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RESEARCH ARTICLE

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In-depth gas chromatography/tandem mass spectrometry fragmentation analysis of formestane and evaluation of mass spectral discrimination of isomeric 3-keto-4-ene hydroxy steroids

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Rationale: The aromatase inhibitor formestane (4-hydroxyandrost-4-ene-3,17-dione) is included in the World Anti-Doping Agency's List of Prohibited Substances in Sport. However, it also occurs endogenously as do its 2-, 6- and 11-hydroxy isomers. The aim of this study is to distinguish the different isomers using gas chromatography/electron ionization mass spectrometry (GC/EI-MS) for enhanced confidence in detection and selectivity for determination.

Methods: Established derivatization protocols to introduce [$^2H_9$]TMS were followed to generate perdeuterotrimethylsilylated and mixed deuterated derivatives for nine different hydroxy steroids, all with 3-keto-4-ene structure. Formestane was additionally labelled with $H_2{}^{18}O$ to obtain derivatives doubly labelled with [$^2H_9$]TMS and ^{18}O. GC/EI-MS spectra of labelled and unlabelled TMS derivatives were compared. Proposals for the generation of fragment ions were substantiated by high-resolution MS (GC/QTOFMS) and tandem mass spectrometry (MS/MS) experiments.

Results: Subclass-specific fragment ions include *m/z* 319 for the 6-hydroxy and *m/z* 219 for the 11-hydroxy compounds. Ions at *m/z* 415, 356, 341, 313, 269 and 267 were indicative for the 2- and 4-hydroxy compounds. For their discrimination the transition *m/z* 503 → 269 was selective for formestane. In 2-, 4- and 6-hydroxy steroids loss of a TMSO radical takes place as cleavage of a TMS-derived methyl radical and a neutral loss of $(CH_3)_2SiO$. Further common fragments were also elucidated.

Conclusions: With the help of stable isotope labelling, the structures of postulated diagnostic fragment ions for the different steroidal subclasses were elucidated. ^{18}O-labelling of the other compounds will be addressed in future studies to substantiate the obtained findings. To increase method sensitivity MS^3 may be suitable in future bioanalytical applications requiring discrimination of the 2- and 4-hydroxy compounds.

1 | INTRODUCTION

In mass spectrometry stable isotope labelling is a helpful tool for fragment structure characterization of steroids and has been employed since the 1960s.[1–7] In particular, deuterium and ^{18}O were used to selectively label the steroid backbone and derive important information from the respective mass shifts of +1 (deuterium) and +2 (oxygen 18) compared with the mass spectra of non-labelled compounds. ^{18}O-labelling is no longer commonly used for steroid research, but it is still used in other fields, for example metabolomics or peptide mass spectrometry.[8,9]

With the use of labelled derivatization agents such as *N,O*-bis(trimethyl-[$^2H_9$]-silyl)acetamide ([$^2H_{18}$]-BSA)[10–12] or [$^2H_9$]-MSTFA[13–15] the structure and origin of diagnostically important (TMS) fragment ions can be further elucidated in gas chromatography/(tandem) mass spectrometry (GC/MS(/MS)) analysis. Combined with $H_2{}^{18}O$ for selective labelling of oxo functions and modern high-resolution mass spectrometers, a better understanding of fragmentation behavior can be expected.

The aromatase inhibitor formestane (**2**) is a 3-keto-4-ene hydroxy steroid included in the World Anti-Doping Agency's Prohibited List,[16] but trace amounts also occur endogenously in humans.[17] It does not exhibit much fragmentation in electron ionization as a per-TMS derivative and thus only the $[M]^{\bullet+}$ and $[M - 15]^+$ ions are used as precursors for multiple reaction monitoring (MRM) transitions in GC/MS/MS.[18] Its isomer 2α-hydroxyandrostenedione (**1**) is a minor and the respective stereoisomer 2β-hydroxyandrostenedione (**10**) a medium abundance metabolite of androstenedione.[19] The 3,5-dienol-TMS derivatives of both analytes, 2α-hydroxyandrostenedione (**1**) and formestane (**2**), show very similar mass spectra with only a few characteristic peaks, making it difficult to distinguish these steroids in anti-doping control.[17] Similarly, the 3,5-dienol-TMS derivatives of the androstenedione metabolite 6-hydroxyandrostenedione (**4**) and the third analyzed formestane isomer 11-hydroxyandrostenedione (**5**) do not produce prominent diagnostic fragment ions but can be discriminated from **1**, **2** and **10** by the respective retention times under routinely applied conditions.[20]

To discriminate between androstenedione and formestane (**2**) administration, a number of metabolites such as testosterone, epitestosterone, androsterone and etiocholanolone in the case of androstenedione,[18] and 4α-hydroxyepiandrosterone (4-OH-EA) as the main metabolite of formestane,[21] can be used as biomarkers in urine samples.[18] The ratio between 4-OH-EA and formestane as parent compound can also serve to predict its endogenous or exogenous origin[18] for which generally costly combustion-isotope ratio mass spectrometry (GC/C-IRMS) methods are required in anti-doping analysis.[17,22,23] In order to avoid overestimation of the formestane concentration due to co-elution of formestane in the chromatogram with 2α-hydroxyandrostenedione (**1**), GC/MS/MS and specific MRM transitions were used, and the detection improved.[18] The distinction between oral and transdermal formestane intake can be achieved by use of the ratio of the 4-OH-EA and 4-hydroxyandrosterone (4-OH-A) concentrations in urine,[17] and the occurrence of androst-4-ene-3,6,17-trione (6-OXO) or 6α-OH-testosterone is suitable to distinguish between 6-OXO and androstenedione administration.[24] The endogenous steroid androst-4-ene-3,11,17-trione (11-oxoandrostenedione, 11-OXO) together with its corresponding conversion product 11β-hydroxyandrostenedione (**5**) plays a role in corticosteroid metabolism and its misuse can be detected by GC/C-IRMS.[25] Because all the metabolites found in urine retained an oxo or hydroxy group at C-11, no confounding interferences regarding formestane and/or androstenedione metabolism have been described.

For unequivocal structural elucidation of steroid metabolites by means of GC/MS, retention times and mass spectra of reference standards are required for comparison with the obtained data.[26,27] In some cases, additional nuclear magnetic resonance (NMR) spectroscopy data is helpful for e.g. stereochemical characterization of specific functional groups.[21] However, especially in the case of the structural elucidation of low concentration metabolites with concomitantly limited availability of specimen, the amount required for NMR analyses hampers its reasonable utilization. Even when utilizing a high-resolution microcoil, the limit of detection was reported as 19 ng for a pure reference material of sucrose.[28] The analysis of mixtures or direct combination of chromatographic separation with NMR detection generally requires much higher amounts of analyte.[29–31] Similarly, many other potential (physico-) chemical methods for structural elucidation would require pre-purification of considerably high amounts of analytes and are therefore considered inferior for bioanalytical applications.

The retention times of unknown analytes are dependent on the GC method used. Usually, the method is specifically adapted according to the respective reference standards in order to prevent co-elution of analytes. If reference standards are not commercially available, they must be synthesized, which is often laborious and time-consuming. The isotopic labelling methods presented herein can help to narrow down the number of reference standards needed for metabolite identification or even replace them in uncomplicated set-ups, because stable isotope labelling provides valuable information: ^{18}O-labelling predicts the number of oxo groups and perdeuterotrimethylsilylation the total number of functional groups (e.g. hydroxy and oxo groups) in the compound. With the help of mixed deuterated derivatives, TMS-enol ethers can be distinguished from TMS-ether groups. In summary, the presented isotopic labelling approach is fast and straightforward and can easily be integrated as an additional preparational step into standard steroid GC/MS as well as LC/MS (only applicable for ^{18}O-labelling) methods as no special equipment is needed, and the obtained mass spectra with the typical mass shifts are self-explanatory. In fact, isotopic labelling can be used to distinguish the analyte from undesired signals, such as fragment ions derived from column bleed for example, because only the analyte bears the isotopic label. The two methods presented in this paper were developed for low amounts of analyte (minimum of 1 mg for ^{18}O-labelling and 10 µg for derivatization with ([$^2H_{18}$]-BSA) and are not limited to steroids but can also be adapted for mass spectral characterization of other substance classes.[11]

The aim of this work is to better characterize and substantiate GC/MS fragmentation proposals of nine different 3-keto-4-ene hydroxy steroids with the help of isotopic labelling. Each steroid contains one hydroxy group in position C-2, C-4, C-6 or C-11 and either an oxo or a hydroxy group in position C-17 (Tables 1 and 2).

2 | EXPERIMENTAL

2.1 | Steroid standards

The reference standards 2α-hydroxyandrost-4-ene-3,17-dione (2α-hydroxyandrostenedione, **1**), 2β-hydroxyandrost-4-ene-3,17-dione (2β-hydroxyandrostenedione, **10**), 6β-hydroxyandrost-4-ene-3,17-dione (6β-hydroxyandrostenedione, **4**), 2α,17β-dihydroxyandrost-4-en-3-one (2α-hydroxytestosterone, **6**), 6β,17β-dihydroxyandrost-4-en-3-one (6β-hydroxytestosterone, **8**) and 11β,17β-dihydroxyandrost-4-en-3-one (11β-hydroxytestosterone, **9**) were from Steraloids (Newport, RI, USA) and 4-hydroxyandrost-4-ene-3,17-dione (formestane, **2**) was from Sigma-Aldrich (Milan, Italy). 11β-Hydroxyandrost-4-ene-3,17-dione (11β-hydroxyandrostenedione, **5**) was purchased from Sigma-Aldrich GmbH (Steinheim, Germany) and 4,17β-dihydroxyandrost-4-en-3-one (4-hydroxytestosterone, **7**) from NMIA (Pymble, Australia). 4-Hydroxyandrost-4-ene-3,17-[$^{18}O_2$]-dione, ([$^{18}O_2$]-formestane, **3**) was synthesized from **2** as described below.

2.2 | Reagents and chemicals

Water-^{18}O (97 atom % ^{18}O), acetonitrile (ACN, anhydrous, 99.8%), ethanethiol (97%), 2-mercaptoethanol (99.0%) and ammonium iodide (NH_4I, 99.9%) were obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany). Toluene, isopropylamine (99+%) and titanium tetrachloride ($TiCl_4$, 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-[$^2H_9$]-silyl)acetamide ([$^2H_{18}$]-BSA) from abcr GmbH (Karlsruhe, Germany).

2.3 | ^{18}O-labelling of formestane

To obtain 4-hydroxyandrost-4-ene-3,17-[$^{18}O_2$]-dione ([$^{18}O_2$]-formestane, **3**), the two oxo functions of formestane (**2**) were isotopically labelled utilizing $H_2{}^{18}O$ via an imine intermediate as previously described.[11,12] Formestane (1.5 mg) was dissolved in toluene (900 μL). At a temperature of 0°C, $TiCl_4$/toluene (1:2, v:v; 18 μL) and isopropylamine (45 μL) were added while stirring. The mixture was incubated at 100°C for 15 min. After cooling, water (900 μL) was 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_2O (control) or $H_2{}^{18}O$ were evaporated at 100°C in a heating block. The residue was derivatized as described below.

2.4 | Derivatization

Individual stock solutions (1 mg/mL) of compounds **1**, **2** and **4**–**10** were prepared in ACN and stored at −18°C prior to GC/MS analysis. Aliquots of 10 μL of each stock solution were evaporated at 100°C and the respective residues derivatized with either MSTFA or [$^2H_{18}$]-BSA, or both, using four different derivatization protocols[12]:

- pertrimethylsilylation: 90 μL MSTFA/TMIS reagent (MSTFA/NH_4I/ethanethiol, 1,000:2:3, v/w/v) at 60°C for 15 min (to obtain derivatives **#.1**, Tables 3 and 4)
- perdeuterotrimethylsilylation: 80 μL [$^2H_{18}$]-BSA/TMIS reagent (5 μL of a saturated NH_4I solution in 2-mercaptoethanol added to 100 μL [$^2H_{18}$]-BSA) at 90°C for 30 min (to obtain derivatives **#.2**, Tables 3 and 4)
- mixed deuterated derivatives:
  15 μL of [$^2H_{18}$]-BSA at 90°C for 30 min, evaporation in a gentle stream of nitrogen, 65 μL MSTFA/TMIS reagent at 60°C for 15 min (to obtain derivatives **#.3**, Tables 3 and 4)
  15 μL MSTFA at 60°C for 15 min, evaporation in a gentle stream of nitrogen, 65 μL [$^2H_{18}$]-BSA/TMIS reagent at 90°C for 30 min (to obtain derivatives **#.4**, Tables 3 and 4)

2.5 | Instrumentation

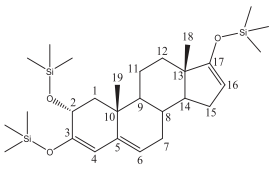
2.5.1 | GC/EI-MS experiments

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 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: 2.5 min 188°C, +3°C/min, 2 min 211°C, +10°C/min, 0 min 238°C, +40°C/min, 3.2 min 320°C; injection volume: 1 μL; split 1:15′ injection temperature: 280°C; and ionization: 70 eV, EI.

2.5.2 | High-resolution accurate mass analyses

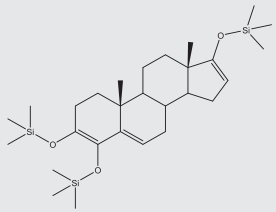
High-resolution accurate mass spectrometry was used for structural confirmation of fragment ions and fragment ion pathway elucidation. Electron ionization quadrupole time-of-flight mass spectrometry (EI-QTOFMS) analyses were performed on a model 7890 gas chromatograph hyphenated to a model 7200 QTOF mass spectrometer (Agilent Technologies, mass resolving power R = 14,522). Chromatography was performed using the same column and the same conditions as described for GC/EI-MS. The analyses were carried out in full scan MS as well as product ion scan (targeted MS/MS) mode (mass range m/z 50–450). Different precursor ions were selected for the differently labelled derivatives (Table S3,

TABLE 1 Experimental mass data of GC/MS/MS product ions and chemical structures of standards **1–5** and **10**. Precursor ions are the respective $[M]^{\bullet+}$ ions (**#.1-4A**), data in italics recorded in scan mode (abundance too low in MS/MS spectra). Calculated experimental error is given in brackets. Abbreviations: $[M - 73]^+ = [M - TMS]^+$, $[M - 82]^+ = [M - [^2H_9]\text{-}TMS]^+$, $[M - CH_3 - 73]^{\bullet+} = [M - CH_3 - TMS]^{\bullet+}$, $[M - CH_3 - 82]^{\bullet+} = [M - CH_3 - [^2H_9]\text{-}TMS]^{\bullet+}$, $[M - 89]^+ = [M - TMSO]^+$, $[M - 91]^+ = [M - TMS^{18}O]^+$, $[M - 98]^+ = [M - [^2H_9]\text{-}TMSO]^+$, $[M - 100]^+ = [M - [^2H_9]\text{-}TMS^{18}O]^+$, $[M - 90]^{\bullet+} = [M - TMSOH]^{\bullet+}$, $[M - 99]^{\bullet+} = [M - [^2H_9]\text{-}TMSOH]^{\bullet+}$, $[M - CH_3 - 90]^+ = [M - CH_3 - TMSOH]^+$, $[M - CH_3 - 92]^+ = [M - CH_3 - TMS^{18}OH]^+$, $[M - CH_3 - 99]^+ = [M - CH_3 - [^2H_9]\text{-}TMSOH]^+$, $[M - CH_3 - 101]^+ = [M - CH_3 - [^2H_9]\text{-}TMS^{18}OH]^+$, $[M - CH_3 - 2 \times 90]^+ = [M - CH_3 - 2 \times TMSOH]^+$, $[M - CH_3 - 90 - 99]^+ = [M - CH_3 - TMSOH - [^2H_9]\text{-}TMSOH]^+$, $[M - CH_3 - 2 \times 99]^+ = [M - CH_3 - 2 \times [^2H_9]\text{-}TMSOH]^+$, $[M - CH_3 - 90 - 92]^+ = [M - CH_3 - TMSOH - TMS^{18}OH]^+$, $[M - CH_3 - 2 \times 92]^+ = [M - CH_3 - 2 \times TMS^{18}OH]^+$, $[M - CH_3 - 99 - 92]^+ = [M - CH_3 - [^2H_9\text{-}TMSOH - TMS^{18}OH]^+$, $[M - CH_3 - 90 - 101]^+ = [M - CH_3 - TMSOH - [^2H_9]\text{-}TMS^{18}OH]^+$, $[M - CH_3 - 99 - 101]^+ = [M - CH_3 - [^2H_9]\text{-}TMSOH - [^2H_9]\text{-}TMS^{18}OH]^+$,$[M - CH_3 - 2 \times 101]^+ = [M - CH_3 - 2 \times [^2H_9]\text{-}TMS^{18}OH]^+$

No.	Steroid		Ion	#.1: Tris-TMS	#.2: Tris-$[^2H_9]$-TMS	#.3: 2/4/6/11-$[^2H_9]$-TMS, 3,17-bis-TMS	#.4: 2/4/6/11-TMS, 3,17-bis-$[^2H_9]$-TMS
1		A	$[M]^{\bullet+}$	518.3050 (2.32 ppm)	545.4767 (1.83 ppm)	527.3625 (0.38 ppm)	536.4220 (5.22 ppm)
		B	$[M - CH_3]^+$	503.2814 (2.78 ppm)	530.4503 (3.58 ppm)	512.3376 (3.12 ppm)	521.3978 (4.03 ppm)
			$[M - CD_3]^+$		*527.4330 (0.76 ppm)*	*509.3209 (0.98 ppm)*	*518.3790 (4.05 ppm)*
		C	$[M - 73]^+$	*445.2586 (0.67 ppm)*		*454.3154 (0 ppm)*	*463.3727 (1.73 ppm)*
			$[M - 82]^+$		*463.3715 (0.86 ppm)*	*445.2592 (0.67 ppm)*	*454.3166 (2.64 ppm)*
		E	$[M - 89]^+$	429.2633 (1.63 ppm)		*438.3204 (0.23 ppm)*	447.3783 (3.13 ppm)
			$[M - 98]^+$		447.3755 (3.13 ppm)	429.2617 (5.36 ppm)	438.3210 (1.14 ppm)
		F	$[M - 90]^{\bullet+}$	428.2551 (2.34 ppm)		437.3151 (5.72 ppm)	446.3711 (4.48 ppm)
			$[M - 99]^{\bullet+}$		446.3671 (4.48 ppm)	428.2556 (1.17 ppm)	437.3154 (6.40 ppm)
		G	$[M - CH_3 - 90]^+$	413.2326 (0.24 ppm)		422.2863 (6.87 ppm)	431.3468 (2.78 ppm)
			$[M - CH_3 - 99]^+$		431.3436 (4.64 ppm)	413.2327 (0 ppm)	422.2911 (4.50 ppm)
		H	$[M - CH_3 - 2 \times 90]^+$	323.1829 (0.93 ppm)		332.2376 (4.51 ppm)	
			$[M - CH_3 - 90 - 99]^+$			323.1819 (2.17 ppm)	332.2405 (4.21 ppm)
			$[M - CH_3 - 2 \times 99]^+$		332.2373 (5.42 ppm)		323.1814 (3.71 ppm)
		I	***m/z* 415**	415.2470 (3.13 ppm)		*415.2459 (5.78 ppm)*	424.3070 (5.18 ppm)
					433.3600 (3.00 ppm)	*424.3036 (2.83 ppm)*	433.3620 (1.62 ppm)
		J	***m/z* 356**	356.2155 (3.09 ppm)	365.2720 (3.01 ppm)	356.2149 (4.77 ppm)	365.2746 (4.11 ppm)
		K	***m/z* 341**	341.1924 (2.05 ppm)	350.2482 (4.00 ppm)	341.1919 (3.52 ppm)	350.2513 (4.85 ppm)
		L	***m/z* 313**	313.1976 (1.92 ppm)	322.2532 (4.65 ppm)	313.1974 (2.55 ppm)	322.2561 (4.34 ppm)
		M	***m/z* 269**	269.1372 (5.94 ppm)	287.2505 (4.18 ppm)	278.1944 (3.24 ppm)	278.1953 (0 ppm)
		N	***m/z* 267**	267.1222 (3.37 ppm)	285.2346 (5.26 ppm)	276.1789 (2.53 ppm)	276.1804 (2.90 ppm)
		O	***m/z* 147**	147.0647 (5.44 ppm)		153.1039 (3.92 ppm)	153.1040 (4.57 ppm)
					162.1592 (3.08 ppm)	156.1215 (3.84 ppm)	156.1225 (2.56 ppm)

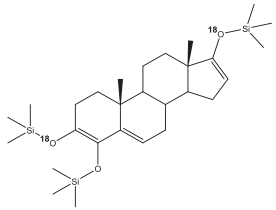
(Continues)

TABLE 1 (Continued)

No.	Steroid		Ion	#.1: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 2/4/6/11-[$^2H_9$]-TMS, 3,17-bis-TMS	#.4: 2/4/6/11-TMS, 3,17-bis-[$^2H_9$]-TMS
2		A	$[M]^{\bullet+}$	518.3067 (0.96 ppm)	545.4746 (2.02 ppm)	527.3632 (0.95 ppm)	536.4182 (1.86 ppm)
		B	$[M - CH_3]^+$	503.2820 (1.59 ppm)	530.4486 (6.79 ppm)	512.3384 (1.56 ppm)	521.3959 (0.38 ppm)
			$[M - CD_3]^+$		*527.4338 (0.76 ppm)*	*509.3203 (0.20 ppm)*	*518.3774 (3.09 ppm)*
		C	$[M - 73]^+$	*445.2587 (0.45 ppm)*		*454.3140 (3.08 ppm)*	*463.3728 (1.94 ppm)*
			$[M - 82]^+$		*463.3723 (0.86 ppm)*		*454.3159 (1.10 ppm)*
		D	$[M - CH_3 - 73]^{\bullet+}$	430.2425 (16.50 ppm)		439.3067 (33.69 ppm)	
			$[M - CH_3 - 82]^{\bullet+}$		448.3790 (68.25 ppm)		439.2986 (15.25 ppm)
		E	$[M - 89]^+$	429.2632 (1.86 ppm)		438.3197 (1.83 ppm)	*447.3774 (1.12 ppm)*
			$[M - 98]^+$		447.3747 (4.92 ppm)	429.2642 (0.47 ppm)	*438.3191 (3.19 ppm)*
		G	$[M - CH_3 - 90]^+$	413.2318 (2.18 ppm)		422.2882 (2.37 ppm)	*431.3459 (0.70 ppm)*
			$[M - CH_3 - 99]^+$		431.3457 (0.23 ppm)	413.2348 (5.08 ppm)	422.2885 (1.66 ppm)
		H	$[M - CH_3 - 2 \times 90]^+$	323.1816 (3.09 ppm)		332.2404 (3.91 ppm)	
			$[M - CH_3 - 90 - 99]^+$			323.1834 (2.48 ppm)	*332.2386 (1.50 ppm)*
			$[M - CH_3 - 2 \times 99]^+$		332.2387 (1.20 ppm)		323.1811 (4.64 ppm)
		J	***m/z* 356**	356.2168 (0.56 ppm)	365.2748 (4.65 ppm)	*356.2184 (5.05 ppm)*	*365.2735 (1.10 ppm)*
		K	***m/z* 341**	341.1921 (2.93 ppm)	350.2477 (5.42 ppm)	341.1924 (2.05 ppm)	350.2498 (0.57 ppm)
		L	***m/z* 313**	*313.1975 (2.24 ppm)*	322.2556 (2.79 ppm)	*313.1968 (4.47 ppm)*	*322.2544 (0.93 ppm)*
		M	***m/z* 269**	269.1386 (0.74 ppm)	287.2515 (0.70 ppm)	278.1944 (3.24 ppm)	278.1944 (3.24 ppm)
		N	***m/z* 267**	267.1221 (3.64 ppm)	285.2377 (5.61 ppm)	276.1795 (0.36 ppm)	276.1794 (0.72 ppm)
		O	***m/z* 147**	147.0658 (1.36 ppm)		153.1033 (0 ppm)	153.1031 (1.31 ppm)
					162.1594 (1.85 ppm)	156.1216 (3.20 ppm)	156.1218 (1.92 ppm)

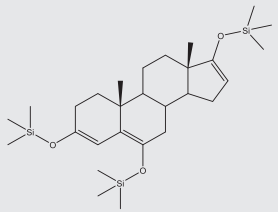
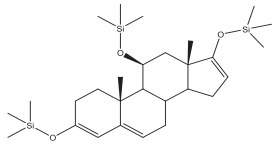
(Continues)

TABLE 1 (Continued)

No.	Steroid		Ion	#.1: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 2/4/6/11-[$^2H_9$]-TMS, 3,17-bis-TMS	#.4: 2/4/6/11-TMS, 3,17-bis-[$^2H_9$]-TMS
3		A	$[M]^{\bullet+}$	522.3163 (3.06 ppm)	549.4822 (3.64 ppm)	531.3706 (1.13 ppm)	540.4280 (0.56 ppm)
		B	$[M - CH_3]^+$	507.2916 (0.79 ppm)	534.4605 (0.37 ppm)	*516.3468 (1.74 ppm)*	*525.4041 (0.19 ppm)*
			$[M - CD_3]^+$		*531.4416 (0.56 ppm)*	*513.3285 (0.78 ppm)*	*522.3861 (1.34 ppm)*
		C	$[M - 73]^+$	*449.2695 (4.67 ppm)*		*458.3248 (1.96 ppm)*	
			$[M - 82]^+$		*467.3824 (4.49 ppm)*		*458.3256 (3.71 ppm)*
		D	$[M - CH_3 - 73]^{\bullet+}$	434.2439 (10.13 ppm)			
			$[M - CH_3 - 82]^{\bullet+}$		452.3569 (63.00 ppm)	434.2541 (17.96 ppm)	443.2969 (7.90 ppm)
		E	$[M - 89]^+$	433.2713 (2.77 ppm)			*451.3857 (0.66 ppm)*
			$[M - 91]^+$	431.2686 (0.93 ppm)		440.3236 (2.50 ppm)	
			$[M - 98]^+$		451.3832 (4.87 ppm)	*433.2727 (0.46 ppm)*	
			$[M - 100]^+$		449.3825 (2.89 ppm)		*440.3245 (0.45 ppm)*
		G	$[M - CH_3 - 90]^+$	*417.2434 (5.27 ppm)*			*435.3542 (0.23 ppm)*
			$[M - CH_3 - 92]^+$	415.2395 (6.26 ppm)		*424.2939 (1.18 ppm)*	
			$[M - CH_3 - 99]^+$		435.3562 (4.82 ppm)	417.2388 (5.75 ppm)	
			$[M - CH_3 - 101]^+$		433.3516 (3.92 ppm)		424.2916 (4.24 ppm)
		H	$[M - CH_3 - 90 - 92]^+$	*325.1859 (2.77 ppm)*			
			$[M - CH_3 - 2 \times 92]^+$	*323.1781 (13.92 ppm)*		*332.2347 (13.24 ppm)*	
			$[M - CH_3 - 99 - 92]^+$			*325.1865 (0.92 ppm)*	
			$[M - CH_3 - 90 - 101]^+$				*334.2423 (2.99 ppm)*
			$[M - CH_3 - 99 - 101]^+$		*334.2439 (1.80 ppm)*		
			$[M - CH_3 - 2 \times 101]^+$		*332.2385 (1.81 ppm)*		*323.1849 (7.12 ppm)*
		J	***m/z* 356**	*358.2214 (1.40 ppm)*	*369.2808 (2.17 ppm)*	-	*369.2808 (2.17 ppm)*
				360.2224 (7.50 ppm)	*367.2774 (0.27 ppm)*	-	*367.2774 (0.27 ppm)*
		K	***m/z* 341**	343.1959 (4.37 ppm)	352.2611 (6.25 ppm)	343.1997 (6.70 ppm)	*352.2559 (5.68 ppm)*
				345.2024 (2.32 ppm)	-	-	*354.2595 (3.95 ppm)*
		L	***m/z* 313**	*315.2035 (3.17 ppm)*	324.2583 (2.16 ppm)	*315.2026 (0.32 ppm)*	*324.2589 (0.31 ppm)*
		M	***m/z* 269**	271.1429 (0.37 ppm)	289.2566 (2.07 ppm)	280.1994 (0.36 ppm)	280.1997 (0.71 ppm)
		N	***m/z* 267**	269.1282 (2.97 ppm)	287.2402 (0.35 ppm)	278.1830 (2.88 ppm)	278.1830 (2.88 ppm)
		O	***m/z* 147**	147.0661 (3.40 ppm)		153.1034 (0.65 ppm)	*153.1031 (1.31 ppm)*
				149.0698 (0 ppm)		155.1076 (0.64 ppm)	155.1068 (4.51 ppm)
					162.1593 (2.47 ppm)	156.1211(6.41 ppm)	*156.1213 (5.12 ppm)*
					164.1636 (2.44 ppm)	158.1261 (1.26 ppm)	158.1258 (3.16 ppm)

(Continues)

TABLE 1 (Continued)

No.	Steroid		Ion	#.1: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 2/4/6/11-[$^2H_9$]-TMS, 3,17-bis-TMS	#.4: 2/4/6/11-TMS, 3,17-bis-[$^2H_9$]-TMS
4		A	$[M]^{\bullet+}$	518.3048 (3.09 ppm)	545.4743 (2.57 ppm)	527.3635 (1.52 ppm)	536.4172 (3.73 ppm)
		B	$[M - CH_3]^+$	503.2815 (2.58 ppm)	530.4503 (3.58 ppm)	512.3391 (0.20 ppm)	521.3978 (4.03 ppm)
			$[M - CD_3]^+$		*527.4336 (0.38 ppm)*	*509.3206 (0.39 ppm)*	*518.3774 (3.09 ppm)*
		C	$[M - 73]^+$	*445.2587 (0.45 ppm)*	*463.3720 (0.22 ppm)*	*454.3151 (0.66 ppm)*	463.3715 (2.59 ppm)
			$[M - 82]^+$			*445.2587 (0.45 ppm)*	454.3162 (1.76 ppm)
		E	$[M - 89]^+$	429.2643 (0.7 ppm)	*447.3751 (4.02 ppm)*	*438.3180 (5.70 ppm)*	*447.3743 (5.81 ppm)*
			$[M - 98]^+$			*429.2634 (1.40 ppm)*	*438.3181 (5.48 ppm)*
		F	$[M - 90]^{\bullet+}$	428.2571 (2.34 ppm)	*446.3663 (6.27 ppm)*	*437.3102 (5.49 ppm)*	446.3694 (0.67 ppm)
			$[M - 99]^{\bullet+}$			*428.2554 (1.63 ppm)*	437.3152 (5.95 ppm)
		G	$[M - CH_3 - 90]^+$	413.2318 (2.18 ppm)	431.3438 (4.17 ppm)	422.2887 (1.18 ppm)	431.3432 (5.56 ppm)
			$[M - CH_3 - 99]^+$			413.2340 (3.15 ppm)	422.2884 (1.89 ppm)
		H	$[M - CH_3 - 2 \times 90]^+$	323.1817 (2.78 ppm)		332.2380 (3.31 ppm)	
			$[M - CH_3 - 90 - 99]^+$			323.1822 (1.24 ppm)	332.2380 (3.31 ppm)
			$[M - CH_3 - 2 \times 99]^+$		332.2378 (3.91 ppm)		323.1834 (2.48 ppm)
		P	***m/z* 319**	319.1537 (2.19 ppm)	337.2665 (2.67 ppm)	328.2106 (0.91 ppm)	328.2111 (0.61 ppm)
5		A	$[M]^{\bullet+}$	518.3052 (1.93 ppm)	545.4737 (3.67 ppm)	527.3606 (3.98 ppm)	536.4202 (1.86 ppm)
		B	$[M - CH_3]^+$	503.2820 (1.59 ppm)	*530.4516 (1.13 ppm)*	512.3359 (6.44 ppm)	*521.3968 (2.11 ppm)*
			$[M - CD_3]^+$		*527.4329 (0.95 ppm)*	*509.3206 (0.39 ppm)*	*518.3774 (3.09 ppm)*
		C	$[M - 73]^+$	*445.2589 (0 ppm)*		*454.3145 (1.98 ppm)*	*463.3713 (1.29 ppm)*
			$[M - 82]^+$		*463.3715 (0.86 ppm)*	*445.2585 (0.90 ppm)*	*454.3173 (4.18 ppm)*
		F	$[M - 90]^{\bullet+}$	428.2548 (3.04 ppm)		437.3103 (5.26 ppm)	*446.3698 (1.57 ppm)*
			$[M - 99]^{\bullet+}$		446.3674 (3.81 ppm)	428.2546 (3.50 ppm)	437.3114 (2.74 ppm)
		G	$[M - CH_3 - 90]^+$	413.2314 (3.15 ppm)		422.2877 (3.55 ppm)	431.3460 (0.93 ppm)
			$[M - CH_3 - 99]^+$		431.3440 (3.71 ppm)	413.2321 (1.45 ppm)	422.2874 (4.26 ppm)
		H	$[M - CH_3 - 2 \times 90]^+$	323.1819 (2.17 ppm)		332.2390 (0.30 ppm)	
			$[M - CH_3 - 90 - 99]^+$			323.1807 (5.88 ppm)	332.2369 (6.62 ppm)
			$[M - CH_3 - 2 \times 99]^+$		332.2378 (3.91 ppm)		323.1817 (2.78 ppm)
		R	***m/z* 387**	387.2157 (3.36 ppm)		387.2167 (0.77 ppm)	396.2747 (3.03 ppm)
					405.3287 (3.21 ppm)	396.2727 (2.02 ppm)	*405.3309 (2.22 ppm)*
		S	***m/z* 234**	234.1428 (2.56 ppm)		234.1428 (2.56 ppm)	*234.1433 (0.43 ppm)*
					243.1988 (4.52 ppm)	243.1995 (1.64 ppm)	243.1992 (2.88 ppm)
		T	***m/z* 219**	219.1194 (2.74 ppm)		219.1195 (2.28 ppm)	*219.1199 (0.46 ppm)*
					228.1756 (3.94 ppm)	228.1758 (3.07 ppm)	228.1755 (4.38 ppm)

(Continues)

TABLE 1 (Continued)

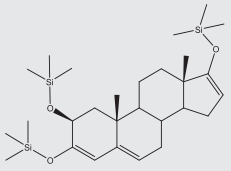
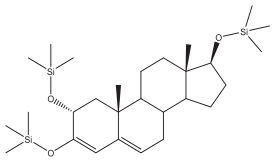
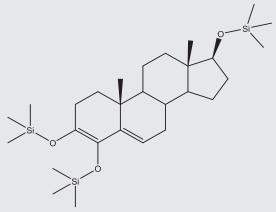
No.	Steroid		Ion	#.1: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 2/4/6/11-[$^2H_9$]-TMS, 3,17-bis-TMS	#.4: 2/4/6/11-TMS, 3,17-bis-[$^2H_9$]-TMS
10		A	$[M]^{\bullet+}$	518.3050 (2.32 ppm)	545.4754 (3.67 ppm)	527.3632 (0.95 ppm)	536.4198 (1.12 ppm)
		B	$[M - CH_3]^+$	503.2826 (0.40 ppm)	530.4517 (0.94 ppm)	512.3379 (2.54 ppm)	*521.3969 (2.30 ppm)*
			$[M - CD_3]^+$		*527.4335 (0.19 ppm)*	*509.3199 (0.98 ppm)*	*518.3783 (2.70 ppm)*
		C	$[M - 73]^+$	*445.2565 (5.39 ppm)*			*463.3715 (0.86 ppm)*
			$[M - 82]^+$		*463.3710 (1.94 ppm)*	*445.2588 (0.22 ppm)*	*454.3174 (4.40 ppm)*
		E	$[M - 89]^+$	429.2654 (3.26 ppm)		*438.3161 (10.04 ppm)*	*447.3747 (4.92 ppm)*
			$[M - 98]^+$		447.3749 (4.47 ppm)	*429.2590 (11.65 ppm)*	438.3268 (14.37 ppm)
		F	$[M - 90]^{\bullet+}$	428.2549 (2.80 ppm)		437.3128 (0.46 ppm)	446.3671 (4.48 ppm)
			$[M - 99]^{\bullet+}$		446.3678 (2.91 ppm)	*428.2578 (3.97 ppm)*	*437.3156 (6.86 ppm)*
		G	$[M - CH_3 - 90]^+$	413.2327 (0 ppm)		*422.2896 (0.95 ppm)*	*431.3470 (3.25 ppm)*
			$[M - CH_3 - 99]^+$		431.3450 (1.39 ppm)	*413.2327 (0 ppm)*	422.2885 (1.66 ppm)
		H	$[M - CH_3 - 2 \times 90]^+$	323.1812 (4.33 ppm)		*332.2396 (1.50 ppm)*	
			$[M - CH_3 - 90 - 99]^+$			*323.1829 (0.93 ppm)*	*332.2390 (0.30 ppm)*
			$[M - CH_3 - 2 \times 99]^+$		*332.2388 (0.90 ppm)*		*323.1860 (10.52 ppm)*
		L	***m/z* 313**	313.2002 (6.39 ppm)	322.2571 (7.45 ppm)	*313.1940 (13.41 ppm)*	322.2534 (4.03 ppm)
		M	***m/z* 269**	269.1372 (5.94 ppm)	287.2488 (10.10 ppm)	-	278.1941 (4.31 ppm)
		N	***m/z* 267**	267.1229 (0.75 ppm)	285.2356 (1.75 ppm)	276.1790 (2.17 ppm)	276.180 (5.07 ppm)
		O	***m/z* 147**	*147.0655 (0.68 ppm)*	*162.1595 (1.23 ppm)*	*153.1030 (1.96 ppm)*	*153.1037 (2.61 ppm)*

TABLE 2 Experimental mass data of GC/MS/MS product ions and chemical structures of standards **6–9**. Precursor ions are the respective $[M]^{\bullet+}$ ions (**#.1–4A**), data in italics recorded in scan mode. Calculated experimental error is given in brackets. For abbreviations, see Table 1

No.	Steroid		Ion	#.1: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 3-TMS, 2α/4/6/11β,17β-bis-[$^2H_9$]-TMS	#.4: 3-[$^2H_9$]-TMS, 2α/4/6/11β,17β-bis-TMS
6		A	$[M]^{\bullet+}$	520.3205 (2.69 ppm)	547.4903 (1.83 ppm)	538.4334 (2.79 ppm)	529.3784 (0.19 ppm)
		B	$[M - CH_3]^+$	505. 2976 (1.58 ppm)	532.4668 (2.07 ppm)	523.4102 (2.29 ppm)	*514.3551 (0.39 ppm)*
			$[M - CD_3]^+$		*529.4494 (0.76 ppm)*	*520.3923 (0.58 ppm)*	*511.3359 (0.39 ppm)*
		C	$[M - 73]^+$	447.2740 (1.12 ppm)			456.3309 (0.22 ppm)
			$[M - 82]^+$		465.3880 (1.07 ppm)	456.3310 (0 ppm)	
		E	$[M - 89]^+$	431.2783 (3.01 ppm)		449.3907 (4.23 ppm)	440.3345 (3.63 ppm)
			$[M - 98]^+$		449.3922 (0.89 ppm)	*440.3341 (4.54 ppm)*	431.2789 (1.62 ppm)
		F	$[M - 90]^{\bullet+}$	430.2718 (0 ppm)		448.3827 (4.68 ppm)	439.3282 (0.23 ppm)
			$[M - 99]^{\bullet+}$		448.3844 (0.89 ppm)	439.3274 (2.05 ppm)	*430.2715 (0.70 ppm)*
		G	$[M - CH_3 - 90]^+$	415.2468 (3.61 ppm)		433.3600 (3.00 ppm)	424.3047 (0.24 ppm)
			$[M - CH_3 - 99]^+$		*433.3616 (0.69 ppm)*	424.3052 (0.94 ppm)	*415.2480 (0.72 ppm)*
		H	$[M - CH_3 - 2 \times 90]^+$	325.1963 (5.84 ppm)			
			$[M - CH_3 - 90 - 99]^+$			*334.2544 (0.90 ppm)*	325.1976 (1.85 ppm)
			$[M - CH_3 - 2 \times 99]^+$		334.2542 (1.50 ppm)	325.1972 (3.08 ppm)	
		I	***m/z* 417**	417.2622 (4.31 ppm)	435.3759 (2.30 ppm)	435.3751 (4.13 ppm)	*417.2637 (0.72 ppm)*
						426.3228 (5.40 ppm)	*426.3202 (0.70 ppm)*
		J	***m/z* 358**	358.2309 (3.91 ppm)	367.2870 (4.63 ppm)	367.2881 (1.63 ppm)	*358.2323 (0 ppm)*
		K	***m/z* 343**	343.2082 (1.75 ppm)	352.2641 (3.41 ppm)	352.2640 (3.69 ppm)	*343.2088 (0 ppm)*
		L	***m/z* 315**	315.2130 (2.86 ppm)	324.2688 (4.93 ppm)	324.2703 (0.31 ppm)	*315.2134 (1.59 ppm)*
		M	***m/z* 269**	269.1387 (0.37 ppm)	287.2503 (4.87 ppm)	278.1937 (5.75 ppm)	*278.1935 (6.47 ppm)*
		N	***m/z* 267**	267.1227 (1.50 ppm)	285.2360 (0.35 ppm)	276.1789 (2.53 ppm)	*276.1794 (0.72 ppm)*
		O	***m/z* 147**	147.0654 (1.36 ppm)		153.1030 (1.96 ppm)	153.1031 (1.31 ppm)
					162.1590 (4.32 ppm)	156.1218 (1.92 ppm)	156.1220 (0.64 ppm)

(Continues)

TABLE 2 (Continued)

No.	Steroid		Ion	#.1: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 3-TMS, 2α/4/6/11β,17β-bis-[$^2H_9$]-TMS	#.4: 3-[$^2H_9$]-TMS, 2α/4/6/11β,17β-bis-TMS
7		A	$[M]^{\bullet+}$	520.3203 (3.08 ppm)	547.4896 (3.11 ppm)	538.4338 (2.04 ppm)	529.3770 (2.64 ppm)
		B	$[M - CH_3]^+$	505.2971 (2.57 ppm)	532.4663 (3.0 ppm)	523.4107 (1.34 ppm)	514.3536 (2.53 ppm)
			$[M - CD_3]^+$	*529.4488 (0.38 ppm)*		*520.3931 (0.96 ppm)*	*511.3362 (0.20 ppm)*
		C	$[M - 73]^+$	447.2744 (0.22 ppm)			456.3309 (0.22 ppm)
			$[M - 82]^+$		465.3866 (1.93 ppm)	456.3309 (0.22 ppm)	
		D	$[M - CH_3 - 73]^{\bullet+}$	432.2495 (3.47 ppm)			
			$[M - CH_3 - 82]^{\bullet+}$		450.3977 (74.83 ppm)	441.3045 (6.80 ppm)	432.2487 (5.32 ppm)
		E	$[M - 89]^+$	431.2787 (2.09 ppm)			
			$[M - 98]^+$		449.3908 (4.01 ppm)	440.3332 (6.59 ppm)	431.2784 (2.78 ppm)
		F	$[M - 90]^{\bullet+}$	430.2705 (3.02 ppm)		448.3878 (6.69 ppm)	439.3291 (1.82 ppm)
			$[M - 99]^{\bullet+}$		448.3868 (4.46 ppm)	439.3294 (2.50 ppm)	430.2713 (1.16 ppm)
		G	$[M - CH_3 - 90]^+$	415.2479 (0.96 ppm)		433.3591 (5.08 ppm)	424.3056 (1.89 ppm)
			$[M - CH_3 - 99]^+$		433.3599 (3.23 ppm)	*424.3050 (0.47 ppm)*	415.2472 (2.65 ppm)
		H	$[M - CH_3 - 2 \times 90]^+$	325.1974 (2.46 ppm)			334.2530 (5.09 ppm)
			$[M - CH_3 - 90 - 99]^+$			*334.2548 (0.30 ppm)*	325.1974 (2.46 ppm)
			$[M - CH_3 - 2 \times 99]^+$		334.2551 (1.20 ppm)	*325.1972 (3.08 ppm)*	
		J	***m/z* 358**	358.2312 (3.07 ppm)	367.2876 (2.99 ppm)	367.2887 (0 ppm)	358.2315 (2.23 ppm)
		K	***m/z* 343**	343.2072 (4.66 ppm)	*352.2648 (1.42 ppm)*	*352.2655 (0.57 ppm)*	343.2101 (3.79 ppm)
		L	***m/z* 315**	315.2129 (3.17 ppm)	*324.2690 (4.32 ppm)*	324.2701 (0.93 ppm)	*315.2133 (1.90 ppm)*
		M	***m/z* 269**	269.1373 (5.57 ppm)	*287.2509 (2.79 ppm)*	*278.1948 (1.80 ppm)*	278.1943 (3.59 ppm)
		N	***m/z* 267**	267.1224 (2.62 ppm)	285.2359 (0.70 ppm)	*276.1797 (0.36 ppm)*	276.1788 (2.90 ppm)
		O	***m/z* 147**	147.0655 (0.68 ppm)		153.1032 (0.65 ppm)	153.1029 (2.61 ppm)
					162.1592 (3.08 ppm)	156.1213 (5.12 ppm)	156.1215 (3.84 ppm)

(Continues)

TABLE 2 (Continued)

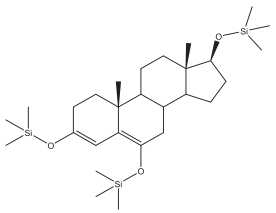
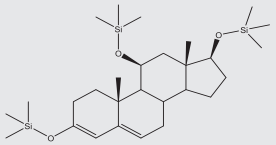
No.	Steroid		Ion	#.1: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 3-TMS, 2α/4/6/11β,17β-bis-[$^2H_9$]-TMS	#.4: 3-[$^2H_9$]-TMS, 2α/4/6/11β,17β-bis-TMS
8		A	$[M]^{\bullet+}$	520.3207 (2.31 ppm)	547.4927 (2.56 ppm)	538.4349 (3.16 ppm)	529.3796 (2.27 ppm)
		B	$[M - CH_3]^+$	505.2972 (2.37 ppm)	532.4668 (2.07 ppm)	*523.4132 (3.44 ppm)*	*514.3561 (2.33 ppm)*
			$[M - CD_3]^+$		*529.4497 (1.32 ppm)*	*520.3925 (0.19 ppm)*	*511.3359 (0.39 ppm)*
		C	$[M - 73]^+$	447.2742 (0.67 ppm)		465.3877 (0.43 ppm)	456.3308 (0.44 ppm)
			$[M - 82]^+$		465.3881 (1.29 ppm)	456.3311 (0.22 ppm)	*447.2767 (4.92 ppm)*
		E	$[M - 89]^+$	431.2794 (0.46 ppm)			*440.3337 (5.45 ppm)*
			$[M - 98]^+$		449.3825 (22.47 ppm)	*440.3319 (9.54 ppm)*	
		F	$[M - 90]^{\bullet+}$	*430.2717 (0.23 ppm)*		*448.3811 (8.25 ppm)*	*439.3277 (1.37 ppm)*
			$[M - 99]^{\bullet+}$		*448.3842 (1.34 ppm)*	*439.3283 (0 ppm)*	
		G	$[M - CH_3 - 90]^+$	415.2475 (1.93 ppm)		*433.3620 (1.62 ppm)*	*424.3055 (1.65 ppm)*
			$[M - CH_3 - 99]^+$		*433.3621 (0.23 ppm)*	*424.3061 (3.06 ppm)*	*415.2487 (0.96 ppm)*
		H	$[M - CH_3 - 2 \times 90]^+$	325.1971 (3.38 ppm)			
			$[M - CH_3 - 90 - 99]^+$			*334.2553 (1.80 ppm)*	325.1986 (1.23 ppm)
			$[M - CH_3 - 2 \times 99]^+$		334.2536 (3.29 ppm)	*325.1993 (3.38 ppm)*	
		P	***m/z* 319**	319.1540 (1.25 ppm)	337.2666 (2.37 ppm)	328. 2117 (2.44 ppm)	328.2115 (1.83 ppm)
9		A	$[M]^{\bullet+}$	520.3207 (2.31 ppm)	547.4901 (2.19 ppm)	538.4330 (3.53 ppm)	529.3778 (1.13 ppm)
		B	$[M - CH_3]^+$	*505.2984 (0 ppm)*	*532.4669 (1.88 ppm)*	523.4105 (1.72 ppm)	*514.3571 (4.28 ppm)*
			$[M - CD_3]^+$		*529.4484 (1.13 ppm)*	*520.3923 (0.58 ppm)*	*511.3359 (0.39 ppm)*
		C	$[M - 73]^+$	447.2735 (2.24 ppm)			456.3310 (0 ppm)
			$[M - 82]^+$		*465.3864 (2.36 ppm)*	456.3304 (1.31 ppm)	
		F	$[M - 90]^{\bullet+}$	430.2712 (1.39 ppm)		448.3824 (5.35 ppm)	*439.3297 (3.19 ppm)*
			$[M - 99]^{\bullet+}$		*448.3845 (0.67 ppm)*	439.3266 (3.87 ppm)	430.2694 (5.58 ppm)
		G	$[M - CH_3 - 90]^+$	415.2477 (1.44 ppm)		*433.3614 (1.62 ppm)*	424.3051 (0.71 ppm)
			$[M - CH_3 - 99]^+$		433.3596 (3.92 ppm)	424.3026 (5.18 ppm)	415.2457 (6.26 ppm)
		H	$[M - CH_3 - 2 \times 90]^+$	325.1976 (1.85 ppm)			
			$[M - CH_3 - 90 - 99]^+$			334.2535 (3.59 ppm)	325.2000 (5.54 ppm)
			$[M - CH_3 - 2 \times 99]^+$		334.2535 (3.59 ppm)	325.1965 (5.23 ppm)	
		Q	***m/z* 389**	389.2312 (3.85 ppm)		*398.2886 (1.51 ppm)*	*389.2325 (0.51 ppm)*
					407.3434 (5.40 ppm)	407.3446 (2.45 ppm)	*398.2891 (4.17 ppm)*
		R	***m/z* 387**	387.2165 (1.29 ppm)		396.2731 (1.01 ppm)	*387.2187 (4.39 ppm)*
					405.3277 (5.67 ppm)	405.3295 (1.23 ppm)	*396.2737 (0.50 ppm)*
		S	***m/z* 234**	234.1428 (2.56 ppm)		234.1422 (5.13 ppm)	234.1421 (5.55 ppm)
					243.1990 (3.70 ppm)	243.2000 (0.41 ppm)	*243.1999 (0 ppm)*
		T	***m/z* 219**	219.1194 (2.74 ppm)		219.1190 (4.56 ppm)	*219.1191 (4.11 ppm)*
					228.1760 (2.19 ppm)	228.1754 (4.82 ppm)	*228.1765 (0 ppm)*

TABLE 3 Structures of unlabelled (**#.1**) and isotopically labelled TMS derivatives of steroid standards **1–5**. Structure **5.1** shows suitable hydrogen atoms within bonding distance (< 3.2 Å) of the C-11 TMSO group. Distances calculated by ChemDraw (version 15.0) displayed as mean values (± 0.5 Å)

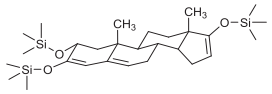
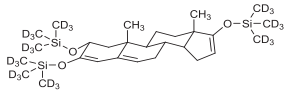
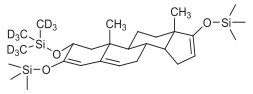
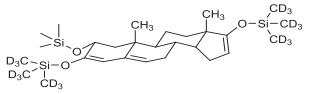
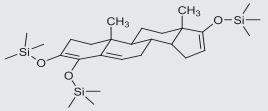
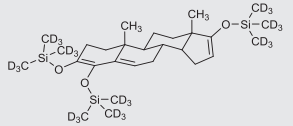
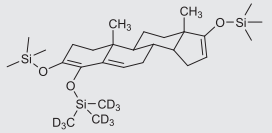
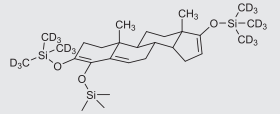
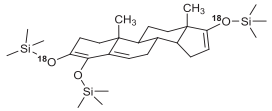
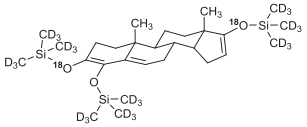
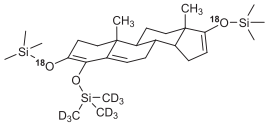
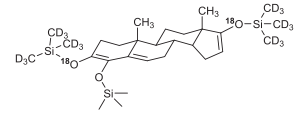
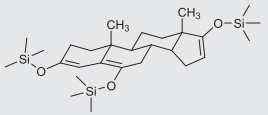
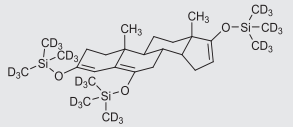
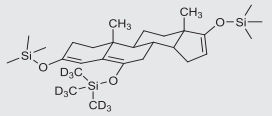
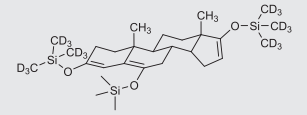
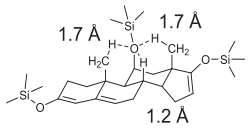
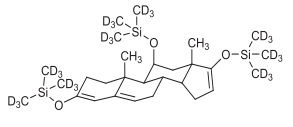
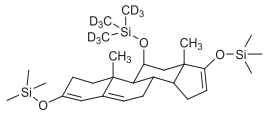
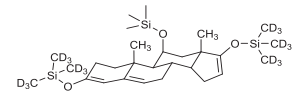
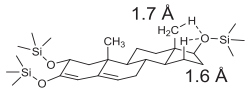
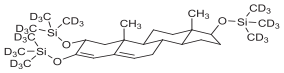
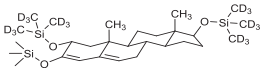
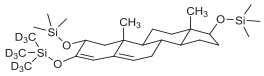
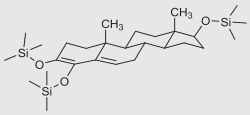
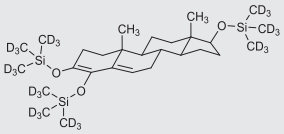
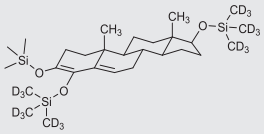
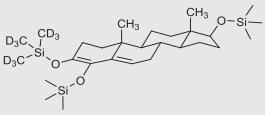
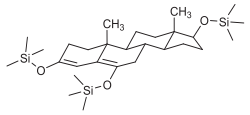
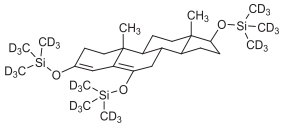
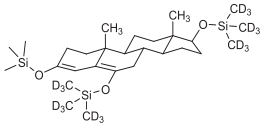
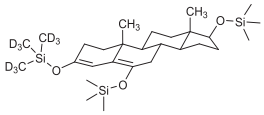
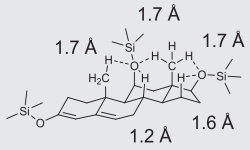
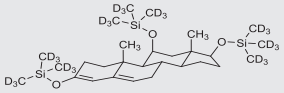
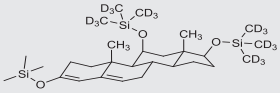
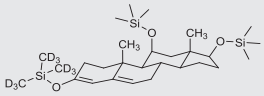
NO.	#.1: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 2/4/6/11-[$^2H_9$]-TMS, 3,17-bis-TMS	#.4: 2/4/6/11-TMS, 3,17-bis-[$^2H_9$]-TMS
1				
2				
3				
4				
5				

TABLE 4 Structures of unlabelled (**#.1**) and isotopically labelled TMS derivatives of steroid standards **6–9** with suitable hydrogen atoms (**6.1** and **9.1** as examples) 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: Tris-TMS	#.2: Tris-[$^2H_9$]-TMS	#.3: 3-TMS, 2α/4/6/11β,17β-bis-[$^2H_9$]-TMS	#.4: 3-[$^2H_9$]-TMS, 2α/4/6/11β,17β-bis-TMS
6				
7				
8				
9				

supporting information) with a precursor ion width of 1 *m/z* unit. For data acquisition and analyses MassHunter B.07.00 software (Agilent Technologies) was used. The elemental composition of fragment ions was calculated based on the high-resolution accurate mass data and the mass error (Δ*m/z*) was calculated automatically. A mass error ≤5 ppm was considered as acceptable in this work.

3 | RESULTS AND DISCUSSION

The observed retention times of all tris-TMS-derivatized analytes ranged between 15.022 min for 2β-hydroxyandrostenedione (**10**) and 15.482 min for 4-hydroxytestosterone (**7**), and are summarized in Table S2 (supporting information). Co-elution occurred in the case of 2β-hydroxyandrostenedione (**10**) and 6β-hydroxyandrostenedione (**4**), formestane (**2**) and 2α-hydroxyandrostenedione (**1**), and 2α-hydroxytestosterone (**6**) and 4-hydroxytestosterone (**7**), which can be explained by the similarities in their structures and chromatographic behavior, and was also described for these analytes in earlier studies with the suggestion of using MS/MS to improve selectivity.[18,19]

The mass spectra of the three derivatives (**#.2**, **#.3**, **#.4**) of each compound **1**–**10**, differently labelled with [$^2H_9$]-TMS groups, were compared with the respective unlabelled derivatives (**#.1**). Proposals for generation of fragment ions were substantiated with accurate mass data and MS/MS experiments, summarized in Tables 1 and 2 and discussed below.

3.1 | Fragment ion $[M - CH_3]^+$

Cleavage of a methyl radical from a TMS-derivatized steroid can either take place from the steroid backbone at position C-18 or C-19 (angular methyl groups) or from one of the TMS groups. In the mass spectra of all perdeuterotrimethylsilylated and mixed deuterated standards (**#.2B**, **#.3B**, **#.4B**, Tables 1 and 2) mainly $[M - CH_3]^+ = [M - 15]^+$ fragment ions were observed and only a very low abundance of $[M - CD_3]^+ = [M - 18]^+$ ions. This finding was confirmed with MS/MS experiments ($[M]^{\bullet+}$ as precursor) and complies with the assumption that in enol-TMS and Δ4 steroids methyl radical loss originates predominantly from the steroid nucleus as reported in the literature.[12,32]

In the case of tris-TMS-formestane (**2**) and 2-hydroxyandrostenedione (**1**), the $[M]^{\bullet+}$ ion (*m/z* 518, **1.1A**, **2.1A**, Table 1) and the $[M - CH_3]^+$ fragment ion (*m/z* 503, **2.1B**, **1.2B**, Table 1 and Figure 1) both generated *m/z* 169 in MS/MS experiments (**1.1**, Figures 2 and 3), which is described as characteristic for oxo

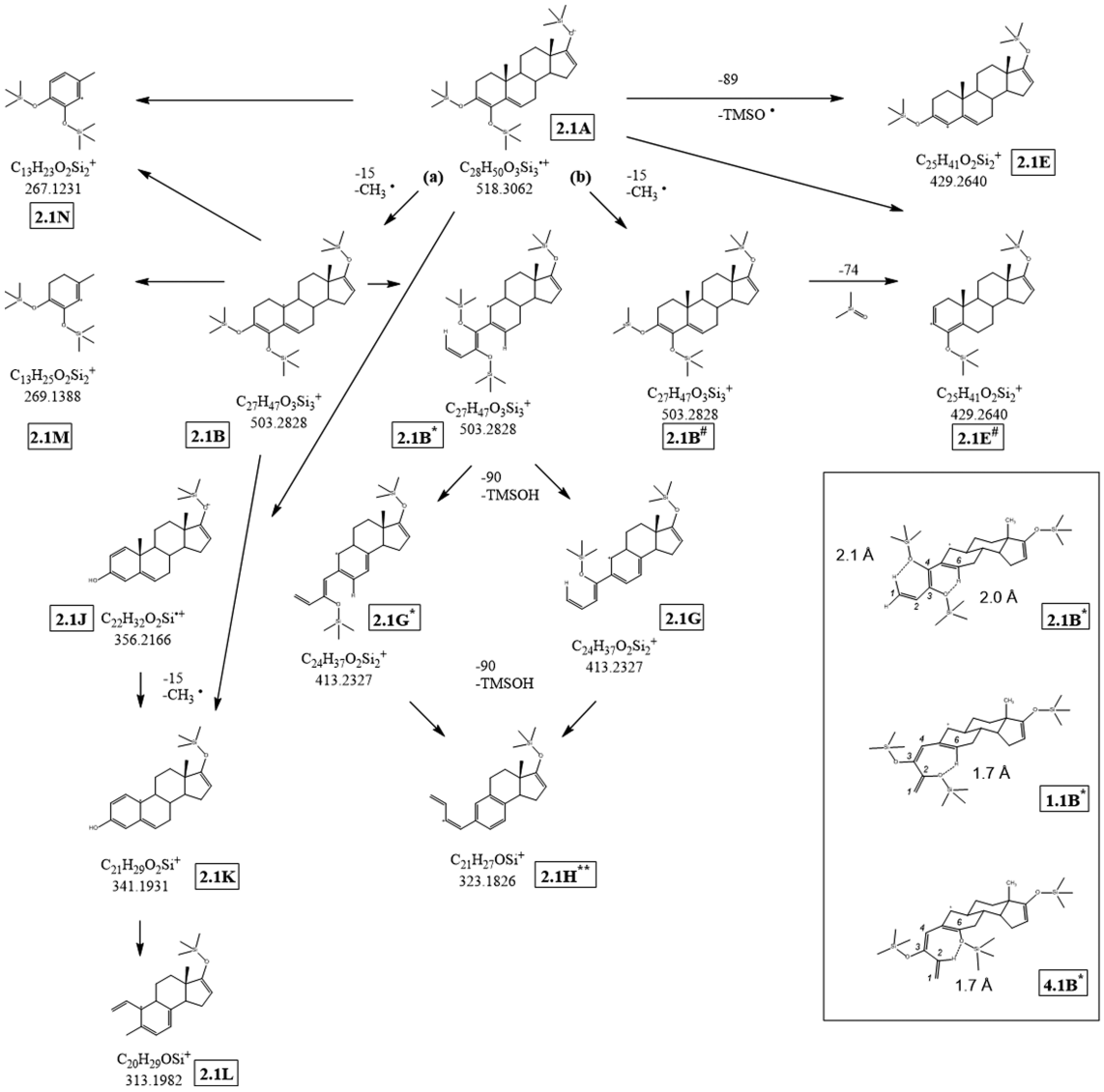


FIGURE 1 Proposed MS/MS fragmentation pathway of formestane-tris-TMS (**2.1A**). Pathway (a): Methyl cleavage from C-19 resulting in **2.1B** and **2.1B***. Pathway (b): Methyl cleavage from the TMS group resulting in **2.1B**$^{\#}$ and **2.1E**$^{\#}$. **2.1G*** predominantly formed compared with **2.1G**, structure shown for **2.1H**** most likely but the TMS group can also be located at C-3 or C-4. Box: Proposed stereochemical structures of fragment ion *m/z* 503 (**2.1B***, **1.1B*** and **4.1B***) with hydrogen atoms within bonding distance (< 3.2 Å) of the C-2/3/4/6 TMSO group for the formation and loss of TMSOH. Distances calculated by ChemDraw (version 15.0) displayed as mean values (± 0.5 Å)

groups in the steroidal D-ring.[33,34] This was also found for the MS/MS experiments of the other 17-oxo steroids **4** and **5**. As the presence of the angular C-18 methyl group is a prerequisite for the formation of the *m/z* 169 ion,[34] methyl loss from *m/z* 518 ([M]$^{\bullet+}$) to form *m/z* 503 probably takes place from the only other methyl group of the steroid backbone (position C-19). Further confirmation is planned in future studies.

3.2 | Fragment ion [M – TMS]$^{+}$

In all the studied mass spectra a [M – 73]$^{+}$ ion was detected with low abundance. It is proposed to result from TMS radical cleavage, and this is further substantiated by labelling experiments (**#.1-4C**, Tables 1 and 2). However, in MS/MS experiments of the respective [M]$^{\bullet+}$ precursor ions, the [M – 73]$^{+}$ ion was undetectable, probably due to its very low abundance. The mass spectra of the mixed deuterated derivatives of the 17-hydroxy steroids **6**, **8** and **9** suggest that the TMS group in position C-3 is the least likely to be cleaved off as a TMS radical because only the *m/z* 456 fragment ion is observed in the case of the **#.3** (**#.3C**, [M – 82]$^{+}$ = [M – [$^{2}H_{9}$]-TMS]$^{+}$) and **#.4** (**#.4C**, Table 2, [M – 73]$^{+}$ = [M – TMS]$^{+}$) derivatives. In the case of **7** (**7.3C**, **7.4C**, Table 2) and the 17-oxo steroids **1–5** and **10**, however, mass spectra of the mixed deuterated derivatives contain both fragment ions, [M – 73]$^{+}$ (**#.3C** *m/z* 454, **#.4C** *m/z* 463) and [M – 82]$^{+}$ (**#.3C** *m/z* 445, **#.4C** *m/z* 454, Table 1), which implies cleavage from any TMS group.

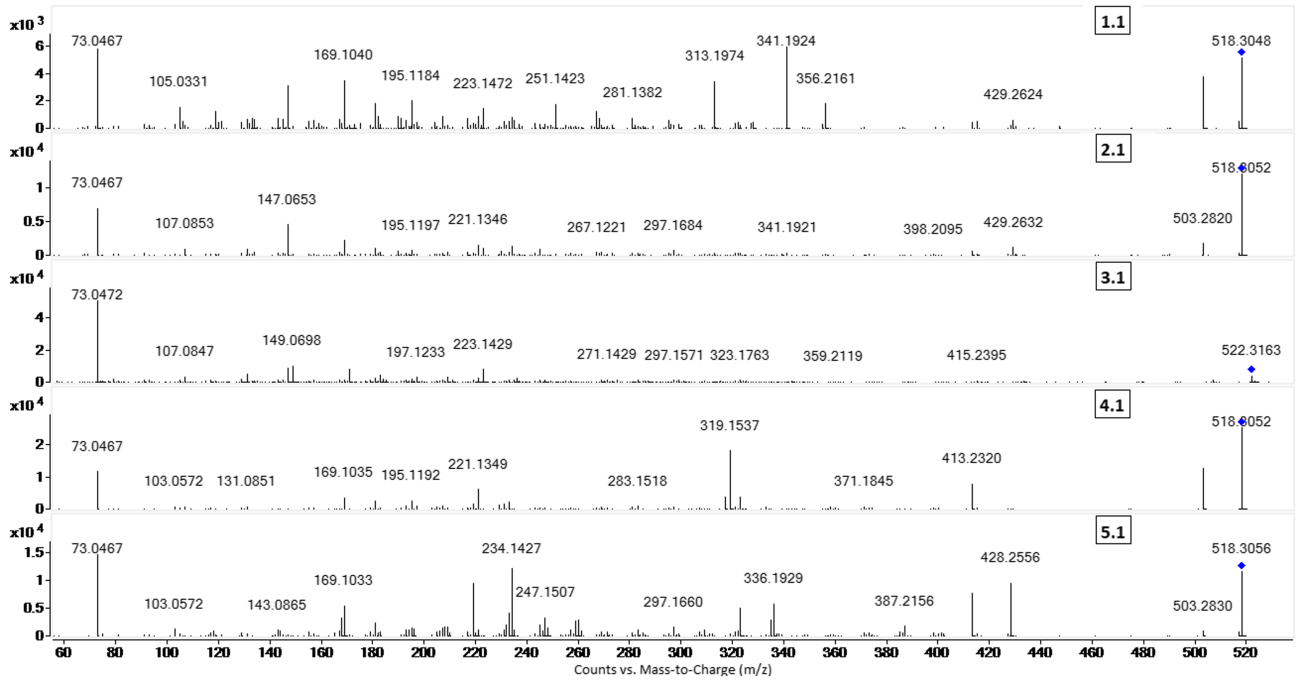


FIGURE 2 MS/MS spectra of standards 2α-hydroxyandrostendione-tris-TMS (**1.1**), formestane-tris-TMS (**2.1**), 3,17-[$^{18}O_2$]-formestane-tris-TMS (**3.1**), 6β-hydroxyandrostendione-tris-TMS (**4.1**) and 11β-hydroxyandrostenedione-tris-TMS (**5.1**). Precursor $[M]^{\bullet+}$ (**#.1A**), collision energy 30 eV [Color figure can be viewed at wileyonlinelibrary.com]

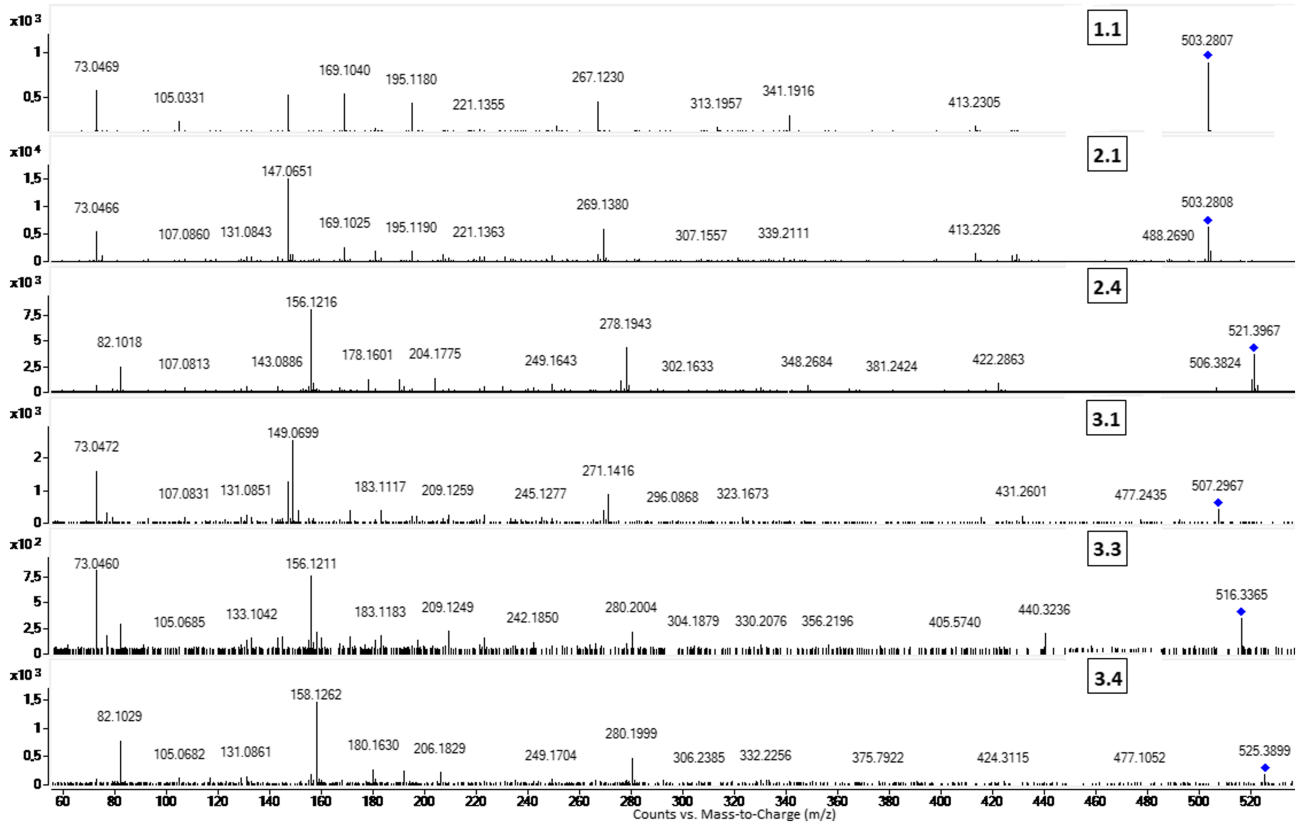


FIGURE 3 MS/MS spectra of standards 2α-hydroxyandrostendione-tris-TMS (**1.1**, 20 eV), formestane-tris-TMS (**2.1**, 15 eV), formestane-4-TMS, 3,17-bis-[$^{2}H_9$]-TMS (**2.4**, 15 eV), 3,17-[$^{18}O_2$]-formestane-tris-TMS (**3.1**, 20 eV), 3,17-[$^{18}O_2$]-formestane-4-[$^{2}H_9$]-TMS, 3,17-bis-TMS (**3.3**, 20 eV) and 3,17-[$^{18}O_2$]-formestane-4-TMS, 3,17-bis-[$^{2}H_9$]-TMS (**3.4**, 20 eV). Precursor $[M - CH_3]^+$ [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Fragment ions [M – TMSO]$^+$ and [M – TMSOH]$^{\bullet+}$

Loss of trimethylsilanol (TMSOH) is a typical feature observed in many GC/MS spectra of different hydroxy steroids. This loss is dependent on the steric characteristics of the steroid backbone as well as hydrogen availability.[4,12] Suitable hydrogen atoms are located in the 1,3-diaxial position from the TMS oxygen and the bonding distance needs to be less than 3.2 Å[35] (**#.1**, Tables 3 and 4). The dependence of TMSOH loss on hydrogen availability and bonding distance was recently demonstrated via ^{18}O-labelling for different hydroxy steroids, for example 5α- and 5β-androstanediols.[12]

For 4-hydroxy-3-keto-4-ene-steroids such as formestane (**2.1**, **3.1**, Table 3) and its 2α- and 6-hydroxy isomers (**1.1**, **4.1**, Table 3) no 1,3-diaxial hydrogen is available within bonding distance to the TMS oxygen in steroidal ring A or B. This is due to the conjugated double bonds after enolization and sp^2 hybridization of C-3-6 or quaternary C-10. When no steroidal ring opening occurs upon electron ionization, TMS groups can thus only be cleaved off as TMSO$^\bullet$ radicals[32] which is observed in the mass spectra as formation of the *m/z* 429 fragment ion ([M – TMSO]$^+$, **1.1E**, **2.1E**, **4.1E**, Table 1 and Figure 1). The positive charge of the resulting cation can be stabilized throughout the conjugated double-bond system in rings A and B. Loss of a TMSO$^\bullet$ radical from position C-17 is not postulated because it cannot be observed for other 3-keto-4-ene steroids, e.g. androstenedione, and the resulting cation would be less stable. For the 17β-hydroxy steroids **6–9**, however, the 15β hydrogen is close enough for binding with the C-17 TMS group and elimination of TMSOH from this position is possible (**6.1**, **9.1**, Table 4; **#.1F**, Table 2) in parallel to the additional loss of a TMSO$^\bullet$ radical from the A- or B-ring (**6.1E**, **7.1E**, **8.1E**, Table 2).

In order to elucidate which of the two TMSO groups in ring A, at position C-3 or position C-4 from formestane (**2**), is cleaved off, mixed deuterated derivatives have been prepared and analyzed. In the mass spectrum of the 3,17-bis-TMS, 4-[$^2H_9$]-TMS ([M]$^{\bullet+}$ = *m/z* 527, **2.3A**, Tables 1 and 3) derivative, fragment ions *m/z* 438 ([M – TMSO]$^+$) and 429 ([M – [$^2H_9$]-TMSO]$^+$, **2.3E**, Table 1) were observed. Corresponding fragment ions *m/z* 447 ([M – TMSO]$^+$) and 438 ([M – [$^2H_9$]-TMSO]$^+$, **2.4E**, Table 1) occurred in the case of the 3,17-bis-[$^2H_9$]-TMS, 4-TMS ([M]$^{\bullet+}$ = *m/z* 536, **2.4A**, Tables 1 and 3) derivative. As a result, two different losses of 89 Da (OTMS$^\bullet$) and 98 Da ([$^2H_9$]-OTMS$^\bullet$) were observed from either position C-3 or position C-4, indicating that there is no steric preference.

Because TMS groups are prone to migrate within the molecule,[36] and to further confirm the hypothesis, additional mixed deuterated derivatives of oxygen-labelled 3,17-[$^{18}O_2$]-formestane (**3**) were prepared and analyzed. In the mass spectrum of the 3,17-[$^{18}O_2$]-3,17-bis-TMS, 4-[$^2H_9$]-TMS ([M]$^{\bullet+}$ = *m/z* 531, **3.3A**, Tables 1 and 3) derivative, fragment ions *m/z* 440 ([M – TMS^{18}O]$^+$) and 433 ([M – [$^2H_9$]-TMSO]$^+$, **3.3E**, Table 1) were observed. In the case of the 3,17-[$^{18}O_2$]-3,17-bis-[$^2H_9$]-TMS, 4-TMS ([M]$^{\bullet+}$ = *m/z* 540, **3.4A**, Tables 1 and 3) derivative, ions *m/z* 451 ([M – TMSO]$^+$) and 440 ([M – [$^2H_9$]-TMS^{18}O]$^+$, **3.4E**, Table 1) were generated. Losses of 89 Da (TMSO$^\bullet$), 91 Da (TMS^{18}O$^\bullet$), 98 Da ([$^2H_9$]-TMSO$^\bullet$) and 100 Da ([$^2H_9$]-TMS^{18}O$^\bullet$) observed for the doubly labelled derivatives confirmed that TMSO$^\bullet$ radical loss in formestane takes place from both the C-3 and the C-4 positions.

Interestingly, the MS/MS spectra of [M – CH$_3$]$^+$ (*m/z* 503) also exhibit the [M – TMSO]$^+$ (*m/z* 429) ion. This finding implies methyl radical cleavage from a TMS group followed by a neutral loss of the remaining dimethylsilyloxy group and can be described as [M – CH$_3$ – (CH$_3$)$_2$SiO]$^+$ = [M – 15 – 74]$^+$ (**2.1E$^\#$**, Figure 1, pathway (b)). In MS/MS experiments of the perdeuterotrimethylsilylated derivatives of compounds **1**, **2**, **4** and **6–8** this ion was generated as [M – CD$_3$ – (CD$_3$)$_2$SiO]$^+$ = [M – 18 – 80]$^+$ (*m/z* 447, **#.1E**, Table 1; *m/z* 449, **#.1E**, Table 2). The ^{18}O-labelled derivatives of formestane substantiated the proposed route of formation via loss of the entire C-3 TMS group: ion [M – CH$_3$ – (CH$_3$)$_2$Si^{18}O]$^+$ = [M – 15 – 76]$^+$ was found for **3.1** (*m/z* 431, **3.1E**) and **3.3** (*m/z* 440, **3.3E**), [M – CD$_3$ – (CD$_3$)$_2$Si^{18}O]$^+$ = [M – 18 – 82]$^+$ for **3.2** (*m/z* 449, **3.2E**) and **3.4** (*m/z* 440, **3.4E**, Table 1).

An ion of very low abundance at *m/z* 428 (**1.1F**, **4.1F**, Table 1) is observed in the mass spectra of **1** (2-hydroxy-) and **4** (6-hydroxy-), indicating that there is also a loss of TMSOH from [M]$^{\bullet+}$. This fragment ion is proposed to originate from the A- or B-ring. A comparison of the MS/MS spectra of the isomers 2α- (**1.1)** and 2β-hydroxyandrostenedione (**10.1**, Table 1, and Figure S1, supporting information) with [M]$^{\bullet+}$ = *m/z* 518 as precursor revealed approximately the same abundance of the *m/z* 428 ([M – TMSOH]$^{\bullet+}$) and 429 ([M – TMSO]$^+$) ions in the case of the 2β isomer; however, in the mass spectrum of the 2α isomer, the abundance of the ion at *m/z* 428 was only one-third of that of *m/z* 429. If the TMSO group is in the 2β position it is within bonding distance to the C-19 methyl hydrogen atoms and loss of TMSOH is more likely than for the 2α isomer. The cleavage of a TMSO$^\bullet$ radical is not observed in the mass spectra of the 11-hydroxy steroids (**5** and **9**) because the requirements for the favored formation and neutral loss of TMSOH are met: the TMS group in position C-11 is within bonding distance to the hydrogens of the angular methyl groups in positions C-18 and C-19 and to the β-hydrogen in position C-8 (**5.1**, Table 3 and **9.1**, Table 4).

3.4 | Fragment ion [M – CH$_3$ – TMSOH]$^+$

Interestingly, standards **1–4** produce the fragment ion [M – 15 – 90]$^+$ (*m/z* 413, **#.1G**, Table 1, **2.1G,** Figure 1, pathway (a)) although no separate loss of TMSOH is observed in the mass spectra. If the cleavage of a methyl radical to form the fragment ion [M – 15]$^+$ takes place from position C-19 (as described above) and bond cleavage between C-10 and C-1 occurs, the resulting conformational changes in the steroid backbone obviously lead to better hydrogen availability and allow the TMS groups in rings A and B to be eliminated as TMSOH rather than as TMSO$^\bullet$: rotation of the C-4–C-5 bond enables the TMS groups on positions C-2 and C-3 to interact with the C-6 hydrogen (**2.1B***, **1.1B***), the C-4 TMS group with the C-1 hydrogen (**2.1B***) and the C-6 TMS group with the C-2 hydrogens (**4.1B***, Figure 1).

The spectra of the mixed deuterated standards (**#.3**, **#.4**, Table 3) revealed the two analogous fragment ions, $[M - 105]^+$ and $[M - 114]^+$ (**#.3G**, **#.4G** Table 1), showing that the TMS group in either ring A or B can be eliminated as TMSOH or $[^2H_9]$-TMSOH following or simultaneously with cleavage of the C-19 methyl group. The derivatives of formestane doubly labelled with ^{18}O and $[^2H_9]$-TMS form fragment ions $[M - 105]^+$, $[M - 107]^+$, $[M - 114]^+$ and $[M - 116]^+$ (**3.3G**, **3.4G**, Table 1).

Formation of $[M - 105]^+$ resulting from cleavage of the C-18 methyl and loss of TMSOH from C-17 is considered less favorable because the occurrence of the *m/z* 169 ion in the MS/MS spectrum of the formestane $[M - 15]^+$ ion indicates an intact D-ring. Cleavage of the C-18 methyl and TMSOH elimination from ring A or B are also unlikely, as only $TMSO^•$ as a radical can be cleaved off without the required conformational changes. The resulting $[M - 104]^{•+}$ (= $[M - 15 - 89]^{+·}$) ion was not detected in the respective mass spectra.

In case of the hydroxy steroids **6–9** formation of the $[M - 105]^+$ fragment ion can take place from either the A- or the D-ring. The TMS oxygen at C-17 is within bonding distance to the three C-18 methyl hydrogens and the 15β hydrogen (**#.1**, Table 4). Thus, formation of $[M - 105]^+$ by elimination of TMSOH from the D-ring is possible. 11β-Hydroxyandrostenedione (**5**) can additionally form the $[M - 105]^+$ fragment ion by elimination of TMSOH from position C-11 and subsequent or concomitant cleavage of a methyl radical (probably the C-19 angular methyl group).

3.5 | Fragment ion $[M - CH_3 - 2 \times TMSOH]^+$

Neutral loss of two TMSOH molecules from the $[M - 15]^+$ fragment ion results in the $[M - 195]^+ = [M - CH_3 - 2 \times TMSOH]^+$ ion (**#.1H**, Tables 1 and 2, **2.1H****, Figure 1), which can be described as different chemical structures depending on the position of the remaining TMS group and which was observed for all nine compounds in their respective MS/MS spectra of $[M]^{•+}$ (**#.1A**), $[M - 15]^+$ (**#.1B**) and $[M - CH_3 - TMSOH]^+$ (**#.1G**) as precursors, in the case of the 11-hydroxy steroids **5** and **9** also with precursor $[M - TMSOH]^{•+}$ (**5.1F**, **9.1F**, Tables 1 and 2). Isotopic labelling with ^{18}O revealed two ions for formestane (**3**), which implies that the $[M - CH_3 - 2 \times TMSOH]^+$ ion contains either the TMS group from position C-4 (*m/z* 323, **3.1H** and **3.4H**, *m/z* 332, **3.2H** and **3.3H**) or one of the TMS groups from position C-3 or C-17 (*m/z* 325, **3.1H** and **3.3H**, *m/z* 334, **3.2H** and **3.4H**, Table 1). The latter explanation is considered more likely regarding the proposed formation of the $[M - CH_3 - TMSOH]^+$ ion (**2.1G***, **2.1H****, Figure 1). Due to the double bond between C-16 and C-17 and sp^2 hybridization, the TMS oxygen is far away from any hydrogen for bonding and subsequent loss of TMSOH. The $[M - 195]^+$ fragment ion of the C-17 TMS enol ether of the 17-oxo series (**1–5**) is therefore proposed to predominantly contain the C-17 TMS group. In the case of the 17-hydroxy analogs (**6–9**), isotopic labelling with $[^2H_9]$-TMS did not convey a clear trend as to which TMS group remains in the fragment ion $[M - 195]^+$. In general, MS/MS data of the mixed deuterated and perdeuterotrimethylsilylated derivatives comply with the proposed fragment ion generation of $[M - CH_3 - 2 \times TMSOH]^+$ (**#.3H**, Table 1; **#.4H**, Table 2), $[M - CH_3 - TMSOH - [^2H_9]\text{-}TMSOH]^+$/ $[M - CH_3 - [^2H_9]\text{-}TMSOH - TMSOH]^+$ (**#.3H**, **#.4H**, Tables 1 and 2) and $[M - CH_3 - 2 \times [^2H_9]\text{-}TMSOH]^+$ (**#.2H**, Tables 1 and 2; **#.4H**, Table 1; **#.3H**, Table 2).

3.6 | Fragment ions representing structural selectivity

The mass spectra of the analyzed 3-keto-4-ene steroids are dominated by common fragment ions that contain one or several TMS groups or are generated through their loss or rearrangement (e.g. *m/z* 169, $[M - TMSOH]^{•+}$, $[M - TMSOH - CH_3]^+$). It is therefore challenging to find fragment ions that can be considered specific for a certain subclass, i.e. position of the hydroxy group in the backbone. MS/MS experiments and stable isotope labelling helped to describe and propose structures of the following fragment ions (Table S1, supporting information).

3.6.1 | 2- and 4-Hydroxy steroids

The mass spectra of the analyzed 2- and 4-hydroxy steroids (**1–3**, **6–7**) do not show abundant fragmentation and are very similar. In order to find minor differences in the fragmentation behavior more MS/MS experiments were necessary than for the other steroid subclasses. With the help of isotopic labelling, accurate mass data and MS/MS experiments, a fragmentation pathway for formestane was established (Figure 1). The given structures were considered most likely for the respective elemental composition; calculated errors for each fragment ion are given in Table 1. For 2β-hydroxyandrostenedione (**10**), of the fragment ions **I–K** and **O**, ions **L**, **M** and **N** are only included in Table 1 but they are not discussed below due to their very low abundance. As this steroid can easily be distinguished from **1** and **2** because of its different retention time, the focus of the work therefore was to find differences in the mass spectra of 2α-hydroxyandrostenedione (**1**) and formestane (**2**).

3.6.1.1 | *m/z* 147

The fragment ion *m/z* 147 is indicative of vicinal or other TMS groups close enough in the molecule to interact and form an ion with the elemental composition of $(CH_3)_2Si{=}O^+\text{-}Si(CH_3)_3$.[37,38] This fragment ion is observed more prominently in the mass spectra of the 2- and 4-hydroxy steroids (**1–3**, **6–7**, **#.1O**, Tables 1 and 2) but can also be found in the spectra of the other subclasses with very low abundance.

In the mass spectra of the perdeuterotrimethylsilylated derivatives (**1.2**, **2.2**, **6.2**, **7.2**, Tables 3 and 4) the ion at *m/z* 147 is shifted to *m/z* 162 (+15 *m/z* units, for a total of five CD_3 groups,

$(CD_3)_2Si=O^+$-$Si(CD_3)_3$, **#.2O**, Tables 1 and 2). In the mass spectra of the mixed deuterated derivatives, ions at *m/z* 153 (+6 *m/z* units, for two CD_3-groups, $(CD_3)_2Si=O^+$-$Si(CH_3)_3$) and *m/z* 156 (+9 *m/z* units, for three CD_3-groups, $(CD_3)_2Si=O^+$-$Si(CH_3)_3$)) are observed (**#.3O**, **#.4O**, Tables 1 and 2).

Interestingly, this ion represents the base peak in the MS/MS spectra of formestane when $[M - 15]^+$ (respectively $[M - 18]^+$ in the case of d_9-TMS derivatization) is utilized as the precursor ion (**2.1**, Figure 3). This finding suggests that the *m/z* 147 ion is formed more readily following conformational changes after cleavage of either the C-19 methyl group (see formation of $[M - 15]^+$) or one of the methyl groups from the involved TMS groups. In Figure 3 the MS/MS spectra of the $[M - 15]^+$ ions of the 4-TMS, 3,17-bis-$[^2H_9]$-TMS (**2.4**, Table 3) and the 4-TMS, 3,17$[^{18}O_2]$-bis-$[^2H_9]$-TMS (**3.4**, Table 3) formestane derivatives are compared. The fragment ion at *m/z* 156 (**2.4O**) is shifted to *m/z* 158 by oxygen labelling (**3.4O**, Table 1) implying that the oxygen originates from position C-3 together with its $[^2H_9]$-TMS group and methyl cleavage occurs from the C-4 TMS group. The MS/MS spectra of $[M - CD_3]^+ = [M - 18]^+$ show the ion at *m/z* 153 for both derivatives (**2.4O**, **3.4**, Table 1). In this case the CD_3 radical is cleaved from the $[^2H_9]$-TMS group in position C-3 and the TMS group from position C-4 supplies the (unlabeled) oxygen for fragment ion generation. For the 4-$[^2H_9]$-TMS, 3,17-bis-TMS derivative (**2.3O**) and $[M - CD_3]^+$ ion as precursor, however, a mass shift of +2 *m/z* units to *m/z* 155 (**3.3O**, Table 1) is observed after ^{18}O-labelling because the positions of CD_3 loss (C-4 $[^2H_9]$-TMS) and oxygen (C-3 TMS group) are opposite to those in derivatives **2.4** and **3.4**. As a result, the "intact" TMS group supplies its attached oxygen for the formation of the *m/z* 147 ion.

3.6.1.2 | *m/z* 417 and 415

Fragment ions at *m/z* 417 (**6.1I**, Table 2) and 415 (**1.1I**, Table 1) were observed in the mass spectra of the 2-hydroxy compounds (**1** and **6**), and with a very low abundance also for the 4-hydroxy steroids **2**, **3** and **7**. Deuteration showed that the fragment ions contain two TMS groups because a mass shift of +18 *m/z* units was observed (*m/z* 433, **1.2I**, Table 1 and *m/z* 435, **6.2I**, Table 2). The proposed elemental compositions were substantiated as $C_{24}H_{41}O_2Si_2^+$ (exact mass 417.2640) and $C_{24}H_{39}O_2Si_2^+$ (exact mass 415.2483) via accurate mass calculations. The obtained data for the 4-hydroxy steroids does not comply with these elemental compositions and must therefore be of different origin: the experimental masses were *m/z* 415.2112 (mass error 89.34 ppm) for **2.1**, 419.2191 (mass error 89.92 ppm) for **3.1** (^{18}O-labelled formestane) and 417.2265 (mass error 89.87 ppm) for **7.1**. An elemental composition of $C_{23}H_{35}O_3Si_2^+$ (exact mass 415.2119), however, fits with the observed fragment ions, resulting in mass errors of 1.69 ppm for **2.1**, 3.10 ppm for **3.1** and 2.64 ppm for **7.1,** and might be explained by a methyl cleavage from the fragment ion at *m/z* 430 (see below). MS/MS experiments with $[M]^{\bullet+}$ of the mixed deuterated derivatives of the 2-hydroxy isomers **1.3**, **1.4**, **6.3** and **6.4** (Tables 3 and 4) as the precursor ion imply that either the C-2 and C-17 TMS groups are present in these fragment ions (*m/z* 424 **1.3I**, **1.4I**, *m/z* 435 **6.3I**, *m/z* 417 **6.4I**) or the C-3 and C-17 TMS groups are present (*m/z* 415 **1.3I**, *m/z* 433 **1.4I**, *m/z* 426 **6.3I**, **6.4I**, Tables 1 and 2). When using *m/z* 415 (**1.1I**) and 433 (**1.2I**, Table 1) as precursors, product ions at *m/z* 169 and 178 (*m/z* 169 + 9 *m/z* units for the $[^2H_9]$-TMS group in position C-17) were

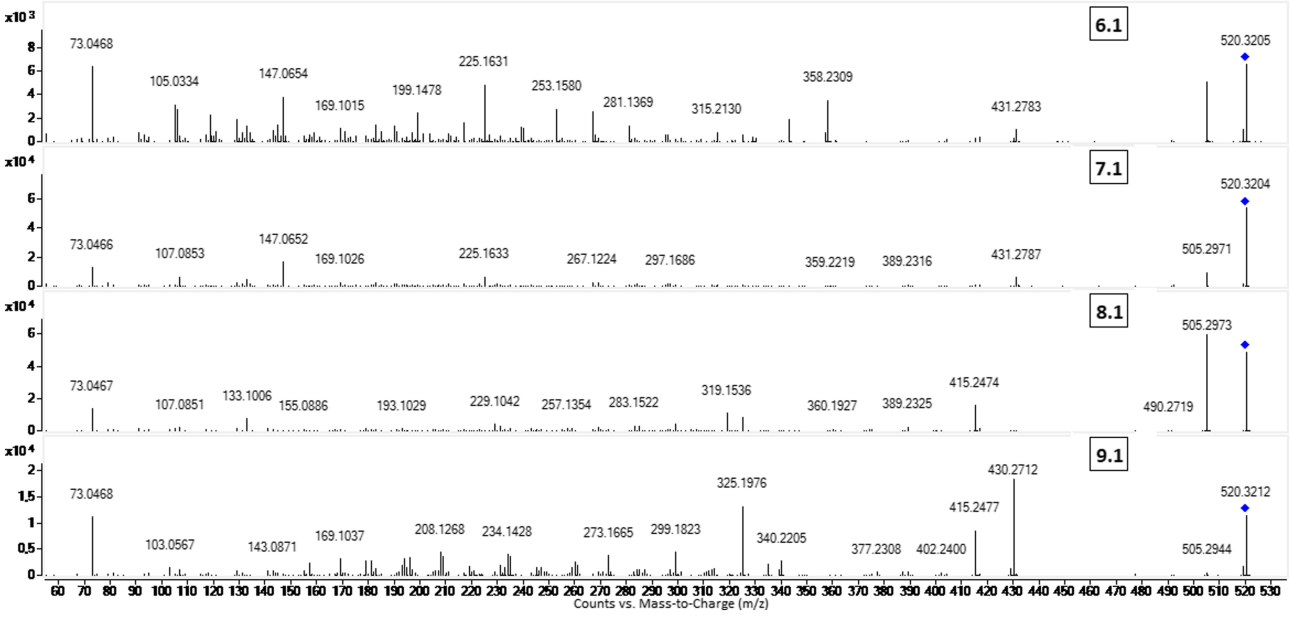


FIGURE 4 MS/MS spectra of standards 2α-hydroxytestosterone-tris-TMS (**6.1**), 4-hydroxytestosterone-tris-TMS (**7.1**), 6β-hydroxytestosterone-tris-TMS (**8.1**) and 11β-hydroxytestosterone-tris-TMS (**9.1**). Precursor $[M]^{\bullet+}$ (**#.1A**), collision energy 30 eV [Color figure can be viewed at wileyonlinelibrary.com]

generated, which is only possible with an intact D-ring (see above). The fragment ion at *m/z* 417 (**6.1I**, Table 2) is considered to be the 17-hydroxy analog to *m/z* 415 with two additional hydrogen atoms in the D-ring. It is therefore unlikely that the ion at *m/z* 417 contains the C-2 and C-3 TMS groups and not the C-17 TMS group.

3.6.1.3 | *m/z* 356, 341 and 313

Fragment ions *m/z* 358, 343 and 315 for the 17-hydroxy steroids **6** and **7** and the respective analogs *m/z* 356, 341 and 313 for the 17-oxo steroids **1–3** (**#.1J**, **1K**, **1L**, Tables 1 and 2; **2.1J**, **2.1K**, **2.1L**, Figure 1) were found to be common for both subclasses, 2- and 4-OH steroids. However, the abundance in the product ion spectra of the 2-hydoxy isomers is much higher than for the 4-hydroxy isomers where only relative intensities below 10% for these fragments are detected (**1.1**, **2.1**, **3.1**, Figure 2; **6.1**, **7.1**, Figure 4). MS/MS experiments for **6** and **7** revealed that cleavage of a methyl group from the ion at *m/z* 358 results in *m/z* 343 and, after a neutral loss of 28 Da (CO) from the A-ring, a product ion at *m/z* 315 (**6.1L**, **7.1L**, Table 2) is generated. The same was seen for the *m/z* 356, 341 and 313 ions for the 17-oxo steroids **1–3**. The fragment ion $[M - 15]^+$ only resulted in *m/z* 343/341 and not *m/z* 358/356 in MS/MS experiments, which indicates that the same methyl group (probably the C-19 angular methyl group) is cleaved off in the formation of $[M - 15]^+$ and is present in *m/z* 358/356.

To obtain more information about these three fragment ions, mass spectra of the isotopically labelled formestane derivatives were compared. In the case of the perdeuterotrimethylsilylated and the 4-TMS, 3,17-bis-[$^2H_9$]-TMS derivative all fragment ions were shifted by +9 *m/z* units to yield *m/z* 365 (**2.2J**, **2.4J**), 350 (**2.2K**, **2.4K**) and 322 (**2.2L**, **2.4L**, Table 1). No mass shift was observed for the 4-[$^2H_9$]-TMS, 3,17-bis-TMS derivative (**2.3J**, **2.3K**, **2.3L**, Table 1). This leads to the conclusion that these three fragment ions only contain one TMS group that is not located in the A-ring and it is therefore proposed to be the one in position C-17 (**2.1J**, **2.1K**, **2.1L**, Figure 1 and Table S1, supporting information).

Mass spectra of ^{18}O-labelled formestane were used to further investigate the position of the remaining hydroxy group in ring A. Mass shifts of both +2 and +4 *m/z* units were observed for fragment ions *m/z* 356 (to *m/z* 358 and 360, **3.1J**) and 341 (to *m/z* 343 and 345, **3.1K**, Table 1) which indicate that the hydroxy group in these two fragment ions originates from either position C-3 or position C-4. For the fragment ion at *m/z* 313 the observed mass shift was +2 *m/z* units to *m/z* 315 (**3.1L**, Table 1) which complies with the assumption that there is only one (labelled) oxygen left in the fragment ion, namely the one at position C-17.

In the case of the formestane derivatives doubly labelled with ^{18}O and [$^2H_9$]TMS, the detected fragment ions were shifted accordingly to *m/z* 367 (*m/z* 356 + 2 + 9, **3.2J**, **3.4J**), 369 (*m/z* 356 + 4 + 9, **3.2J**, **3.4J**), 352 (*m/z* 341 + 2 + 9, **3.2K**, **3.4K**), 354 (*m/z* 341 + 4 + 9, **3.4K**) and 324 (*m/z* 313 + 2 + 9, **3.2L, 3.4L** Table 1).

3.6.1.4 | *m/z* 269 and 267

Two other fragment ions observed in all the 2- and 4-hydroxy steroids are *m/z* 267 (**1.1N**, **2.1N**, **6.1N**, **7.1N**) and its analog *m/z* 269 (**1.1M**, **2.1M**, **6.1M**, **7.1M**, Tables 1 and 2). The latter contains two more hydrogen atoms and is more pronounced in the mass spectra of the 4-hydroxy steroids **2–3** and **7**. These fragment ions are proposed to originate from the A-ring and contain two TMS groups (**2.1N**, **2.1M**, Figure 1). Thus, a mass shift of +18 *m/z* units for the perdeuterotrimethylsilyl (*m/z* 287 **1.2M**, **2.2M**, **6.1M**, **7.1M** and *m/z* 285 **1.2N**, **2.2N**, **6.1N**, **7.1 N**, Tables 1 and 2) derivatives and a mass shift of +9 *m/z* units for the respective mixed deuterated derivatives is observed (*m/z* 278 **1.3M**, **1.4M**, **2.3 M**, **2.4M**, **6.3M**, **6.4M**, **7.3M**, **7.4M** and *m/z* 276 **1.3N**, **1.4N**, **2.3N**, **2.4N**, **6.3N**, **6.4N**, **7.3N**, **7.4N**, Tables 1 and 2). There is an additional mass shift of +2 *m/z* units for the ^{18}O-labelled formestane derivative to *m/z* 269 (*m/z* 267 + 2, **3.1N**) and 271 (*m/z* 269 + 2, **3.1M**, Table 1) which complies with the proposed structure and accurate mass data. In the mass spectra of the doubly labelled formestane derivatives, these two fragment ions were shifted to *m/z* 289 (*m/z* 269 + 2 + 18, **3.2M**), 287 (*m/z* 267 + 2 + 18, **3.2N**), 280 (*m/z* 269 + 2 + 9, **3.3M**, **3.4M**) and 278 (*m/z* 267 + 2 + 9, **3.3N**, **3.4N**, Table 1).

MS/MS analyses showed that $[M - 15]^+$ as precursor ion gives rise to the ions *m/z* 267 for **1** and *m/z* 269 for **2** in high abundance (**1.1**, **2.1**, Figure 3), which could be used to better distinguish these structurally closely related compounds, for example using the MRM transitions *m/z* 503 → *m/z* 269 and *m/z* 267 in addition to the transitions previously described.[17] In the case of the 17-hydroxy analogs, MS/MS of $[M - 15]^+$ showed both product ions *m/z* 269 and 267 in the mass spectrum of **7** (4-hydroxytestosterone) and a product ion at *m/z* 267 in the spectrum of **6** (2-hydroxytestosterone).

3.6.1.5 | *m/z* 430

The loss of 88 Da from $[M]^{\bullet+}$ was only observed in the mass spectra of **2**, **3** and **7** (**#.1D**, Tables 1 and 2) as a very small signal and it must therefore originate from ring A. It was seen at unit resolution but could not be detected using the GC/QTOF mass spectrometer in scan mode. This may be explained by differences in the mass analyzer design and overlap of the isotopic cluster of this ion with the M + 1 ion of the fragment ion at *m/z* 429 ($[M - TMSO]^+$, **2.1E**, Table 1). MS/MS experiments indicated a concerted cleavage of a methyl and a TMS radical with a proposed elemental composition of $C_{24}H_{38}O_3Si_2^{\bullet+}$ (accurate mass 430.2354). The accurate mass of the corresponding M + 1 ion of the fragment ion at *m/z* 429 is 430.2673 ($C_{25}H_{40}DO_2Si_2^+$); the mass difference between these two ions is only 0.0319 *m/z* units. The recorded value in MS/MS experiments was 430.2425 with a mass error of 16.5 ppm (**2.1D**, Table 1) for $[M - CH_3 - TMS]^{\bullet+}$ and 57.64 ppm for the explanation involving the M + 1 ion. Except for perdeuterotrimethylsilylation (**2.2D**, **3.2D**, **7.2D**), the observed masses for the isotopically labelled derivatives revealed lower mass errors for the $[M - CH_3 - TMS]^{\bullet+}$ hypothesis (**#.3D**, **#.4D**, Tables 1 and 2) although still much higher than those obtained for all other fragments

described in Tables 1 and 2. ^{18}O-labelling was helpful in the case of the fragment ion at *m/z* 443 (**3.4D**, Table 1, $C_{24}H_{29}D_9O^{18}O_2Si_2^{\bullet+}$, 443.3004) because the mass difference from its *m/z* 429 counterpart ($C_{25}H_{32}D_9{}^{18}O_2Si_2^+$, 442.3289) was 0.9715 *m/z* units and both fragment ions were observed in the respective mass spectra. To propose a definite structure for the fragment ion at *m/z* 430 (*m/z* 432 for **7**), further experiments, e.g.^{18}O-labelling for 4-hydroxytestosterone (**7**), are needed.

3.6.2 | 6-Hydroxy steroids

The two 6-hydroxy steroids **4** and **8** exhibit a fragment ion *m/z* 319 in their GC/MS spectra (**4.1P**, Table 1; **8.1P**, Table 2) which is not observed for the other subclasses and has been used previously as an identifying fragment ion for screening purposes.[24] Since both compounds (**4** and **8**) yield the same fragment ion, it cannot originate from the D-ring but from other parts of the steroid backbone. After perdeuterotrimethylsilylation the ion was shifted to *m/z* 337 (+18 *m/z* units, **4.2P**, **8.2P**) and to *m/z* 328 (+9 *m/z* units, **4.3P**, **4.4P**, Table 1; **8.3P**, **8.4P**, Table 2) in the mass spectra of the mixed deuterated derivatives. This leads to the conclusion that the fragment ion contains the two TMS groups from ring A and ring B. Its elemental composition was substantiated with accurate mass data. Furthermore, it was also observed in the MS/MS spectra of the respective $[M - 15]^+$ ions. Hence, its structure was proposed as an A/B-ring fragment composed of C1–C11 as displayed in Table S1 (supporting information).

3.6.3 | 11-Hydroxy steroids

There are several fragment ions in the GC/MS spectra of the 11-hydroxy steroids **5** and **9** that are indicative for the position of the hydroxy group of 11-hydroxy steroids. Fragment ions *m/z* 219 (**5.1 T**, **9.1 T**), 234 (**5.1S**, **9.1S**) and 387 (**5.1R**, **9.1R**, Tables 1 and 2) were observed for both compounds, and *m/z* 389 (**9.1Q**, Table 2) for the 17-hydroxylated steroid **9** only. After isotopic labelling with [$^2H_9$]-TMS and accurate mass analyses it was apparent that the fragment ion at *m/z* 387 contains one more double bond than *m/z* 389 and that these two fragment ions bear two TMS moieties: perdeuterotrimethylsilylation resulted in a mass shift of +18 *m/z* units (*m/z* 405, **5.2R**, **9.2R**, Tables 1 and 2 and *m/z* 407, **9.2Q**, Table 2). In the case of **9**, generation of the fragment ion at *m/z* 387 was also observed when using the $[M - 105]^+$ ion (**9.1G**, Table 2) as the precursor in MS/MS measurements and it can thus be explained by a subsequent loss of ethylene $[M - 105 - 28]^+$. Due to its very low abundance, this finding could not be substantiated with the deuterated derivatives. The formation of fragment ions *m/z* 387 from **5** and *m/z* 389 from **9** require an overall loss of $C_6H_{15}OSi^{\bullet}$ ($[M - 131]^+$) from the respective molecular ion, which can be explained by cleavage of C-15–C-17 from the D-ring together with the attached C-17 TMS group. MS/MS experiments of the mixed deuterated derivatives **5.3**, **5.4**, **9.3** and **9.4** (Tables 3 and 4) revealed that fragments **Q** and **R** contain either the C-11 TMS and the C-3 or C-17 group (*m/z* 387 **9.4R**, *m/z* 389 **9.4Q**, *m/z* 396 **5.3R**, **5.4R**, **9.3R**, **9.4R**, *m/z* 398 **9.3Q**, **9.4Q**, *m/z* 405 **9.3R**, *m/z* 407 **9.3Q**) or the C-3 and C-17 TMS group (*m/z* 387 **5.3R**, *m/z* 396 **9.4R**, *m/z* 398 **9.3Q**, **9.4Q**, *m/z* 405 **5.4R**, Tables 1 and 2). These findings suggest that these fragments are not as specific for the 11-hydroxy steroids as initially postulated. In fact, they were also observed in the mass spectra of the other compounds **1–4** (*m/z* 387) and **6–8** (*m/z* 389), although in very low abundance. MS/MS experiments of ^{18}O-formestane (**3.1**, Table 3) substantiated different routes of formation for the fragment ion at *m/z* 387, because mass shifts of *2 m/z* units (*m/z* 389.2239 (mass error 6.68 ppm), C-4 and C-3/C-17 TMS group present) and 4 *m/z* units (*m/z* 391.2248 (mass error 1.79 ppm), C-3 and C-17 TMS group present) were observed.

MS/MS experiments confirmed that *m/z* 219 is produced not only from $[M]^{\bullet+}$ but also from $[M - 15]^+$ and *m/z* 234 which indicates a methyl radical loss in its fragmentation pathway. Only one TMS group remains in the fragment ions *m/z* 219 and 234 because the observed mass shift in the mass spectra is only +9 *m/z* units to *m/z* 228 (**5.2 T**, **9.2 T**) and *m/z* 243 if all the TMS groups in steroids **5** and **9** are labelled with [$^2H_9$]-TMS (**5.2S**, **9.2S**, Tables 1 and 2). The mass spectra of the mixed deuterated derivatives showed two corresponding fragment ions, one with a mass shift of +9 *m/z* units and one without a mass shift (**5.3S**, **5.4S**, **5.3 T**, **5.4 T**, **9.3S**, **9.4S**, **9.3 T**, **9.4 T**, Tables 1 and 2). This finding implies that the TMS group at either position C-3, C-11 or C-17 is present in these fragment ions. Thus, formation of this ion is probably related to a loss of the C-and D-ring, as illustrated in Table S1 (supporting information). Further research, especially more MS/MS experiments and ^{18}O labelling of steroids **5** and **9**, is needed to further evaluate the obtained results.

4 | CONCLUSIONS

In summary, generation of common TMS-derived and additional fragment ions dependent on the different ring hydroxylation sites was proposed and confirmed in MS/MS experiments. TMS-derived fragment ions were common for all the 3-keto-4-ene hydroxy steroids under investigation while some more specific fragment ions were observed for each hydroxy steroid subclass.

These MS/MS transitions can be used in screening and can facilitate the detection of hydroxy steroids with the same mass, similar retention times and only little mass spectral fragmentation. Because the data was obtained using reference standards, caution regarding possible interferences should be exercised when the proposed MRM transitions are applied to urine samples.

The presented study postulates that the loss of a methyl group from 3-keto-4-ene hydroxy steroids occurs mainly from the steroid nucleus (preferably from the C-19 angular methyl group) and even seems to be a prerequisite for the formation of the fragment ion $[M - 105]^+$ from the A/B-ring due to conformational changes in 2-, 4- or 6-hydroxy steroids. As described before, neutral losses such as TMSOH from the derivatized steroid are not a random process but are highly dependent on the steric environment. If no suitable hydrogen for its cleavage is available, especially as in case of the

2- and 4-hydroxy-3-keto-4-enes, a TMSO radical loss is observed in the respective mass spectra. The overall loss of 89 Da may be considered as a sequential or concerted cleavage of a TMS-derived methyl radical and a neutral loss of $(CH_3)_2SiO$. The *m/z* 147 ion can serve as an indicator for vicinal TMS groups (in 2- or 4-hydroxy isomers); the oxygen was shown to originate from the position of the respective intact TMS moiety in ring A.

With the help of deuterated derivatization agents and labelling utilizing $H_2{}^{18}O$, the structures of postulated fragment ions more specific for the different steroid subclasses could be further elucidated, e.g. *m/z* 319 for the 6-hydroxy, *m/z* 417/415, 358/356, 343/341, 315/313, 269 and 267 for the 2- and 4-hydroxy and 219 for the 11-hydroxy compounds. The MS^3 transition *m/z* 503 → 269 was revealed as suitable for selective formestane determination, while 2-hydroxyandrostenedione yielded *m/z* 503 → 267 as a MS^3 transition.

Minor drawbacks of the described approach include low abundance of some product ions in the MS/MS spectra and possible TMS migration upon fragment ion formation (which cannot be ruled out completely). Two out of three functional groups were always derivatized in the same manner, making it more challenging to distinguish between TMS enol ethers on positions C-3 and C-17 for the 17-oxo steroids.

Apart from finding a derivatization procedure to isotopically label every single functional group separately, ^{18}O-labelling will also be conducted for all other standards as well in future studies. In this way, proposed fragmentation pathways and fragment ion structures will be further evaluated and confirmed.

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