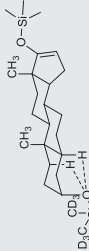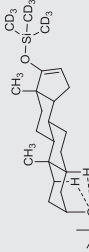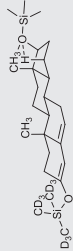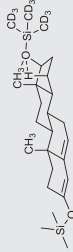
TABLE 3 (Continued)

No.	#.1: 3,17-bis-TMS	#.2: 3/17 ¹⁸O	#.3: 3,17-bis[²H₉]-TMS
12		-	
13		-	

No.	#.4: Other	#.5: 3[²H₉]-TMS, 17-TMS	#.6: 3-TMS, 17[²H₉]-TMS
7			
8			
9	-		
10	See 11		

TABLE 3 (Continued)

No.	#.4: Other	#.5: 3[²H₉]-TMS, 17-TMS	#.6: 3-TMS, 17[²H₉]-TMS
11	-		
12	-		
13	-		

TABLE 4 GC/QTOFMS data of specific fragment ions of standards 5α-DHT (**7**), testosterone (**10**), 16,16,17-[²H₃]-testosterone (**11**) and nandrolone (**13**)

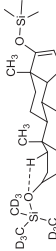| Fragment	Formula	Accurate mass	Exact mass	Mass error
7.1K	$C_7H_{15}OSi^+$	143.0892	143.0887	3.5 ppm
7.3K	$C_6H_6D_9OSi^+$	152.1460	152.1452	5.3 ppm
7.5K	$C_6H_6D_9OSi^+$	152.1450	152.1452	1.3 ppm
7.6K	$C_7H_{15}OSi^+$	143.0886	143.0887	0.7 ppm
7.1L	$C_6H_{11}OSi^+$	127.0579	127.0574	3.9 ppm
7.3L	$C_6H_2D_9OSi^+$	136.1140	136.1139	0.7 ppm
7.5L	$C_6H_2D_9OSi^+$	136.1145	136.1139	4.4 ppm
7.6L	$C_6H_{11}OSi^+$	127.0576	127.0574	1.6 ppm
10.1D	$C_{22}H_{33}OSi^+$	341.2288	341.2295	2.0 ppm
10.3D	$C_{22}H_{24}D_9OSi^+$	350.2851	350.2860	2.6 ppm
10.5D	$C_{22}H_{24}D_9OSi^+$	350.2867	350.2860	2.0 ppm
10.6D	$C_{22}H_{33}OSi^+$	341.2289	341.2295	1.8 ppm
10.1N	$C_{19}H_{29}OSi^+$	301.1976	301.1982	2.0 ppm
10.3N	$C_{19}H_{20}D_9OSi^+$	310.2550	310.2547	1.0 ppm
10.5N	$C_{19}H_{20}D_9OSi^+$	310.2552	310.2547	1.6 ppm
10.6N	$C_{19}H_{29}OSi^+$	301.1998	301.1982	5.3 ppm
10.1O	$C_{12}H_{21}OSi^+$	209.1346	209.1356	4.8 ppm
10.3O	$C_{12}H_{12}D_9OSi^+$	218.1916	218.1921	2.3 ppm
10.5O	$C_{12}H_{12}D_9OSi^+$	218.1922	218.1921	0.5 ppm
10.6O	$C_{12}H_{21}OSi^+$	209.1362	209.1356	2.9 ppm
10.1P	$C_{12}H_{20}OSi^{•+}$	208.1271	208.1278	3.4 ppm
10.3P	$C_{12}H_{11}D_9OSi^{•+}$	217.1842	217.1843	0.5 ppm
10.5P	$C_{12}H_{11}D_9OSi^{•+}$	217.1845	217.1843	1.0 ppm
10.6P	$C_{12}H_{20}OSi^{•+}$	208.1287	208.1278	4.3 ppm
11.1D	$C_{22}H_{30}D_3OSi^+$	344.2472	344.2483	3.2 ppm
11.3D	$C_{22}H_{21}D_{12}OSi^+$	353.3052	353.3048	1.1 ppm
11.5D	$C_{22}H_{21}D_{12}OSi^+$	353.3051	353.3048	0.9 ppm
11.6D	$C_{22}H_{30}D_3OSi^+$	344.2487	344.2483	1.2 ppm
13.1N	$C_{18}H_{27}OSi^+$	287.1826	287.1826	0.0 ppm
13.3N	$C_{18}H_{18}D_9OSi^+$	296.2395	296.2391	1.4 ppm
13.5N	$C_{18}H_{18}D_9OSi^+$	296.2395	296.2391	1.4 ppm
13.6N	$C_{18}H_{27}OSi^+$	287.1834	287.1826	2.8 ppm
13.1P	$C_{11}H_{18}OSi^{•+}$	194.1123	194.1121	1.0 ppm
13.3P	$C_{11}H_9D_9OSi^{•+}$	203.1690	203.1686	2.0 ppm
13.5P	$C_{11}H_9D_9OSi^{•+}$	203.1689	203.1686	1.5 ppm
13.6P	$C_{11}H_{18}OSi^{•+}$	194.1128	194.1121	3.6 ppm

3,17-bis[²H₉]-TMS derivatives (**8.3M**, **9.3M**, Table 2; 9.1–9.3, Figure 3). It is derived from cleavage in the C-ring and contains the angular methyl group in position C-18 (meaning that C-13–C-18 are present in the $C_9H_{17}OSi^+$ ion, **9.1**, Table 3). In the case of the two mixed deuterated derivatives of DHEA (**9**), m/z 169.1 (**9.5 M**) is detected when only the A-ring is labelled with [²H₉]-TMS, and m/z 178.2 (**9.6M**, Table 2) if the D-ring bears the label (9.5, 9.6, Figure 3).

3.7.5 | *m/z* 301, 209 and 208

The mass spectra of the per-TMS derivatives of both 3-oxo-4-ene steroids testosterone (**10**) and 16,16,17-[^{2}H$_3$]-testosterone (**11**) show fragment ions at *m/z* 301.2, 209.1 and 208.1. The *m/z* 301.1 (**10.1N**, Table 2, Table 4) ion can be explained by the loss of C-15-C-17 together with the attached O-TMS group (Table 3). For 16,16,17-[^{2}H$_3$]-testosterone (**11**) an additional fragment ion at *m/z* 302.2 (**11.1N**, Table 2) is observed which may be explained by transfer of one of the deuterium atoms from position C-16 or C-17 during generation of the fragment ion. Different positions of origin for the formation are possible, due to the occurrence of these two ions at *m/z* 301.2 and 302.2. In the mass spectrum of 19-nortestosterone (**13.1N**, Tables 2 and 4) this fragment ion is observed at *m/z* 287.2 (for mass spectra, see supporting information).

The ions at *m/z* 209.1 (**10.1O**) and 208.1 (**10.1P**, Tables 2 and 4) are made up of part of the A- and B-ring[16] when bonds C-9/C-10 and C-7/C-8 are broken, which is facilitated by the Δ5 double bond formed after enolization (**10.1**, Table 3). All three described A-ring fragment ions show the respective mass shifts of +9 *m/z* units to *m/z* 310.3, *m/z* 218.2 and *m/z* 217.2 for the perdeuterotrimethylsilylated (**10.3N,O,P**) and the 3-[^{2}H$_9$]TMS-17-TMS testosterone (**10.5N,O,P**, Tables 2 and 4) and 16,16,17-[^{2}H$_3$]-testosterone (**11.3N,O,P** and **11.5N,O,P**, Table 2) derivatives (for mass spectra, see supporting information). The *m/z* 194.1 fragment ion is found in the mass spectrum of 19-nortestosterone (**13.1P**, Tables 2 and 4) and can be considered analogous to *m/z* 208.1 for testosterone (**10.1P**, Table 2).

3.8 | Summarizing discussion

The formation of the [M – CH$_3$]$^+$, [M – TMSOH]$^{\bullet+}$ and [M – CH$_3$-TMSOH]$^+$ fragment ions in hydroxy steroids was shown to be dependent on steric factors, degree of saturation of the steroid backbone, and hydrogen and O-TMS group availability in the 1,3-diaxial position.

Methyl radical cleavage occurs primarily from the TMS groups in saturated androstanes **1–4**, but from the steroid nucleus in the case of androst-4-ene-3β,17β-diol (**6**) and oxo steroids or α,β-unsaturated steroid ketones (**8–11**). Androst-5-ene-3β,17β-diol (**5**) and 5α-DHT (**7**) cleave off methyl groups from TMS groups and C-18 or C-19 in a ratio of approximately 50:50.

TMSOH is most abundantly eliminated from position C-17 for 5α-androstanes **1–2** and from the 3α position in 5β-androstanes **2–4**. Double bonds in positions Δ4 (**6**) and Δ5 (**5**) direct TMSOH loss to the A-ring due to the increased stability of the resulting [M – 90]$^{\bullet+}$ ion. If an oxo group is present in the molecule (steroids **7–11**) loss of trimethylsilanol predominates from the respective TMS ether and not from the TMS enol function. In 16,16,17-[^{2}H$_3$]-testosterone all three attached deuterium atoms were retained in the D-ring after TMSOH loss from C-17. Similarly, all deuterium atoms in 2,2,3,4,4-[^{2}H$_5$]-androsterone (**8.4**) were also retained, which is in line with the hypothesis of 1,3-loss.

For the formation of the [M – CH$_3$ – TMSOH]$^+$ = [M – 105]$^+$ fragment ion the required methyl radical cleavage predominates from one of the angular methyl groups in position C-18 or C-19 and not from the TMS methyl groups. Fragment ions [M – 90]$^{\bullet+}$ and [M – 105]$^+$ are generated independently from each other and can originate from different TMS groups within the steroid backbone. For steroids **4–7**, however, both fragment ions can be traced back to the same TMS group. Derived from 19-norandrosterone (**14**) spectra, cleavage of CH$_3$-TMSOH including the C-18 methyl group is deduced but this needs confirmation for the other steroids investigated.

The [M – CH$_3$ – (CH$_3$)$_2$SiH-OH]$^+$ = [M – 15 – 76]$^+$ fragment ion may be explained by loss of a methyl radical from a TMS function followed by neutral loss of the remaining dimethylsilyloxy group plus two additional hydrogen atoms from the steroid backbone. In the case of the ^{18}O-labelled derivatives, losses to form both [M – 91]$^+$ and [M – 93]$^+$ = [M – CH$_3$ – (CH$_3$)$_2$SiH-^{18}OH]$^+$ ions are observed, showing that there is no preferred cleavage site (from C-3 or C-17).

For the different steroid subclasses, diagnostic fragment ions at *m/z* 129, [M – 129]$^+$, 127, 143, 169, 301, 209 and 208 were discussed and proposed structures postulated: the *m/z* 129 ion originates from the D-ring in the case of the androstanediols **1–4** and androst-4-ene-3β,17β-diol (**6**). The Δ5 steroids androst-5-ene-3β,17β-diol (**5**) and DHEA (**9**) generate *m/z* 129 from the A-ring and additionally exhibit an [M – 129]$^+$ ion which is formed from the remainder of the molecule including the D-ring. The *m/z* 127 ion is observed in the mass spectrum of 5α-DHT (**7**), derived from the A-ring, and might be analogous to *m/z* 129 with an additional double bond. The *m/z* 143 ion can be described as a resonance-stabilized 3-(O-trimethylsilyl)but-1-ene and is attributed to the A-ring for androst-4-ene-3β,17β-diol (**6**) and 5α-DHT (**7**). The *m/z* 169 ion can be considered as a marker for 17-oxo steroids and is thus derived from the D-ring in androsterone (**8**) and DHEA (**9**). Fragment ions at *m/z* 301, 209 and 208 were observed in the mass spectrum of testosterone (**10**) and 16,16,17-[^{2}H$_3$]-testosterone (**11**): *m/z* 301 is produced by loss of C-15–C-17 together with the attached OTMS group and *m/z* 208 and 209 are made up of part of the A- and B-rings.

4 | CONCLUSIONS

Mass spectral fragmentation analyses showed clear correlation between steroid structure and the obtained mass spectra. Viable and practical approaches for the introduction of [^{2}H$_9$]-TMS and ^{18}O were developed and employed to better characterize structural features of different steroid subclasses in GC/EI-MS spectra. The World Anti-Doping Agency requires both the retention time and unambiguous mass spectrometric characteristics for confirming the identity of compounds by means of GC/MS.[38] The discussed stable isotope labelling procedures are suitable tools to achieve this confidence in mass spectral findings due to the respective mass shifts of +9 *m/z* units for [^{2}H$_9$]-TMS groups and +2 *m/z* units for oxygen-18 even when using unit mass resolution GC/MS alone. Additional GC/QTOF

accurate mass spectra substantiated the elemental compositions of some diagnostic fragment ions but were not mandatory to explain the origin of the ions. In the case of isomer assignments this approach may be more suitable.

Both stable isotope labelling methods enable the detection of structure-related fragment generation in GC/EI-MS which may in turn allow us to propose isomeric assignments that are otherwise almost impossible using MS only. Based on these findings, a considerable reduction in the number of reference isomers needed for proper identification (generally including additional retention time comparison) of unknowns is possible.

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