Mass Spectrometric Analysis of Androstan-17 β -ol-3-one and Androstadiene-17 β -ol-3-one Isomers

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Mass spectrometric identification and characterization of steroids using electrospray ionization and tandem mass spectrometry has advantages in drug testing and doping control analysis attributable to limitations of gas chromatography followed by electron ionization mass spectrometry. Steroids with an androstadiene-17 β -ol-3-one nucleus and double bonds located either at C-1 and C-4, C-4 and C-9, or C-4 and C-6 were used to determine characteristic fragmentation pathways. Diagnostic dissociation routes are proposed using deuterium labeling, MS³ experiments, and analyses of structurally closely related compounds. Steroids such as boldenone (androst-1,4-diene-17 β -ol-3-one) produced characteristic product ions at m/z 121, 135, and 147. Compounds with double bonds at C-4 and C-9 generated abundant product ions at m/z 145 and 147. Conjugated double bonds at C-4 and C-6 gave rise to an intense and characteristic signal at m/z 133. Stereochemical differentiation between 5 α - and 5 β -isomers of androstan-17 β -ol-3-ones was possible because of significant differences in relative abundance of product ions generated by elimination of acetone from α,β -saturated 3-keto steroids. (J Am Soc Mass Spectrom 2005, 16, 1660–1669) © 2005 American Society for Mass Spectrometry

The identification of natural and synthetic compounds using mass spectrometry is of paramount importance for documentation of use of illicit compounds by amateur and professional athletes. Elucidation of dissociation pathways of illegal substances using a variety of ionization and/or dissociation conditions is a robust technique for classification and identification of drugs and related compounds [1].

Mass spectrometric studies on fragmentation of steroids using electron ionization (EI) were performed by Djerassi, Budzikiewicz, and coworkers [2–8]. Detailed studies have also been made employing steroid derivatives such as trimethylsilylated or acetylated compounds [9–18]. Liquid chromatography combined with mass spectrometers with an atmospheric pressure ionization interface enables the detection and characterization of steroids that complement conventional gas chromatographic-mass spectrometric (GC-MS) assays, especially for compounds that are less suitable for GC-MS owing to thermo-labile properties [19]. A very recent example is the so-called “designer steroid” tetrahydrogestrinone [20] (13-ethyl-17-hydroxy-18,19-dinor-17 α -pregn-4,9,11-trien-3-one, THG) that has been identified in several doping control samples by means of methods based on liquid chromatography (LC) and tandem mass spectrometry (MS/MS).

To determine steroid structures, ESI and collision-induced dissociation (CID) were investigated. We describe fragmentation patterns of the isomers androst-1,4-diene-17 β -ol-3-one (1), androst-4,9 (11)-diene-17 β -ol-3-one (6), androst-4,6-diene-17 β -ol-3-one (9), and their analogs as well as 5 α -androstan-17 β -ol-3-one (12), 5 β -androstan-17 β -ol-3-one (13), and related derivatives (Scheme 1). Using stable isotope derivatives and H/D-exchange experiments, fragmentation pathways are proposed that provide classification of steroids by characteristic product ions generated from protonated molecules that include either a 3-keto function or a 3-keto function plus two double bonds.

Experimental

Steroids and Chemicals

Androst-4,6-diene-17 β -ol-3-one, androst-4,9 (11)-diene-17 β -ol-3-one, 17 α -methyl-androst-4,9 (11)-diene-17 β -ol-3-one, and estra-4,6-diene-17 β -ol-3-one were obtained from Steraloids (Newport, RI). 5 α -Androstan-17 β -ol-3-one, 5 β -androstan-17 β -ol-3-one, 17 α -methyl-5 α -androstan-17 β -ol-3-one (mestanolone), 1 α -methyl-5 α -androstan-17 β -ol-3-one (mesterolone), androst-1,4-diene-17 β -ol-3-one (1-dehydro-testosterone, boldenone), methanol-d (99 atom% D), sodium deuterioxide (40% solution in D₂O, 99.9 atom% D), and deuterium oxide (99.9 atom% D) were obtained from Sigma-Aldrich (Steinheim, Germany). 17 α -methyl-androst-1,4-diene-17 β -ol-3-one (metandienone) was supplied by Fluka (Buchs, Switzerland) and 4-chloro-

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Scheme 1. Chemical structures of investigated steroids comprising an androstadiene-17 β -ol-3-one structure: androst-1,4-diene-17 β -ol-3-one (**1**), 17 α -methyl-androst-1,4-diene-17 β -ol-3-one (**2**), 17 α -trideuteriomethyl-androst-1,4-diene-17 β -ol-3-one (**3**), 16,16- $^2\text{H}_2$ -17 α -methyl-androst-1,4-diene-17 β -ol-3-one (**4**), 4-chloro-17 α -methyl-androst-1,4-diene-17 β -ol-3-one (**5**), androst-4,9 (11)-diene-17 β -ol-3-one (**6**), 17 α -methyl-androst-4,9 (11)-diene-17 β -ol-3-one (**7**), 2,2,4,6,6- $^2\text{H}_5$ -17 α -methyl-androst-4,9 (11)-diene-17 β -ol-3-one (**8**), androst-4,6-diene-17 β -ol-3-one (**9**), 2,2,4- $^2\text{H}_3$ -androst-4,6-diene-17 β -ol-3-one (**10**), estra-4,6-diene-17 β -ol-3-one (**11**), 5 α -androstan-17 β -ol-3-one (**12**), 5 β -androstan-17 β -ol-3-one (**13**), 2,2,4,4- $^2\text{H}_4$ -5 α -androstan-17 β -ol-3-one (**14**), 16,16,17- $^2\text{H}_3$ -5 α -androstan-17 β -ol-3-one (**15**), 16,16,17- $^2\text{H}_3$ -5 β -androstan-17 β -ol-3-one (**16**), 17 α -methyl-5 α -androstan-17 β -ol-3-one (**17**), and 1 α -methyl-5 α -androstan-17 β -ol-3-one (**18**).

17 α -methyl-androst-1,4-diene-17 β -ol-3-one by VEB Jena-pharm (Jena, Germany). 17 α -Trideuteriomethyl-androst-1,4-diene-17 β -ol-3-one, 16,16- $^2\text{H}_2$ -17 α -methyl-androst-1,4-diene-17 β -ol-3-one, 16,16,17- $^2\text{H}_3$ -5 α -androstan-17 β -ol-3-one, and 16,16,17- $^2\text{H}_3$ -5 β -androstan-17 β -ol-3-one were synthesized following established procedures [21–24].

*H/D-Exchange to 2,2,4,6,6- $^2\text{H}_5$ -17 α -methyl-androst-4,9 (11)-diene-17 β -ol-3-one (**8**), 2,2,4- $^2\text{H}_3$ -androst-4,6-diene-17 β -ol-3-one (**10**), and 2,2,4,4- $^2\text{H}_4$ -5 α -androstan-17 β -ol-3-one (**14**)*

The exchange of labile hydrogens by deuterium was accomplished by incubation of 5 mg of each steroid in 5

mL of a mixture of CH_3OD , D_2O , and 40% NaOD in D_2O (4/0.95/0.05, vol/vol/vol) for 12 h at 60°C [25, 26]. The hydrogens located at C-2, C-4, and C-6 or C-2 and C-4 were substituted in **7** or **9**, respectively. The efficiency of deuterium exchange was tested by ESI-MS, and the results were as follows: **8** $d_4 = 35\%$, $d_5 = 100\%$; **10** $d_2 = 42\%$, $d_3 = 100\%$; **14** $d_3 = 66\%$, $d_4 = 100\%$.

Mass Spectrometry

Steroids were dissolved in 0.1% acetic acid and acetonitrile (1:1, vol:vol, 1 $\mu\text{g}/\text{mL}$) and analyzed by electrospray ionization (ESI) and collision-induced dissociation (CID) on an Applied Biosystems QTrap 4000

Figure 1. ESI-product ion spectra of the protonated molecules of (a) androst-1,4-diene-17 β -ol-3-one (**1**), ($M + H$)⁺ = 287; (b) androst-4,9(11)-diene-17 β -ol-3-one (**6**), ($M + H$)⁺ = 287; and (c) androst-4,6-diene-17 β -ol-3-one (**9**), ($M + H$)⁺ = 287. The spectra were recorded at collision offset voltages of 30, 40, and 35 V, respectively, using scan speeds of 1000 u/s.

instrument (Darmstadt, Germany) at room temperature. Nitrogen was employed as curtain and collision gas (5.33e-3 Pa) delivered from a Peak Scientific NM30L nitrogen generator (Chicago, IL). Samples were introduced into the mass spectrometer using a Harvard syringe pump (Holliston, MA) at a flow rate of 3 μ L/min. Declustering potentials were optimized for respective protonated molecules, and collision energies

(CE) were adjusted such that the relative abundance of the precursor ion was ca. 10% in product ion spectra. Unit resolution was employed for mass selection in Q1, and the linear ion trap was operated at a scan speed of 1000 u/s from m/z 80 to 400. Ten scans were accumulated per product ion spectrum.

Aliquots of H/D-exchange experiments were dissolved in acetonitrile and introduced into the mass spectrometer using the conditions described above.

Figure 2. ESI-product ion spectra of the protonated molecules of (a) 5 α -androstan-17 β -ol-3-one (**12**), ($M + H$)⁺ = 291; (b) 17 α -methyl-5 α -androstan-17 β -ol-3-one (**17**), ($M + H$)⁺ = 305; and (c) 1 α -methyl-5 α -androstan-17 β -ol-3-one (**18**), ($M + H$)⁺ = 305. The spectra were recorded at collision offset voltages of 35 V using a scan speed of 1000 u/s.

Results and Discussion

The MS/MS spectra of **1**, **6**, and **9** and **12**, **17**, and **18** are representative for investigated classes of steroids (Figures 1 and 2). Product ions of analyzed compounds are listed in Tables 1 and 2. All product ion spectra were recorded from respective protonated molecules, the initial abundance and stability of which was significantly influenced by the presence or absence of conjugated π -electron systems composed by 3-keto functions with or without conjugation to a C-C double-bond (Tables 1 and 2).

Androst-1,4-diene-17 β -ol-3-one and Analogs

m/z 121. The most abundant product ion of **1** (Figure 1a) was found at *m/z* 121. This ion was commonly generated by steroids bearing a 1,4-diene-3-one structure as demonstrated with the Compounds 2–5 (Table 1), the CID spectra of which also contain base peaks at *m/z* 121 (2–4) or accordingly at *m/z* 155 (5). A proposed mechanism for formation of *m/z* 121 is depicted in Scheme 2a starting with the protonation of the oxygen participating in the cross-conjugated π -electron system of the 1,4-diene-3-one functionality (Scheme 2a) followed by the formation of a 3-linked 4-methylphenol residue (Scheme 2b). Migration of the hydrogen from C-8 is proposed to accompany the cleavage of the bond between C-9 and C-10. This mechanism is supported by deuterium labeling experiments by Shapiro and Djerassi using α,β -unsaturated 3-ketosteroids [7]. A temporary charge-transfer to C-8 triggers the fission of the linkage between C-5 and C-6 yielding the fragment ion at *m/z* 121 (Scheme 2c) that stabilized by charge delocalization (Scheme 2d) comparable to structurally related tropylium cations [27]. Support for formation of the product ion at *m/z* 121 from the A-ring was obtained by the analysis of the alkylated, halogenated and alkylated, and analogs labeled with stable isotopes (2–5). While the introduction of a methyl residue at C-17 as well as deuterium labeling at C-16 did not affect the generation of *m/z* 121, an increment by 34 u was observed in the CID spectrum of **5** supporting the proposed fragmentation pathway since **5** bears a chlorine atom at C-4.

m/z 135. Alternative dissociation pathways starting from the protonated species **a** in Scheme 2 give rise to the product ion at *m/z* 135. Two different origins and compositions need to be considered as the formation of the product ion at *m/z* 135 is influenced by modifications of the A-ring as well as the D-ring. Analysis of **2** produced a high intensity peak at *m/z* 149 and a decreased abundance of *m/z* 135 (Table 1), indicating an origin including the steroidal D-ring structure attributable to the increment of *m/z* 135 by 14 u. Deuterium labeling of C-16 (Compound **4**) or substitution of the methyl residue at C-17 by a trideuteromethyl function (Compound **3**) caused a shift of *m/z* 149 by one and two

or three u, respectively (Table 1), and the chlorine atom at C-4 of **5** resulted in an increase of 34 u of *m/z* 135, giving rise to *m/z* 169 (Table 1). Two fragmentation routes leading to product ions at *m/z* 135 are proposed in Scheme 2b. A cleavage of the bond between C-9 and C-10 generated an intermediate ion *e*, which subsequently eliminated the C- and D-ring yielding *m/z* 135, with the suggested structure assigned as *f*. An initial loss of water from the protonated molecule **a** is suggested, which generated *g* at *m/z* 269, producing a product ion at *m/z* 135 (Scheme 2h) by releasing the A- and B-ring as observed in MS [3] experiments.

m/z 147 and 149. The product ion at *m/z* 147 is commonly observed in ESI product ion spectra of steroids that include a 1,4-diene-3-one structure such as **1–4** and many synthetic corticosteroids, e.g., dexamethasone, isoflupredone, prednisolone, and prednylidene. This product ion is proposed to result from the steroidal A-, B-, and C-rings by fissions of the linkages between C-6 and C-7, C-8 and C-9, and C-11

Scheme 2. Proposed fragmentation pathways of androst-1,4-diene-17 β -ol-3-one generating product ions at *m/z* 121 and 135.

Table 1. Relative abundances (%) of selected fragment ions of steroids with androstadiene-17-ol-3-one nucleus studied by electrospray ionization and collision-induced dissociation

No.	Precursor ion (<i>m/z</i>)	CE (eV)	Common fragment ions <i>m/z</i> (relative abundance, %)					
			(<i>M</i> + <i>H</i>) ⁺ – 18	(<i>M</i> + <i>H</i>) ⁺ – 18 – 15	(<i>M</i> + <i>H</i>) ⁺ – 18 – 18	<i>m/z</i> 213	<i>m/z</i> 173	<i>m/z</i> 161
1	287	30	269 (7)	—	—	(3)	(36)	(17)
2	301	30	283 (13)	—	—	(4)	(26)	(20)
3	304	30	286 (21)	—	—	(6)	(31)	<i>m/z</i> 161 (10) <i>m/z</i> 164 (13) ^a <i>m/z</i> 161 (9) <i>m/z</i> 163 (8) ^b
4	303	30	285 (16)	—	—	(3)	(27)	(21)
5	335	30	317 (71)	—	—	<i>m/z</i> 247 (4) ^d	<i>m/z</i> 207 (16) ^d	(7)
6	287	40	269 (38)	254 (51)	251 (6)	(15)	(14)	(17)
7	301	40	283 (97)	268 (66)	265 (7)	(34)	(22)	(13)
8	307	40	<i>m/z</i> 289 (37) <i>m/z</i> 288 (100) ^a	<i>m/z</i> 274 (25) <i>m/z</i> 273 (51) ^e	—	<i>m/z</i> 218 (14) ^f	<i>m/z</i> 175 (13) ^b	(11)
9	287	35	269 (17)	—	251 (6)	—	(23)	(16)
10	290	40	272 (9)	—	254 (3)	—	<i>m/z</i> 176 (19) ^a	<i>m/z</i> 164 (13) ^a
11	273	35	255 (12)	—	237 (6)	(5)	(4)	(7)

- Relative abundance < 3%

^aTriply deuterated.^bDoubly deuterated.^cSingly deuterated.^dPlus 34 u owing to ³⁵Cl.^e-HDO (–19 u).^fFive-fold deuterated.

and C-12 (Scheme 3a). Modifications such as deuterium labeling at C-16 or introduction of a methyl residue at C-17 did not affect the formation of *m/z* 147 but the presence of a chlorine atom at C-4 (5) increased the product ion to *m/z* 181 (Table 1), supporting the presence of the A-ring in the product ion structure. Within the protonated species *a*, a hydrogen transfer from C-9 to C-12 is proposed that generates a double bond between C-9 and C-11, and the subsequent formation of a 4-member ring structure requiring the rearrangement of bonds between C-6 and C-7 as well as C-8 and C-9 yields the

postulated product ion structure at *m/z* 147 labeled as *i*.

The product ion of 1 at *m/z* 149 is presumably composed by the steroidal C- and D-ring with concomitant elimination of water as shown in Scheme 3b. The protonated molecule *a* is the starting point of the proposed charge-driven dissociation pathway that leads to cleavages of the bonds between C-6 and C-7 as well as C-9 and C-10 accompanied by the migration of two hydrogens. Methylation of C-17 (2) leads to a shift of *m/z* 149 to 163 and its triply deuterated analog (3) gives rise to a corresponding product ion at *m/z* 166

Table 2. Relative abundances (%) of selected fragment ions of steroids with androstan-17-ol-3-one nucleus studied by electrospray ionization and collision-induced dissociation

No.	Precursor ion (<i>m/z</i>)	CE (eV)	Common fragment ions <i>m/z</i> (relative abundance, %)				
			(<i>M</i> + <i>H</i>) ⁺ – 18	(<i>M</i> + <i>H</i>) ⁺ – 18 – 18	<i>m/z</i> 215	<i>m/z</i> 213	<i>m/z</i> 201
12	291	35	273 (13)	255 (100)	(71)	(28)	(33)
13	291	35	273 (8)	255 (100)	(9)	(16)	(51)
14	295	35	277 (14)	259 (100)	(64)	<i>m/z</i> 213 (8) <i>m/z</i> 217 (16) ^a <i>m/z</i> 216 (17) ^b	<i>m/z</i> 205 (27) ^a (41)
15	294	35	276 (8)	258 (100)	<i>m/z</i> 218 (68) ^b	<i>m/z</i> 216 (11) ^b	(68)
16	294	35	276 (4)	258 (100)	<i>m/z</i> 218 (6) ^b	<i>m/z</i> 213 (44) <i>m/z</i> 227 (16) ^c <i>m/z</i> 213 (26) <i>m/z</i> 227 (11) ^d	(8)
17	305	35	287 (7)	269 (100)	<i>m/z</i> 229 (83) ^c	<i>m/z</i> 213 (26) <i>m/z</i> 227 (11) ^d	<i>m/z</i> 201 (12) <i>m/z</i> 215 (19) ^d
18	305	35	287 (15)	269 (100)	<i>m/z</i> 215 (19) <i>m/z</i> 229 (96) ^d	<i>m/z</i> 213 (26) <i>m/z</i> 227 (11) ^d	<i>m/z</i> 201 (12) <i>m/z</i> 215 (19) ^d

^aQuadruply deuterated.^bTriply deuterated.^cPlus 14 u owing to 17-methylation.^dPlus 14 u owing to 1-methylation.

Table 1. Continued

<i>m/z</i> 159	<i>m/z</i> 149	<i>m/z</i> 147	<i>m/z</i> 145	<i>m/z</i> 135	<i>m/z</i> 133	<i>m/z</i> 121	Individual fragment ions <i>m/z</i> (relative abundance, %)	
(9)	(10)	(24)	(5)	(54)	(7)	(100)	187 (6)	179 (10)
(6)	(76)	(20)	(5)	(14)	(4)	(100)	187 (14)	227 (6)
(7)	<i>m/z</i> 152 (91) ^a	(19)	(4)	(10)	—	(100)	<i>m/z</i> 187 (5)	227 (6)
							<i>m/z</i> 190 (5) ^a	
(7)	<i>m/z</i> 150 (31) ^c	(19)	(4)	(7)	—	(100)	<i>m/z</i> 187 (4)	227 (4)
	<i>m/z</i> 151 (65) ^b						<i>m/z</i> 189 (4) ^b	
<i>m/z</i> 193 (14) ^d	(13)	<i>m/z</i> 181 (21) ^d	<i>m/z</i> 179 (5) ^d	<i>m/z</i> 169 (12) ^d	<i>m/z</i> 167 (13) ^d	<i>m/z</i> 155 (100) ^d	221 (8)	261 (9)
(22)	(6)	(100)	(59)	(4)	(12)	(37)	239 (33)	211 (26)
(100)	(7)	(81)	(23)	—	(15)	(31)	253 (54)	225 (25)
(65)	—	<i>m/z</i> 150 (42) ^a	(7)	—	—	—	258 (51)	246 (26)
(9)	(6)	(8)	(10)	—	(100)	—	105 (26)	97 (21)
(8)	—	—	(9)	—	(100)	—	105 (39)	<i>m/z</i> 100 (24) ^a
(21)	—	(9)	(6)	—	(100)	—	105 (37)	91 (31)

(data not shown) while 4-chlorination (5, Table 1) does not affect the ion at *m/z* 149.

m/z 161. The fragment ion of 1 at *m/z* 161 is proposed to originate from two different sources since alterations of the D-ring or the A-ring resulted in an increase of *m/z* 161 to respective counterparts, i.e., *m/z* 175 in the case of 2 or *m/z* 195 in the case of 5 (data not shown). Introduction of deuterium atoms into the D-ring (Compounds 3 and 4, Table 1) yielded an unchanged fragment ion at *m/z* 161 and a corresponding analog incremented by three or two Da depending on how many deuterium

atoms were substituted in the molecule. Postulated fragmentation routes of 1 giving rise to *m/z* 161 are depicted in Scheme 3c and d, which are either based on the elimination of the C- and D-ring or the elimination of the A-ring via ion *b* (Scheme 2) yielding the product ions assigned as *k* and *l*, respectively. Elimination of water from *b* (Scheme 3d) causes a shift of the angular C-18 methyl function from C-13 to C-17, and has been observed frequently in particular with 17-methyl-17-hydroxy steroids [1, 28]. Additional support for this mechanism was obtained by the retention of deuterium atoms introduced at C-16 (4), demonstrating the partic-

Table 2. Continued

<i>m/z</i> 199	<i>m/z</i> 173	<i>m/z</i> 161	<i>m/z</i> 159	<i>m/z</i> 147	<i>m/z</i> 145	<i>m/z</i> 133
(52)	(51)	(44)	(82)	(36)	(54)	(34)
(47)	(52)	(46)	(84)	(69)	(56)	(31)
<i>m/z</i> 199 (14)	<i>m/z</i> 173 (27)	(25)	<i>m/z</i> 159 (27)	(26)	(36)	(26)
<i>m/z</i> 203 (17) ^a	<i>m/z</i> 177 (17) ^a		<i>m/z</i> 163 (51) ^a			
<i>m/z</i> 199 (5)	<i>m/z</i> 173 (21)	(33)	(51)	(24)	<i>m/z</i> 145 (19)	(21)
<i>m/z</i> 202 (38) ^b	<i>m/z</i> 176 (27) ^b				<i>m/z</i> 148 (27) ^b	
<i>m/z</i> 199 (16)	<i>m/z</i> 173 (24)	(41)	(52)	(63)	<i>m/z</i> 145 (34)	(28)
<i>m/z</i> 202 (21) ^b	<i>m/z</i> 176 (25) ^b				<i>m/z</i> 148 (33) ^b	
<i>m/z</i> 199 (19)	(42)	(35)	(57)	(29)	(25)	(23)
<i>m/z</i> 213 (44) ^c						
<i>m/z</i> 199 (36)	(83)	(26)	(39)	(40)	(37)	(38)
<i>m/z</i> 213 (26) ^d

Scheme 3. Proposed fragmentation pathways of androst-1,4-diene-17 β -ol-3-one generating product ions at m/z 147, 149, and 161.

ipation of a hydrogen originating from a position different from C-16, suggesting the migration of C-18 to C-17 that allows the introduction of a double bond between C-13 and C-14.

Androst-4,9-diene-17 β -ol-3-one and Analogs

Dissociation pathways of androst-4,9-diene-17 β -ol-3-ones such as Compounds 6 and 7 were dominated by fragmentation routes that differed significantly from those obtained by CID of steroids with an androst-1,4-diene-17 β -ol-3-one structure⁹(Table 1).

m/z 145 and 147. The product ion mass spectrum of 6 (Figure 1b) includes abundant product ions at m/z 145 and 147. Their generation is initiated from the protonated molecule shown in Scheme 4a and referred to as *m*.

The elimination of water, presumably from C-17 accompanied by the cleavage of the bonds between C-9 and C-10 as well as C-6 and C-7 gives rise to the product ion at m/z 145 assigned as *n* in Scheme 4a. Support for the elimination of the A- and B-ring was obtained by the comparative analysis of 7 and 8. The presence of a 17-methyl residue (7) caused a shift of m/z 145 by 14 u to m/z 159, suggesting the presence of the D-ring in the fragment ion *n*, while the introduction of 5 deuterium atoms into the A- and B-ring (8) did not affect the abundance of that ion⁹(Table 1).

In contrast to m/z 145, the base peak at m/z 147 did not contain the D-ring of the molecule as it was not incremented by 14 u by a methyl residue at C-17 (7), and deuterium exchange experiments (8) demonstrated the remaining three deuterium atoms in the corresponding fragment ion, which were shifted to m/z 150. Hence, its generation and final structure is postulated to differ from *i*, the product ion of 1 depicted in Scheme 3a, and a suggested dissociation pathway is shown in Scheme 4b. Starting from the protonated species *m*, the elimination of the entire D-ring (−98 u) by means of a retro-Diels-Alder rearrangement is accompanied by the release of ketene (−42 u) from the A-ring including carbons 2 and 3 yielding the proposed fragment *o* at m/z

Scheme 4. Proposed fragmentation pathways of androst-4,9-diene-17 β -ol-3-one generating product ions at m/z 145, 147, and 254.

Scheme 5. Proposed fragmentation pathways of androst-4,6-diene-17 β -ol-3-one generating product ions at m/z 151 and 133.

147. The resulting cross-conjugated π -electron system is considered the driving force for this dissociation mechanism. Budzikiewicz et al. [26] describe the elimination of ketene from steroids with a 3-keto-4-ene releasing C-2 and C-3 upon electron impact, and migration of C-19 from C-10 to C-1 as a Wagner-Meerwein rearrangement has been proposed in fragmentation patterns of steroids bearing a 3-keto-1-ene structure [19].

$(M + H)^+ - 15$. Another characteristic fragmentation behavior of androst-4,9-diene-17 β -ol-3-ones was the elimination of methyl radicals. Carbon 19 is favored since the resulting radical cation is a conjugated electron system that allows the stabilization of the fragment ion by charge delocalization (Scheme 4c). A concomitant loss of water gave rise to an abundant fragment ion at m/z 254 (Figure 1b), which was identified as precursor ion of m/z 239 by MS [3] experiments, indicating the release of an additional methyl radical that generated an even-electron cation.

Androst-4,6-diene-17 β -ol-3-one and Analogs

m/z 151 and 133. The presence of two double bonds in androstadiene-17 β -ol-3-one, between carbons 4 and 5 as well as 6 and 7, resulted in a specific fragmentation pattern (Figure 1c). The most abundant fragment ion was observed at m/z 133, which is proposed to include the steroidal C- and D-rings. MS [3] experiments provided evidence for its generation from m/z 151 by the elimination of water. Deuterium labeling at C-2 and C-4 yielded Compound 10 [25], substantiating the assumption that the A-ring is not participating in the product ions at m/z 151 and 133 because no mass shift of the respective fragments was observed (Table 1). A postulated dissociation route (Scheme 5) starts with the protonated species *p* undergoing a cleavage of the linkages between C-9 and C-10 as well as C-7 and C-8. Migration of the hydrogen located at C-14 to the leaving group generated the product ion at m/z 151 assigned as

q. The subsequent loss of water gives rise to *r* at m/z 133. Confirmation that carbons 10 and 19 are not involved in the dissociation pathway was obtained by analyzing 11 under comparable fragmentation conditions, where the base peak was observed at m/z 133 (Table 1).

m/z 91, 97, and 105. The product ions at m/z 91 and 105 of 9 (Figure 1c) were identified as fragments generated from m/z 133 by losses of propylene (-42 u) and ethylene (-28 u), respectively, by means of MS [3] experiments. Both ions were not influenced by deuterium labeling (Compound 10) and remained constant also in the case of 11. The product ion at m/z 97, which has been described as diagnostic for 3-keto-4-ene steroids such as testosterone and analogs [29,30], was also observed in the product ion spectrum of 9 (Figure 1c) and was incremented by three mass units following deuterium labeling of 10. This is in agreement the dissociation pathway suggested previously [29], and its absence in the case of 11 substantiates the postulated fragmentation route that includes A- and B-ring.

Androstan-17 β -ol-3-one and Analogs

The fragmentation of 5 α -androstan-17 β -ol-3-one (12) and its analogs (13–18) was considerably different from those described for analogous steroids that included carbon-carbon double bonds. The product ion mass spectrum of the protonated molecule of 12 (Figure 2a) demonstrated the efficient dissociation of the precursor ion upon application of a collision offset voltage of 30 V. In contrast to the earlier depicted dissociation patterns, the saturation of the steroid nucleus resulted in an abundant fragment ion at m/z 255 by elimination of two water molecules [31]. The spectrum was dominated by less diagnostic product ions separated by methylene units (14 u). These have also been observed in lower intensities during analysis of α,β -unsaturated 3-keto steroids (1–11), but the presence of double bonds did promote the formation of specific and characteristic fragment ions.

m/z 215. The fragmentation route to the ion *t* at m/z 215 is considered diagnostic for steroids bearing an androstan-17 β -ol-3-one structure. An elimination of water (-18 u) and acetone (-58 u) is suggested (Scheme 6), and support for this mechanism was ob-

Scheme 6. Proposed fragmentation pathway of androstan-17 β -ol-3-one generating product ion at m/z 215.

tained by the analysis of the methylated analogs (17–18) as well as deuterium labeling of 12 yielding the Compounds 14 and 15. The introduction of methyl residues at C-17 or C-1 incremented the fragment ion at m/z 215 to 229, indicating that these positions remain in the product ion after elimination of a total of 76 u from the precursor ion (Figure 2b and c, Table 2). Deuteration of C-16 and C-17 (15) caused a shift of m/z 215 to 218, providing evidence for the presence of these carbons in the respective product ion, while all four deuterium atoms of 14 located at C-2 and C-4 were eliminated from the steroid with the loss of acetone (Table 2). The release of acetone from 2- and 3-keto steroids has been observed after EI in earlier studies by Budzikiewicz, Djerassi, and coworkers [4, 32], and substantial work has been performed to elucidate the corresponding fragmentation pathway [33]. The participation of the hydrogen located at C-6 in the rearrangement has been identified by deuterium labeling, and a comparable dissociation route after ESI and CID of androstan-17 β -ol-3-ones has been proposed in the present study accordingly.

Formation of the product ion at m/z 215 was strongly influenced by stereochemical properties of the androstan-17 β -ol-3-ones, in particular by the hydrogen orientation at C-5, allowing the mass spectrometric differentiation of stereoisomers. While 5 α -androstan-17 β -ol-3-one (12) gave rise to m/z 215 at a relative abundance of 71%, only 9% of relative abundance is obtained with the corresponding 5 β -isomer 13 under identical mass spectrometric conditions. This observation is in contrast to comparable experiments performed by EI-MS, where the elimination of acetone was favored in the case of 5 β -steroid ketones [4, 32] relative to their 5 α -oriented analogs.

Conclusions

Electrospray ionization followed by tandem mass spectrometry provided detailed information on the position of double bonds in steroids that include a 3-keto-androstadiene-17 β -ol-3-one structure. While a 1,4-diene configuration generated abundant product ions predominantly at m/z 121, 135, and 147, a 4,9-diene structure gave rise to characteristic fragment ions at m/z 145 and 147, and favors the generation of $(M + H)^+ - 18 - 15$. Elimination of a methyl radical is characteristic for steroids with a 4,9-diene structure. The resulting conjugated π -electron system is an anomaly regarding the commonly accepted even-electron rule [34] attributable to the generation of a radical from an even electron cation during gas-phase fragmentation. The conjugation of double bonds located at C-4 and C-6 yielded an intense diagnostic fragment ion at m/z 133 which was not observed with the other compounds investigated. All described fragment ions are subject to interference by modification or derivatization of respective steroids, but the principal dissociation routes provide substantial information that allows a classification of steroidal

structures with sufficient proton affinities by ESI-MS/MS.

The specificity of the fragmentation pathway of 3-keto steroids without carbon-carbon double bonds is limited to the consecutive loss of water and acetone, but this particular dissociation enabled the distinction of these steroids from those bearing unsaturated structures, and the differentiation of corresponding 5 α - and 5 β -stereoisomers by the preference of acetone elimination for 5 α -androstanolones.

Data presented here demonstrate the utility of ESI-MS/MS for the identification and characterization of unknown or modified steroids, which is of particular interest in sports medicine for the detection and identification of designer steroids that have required an enormous effort in terms of structural elucidation of abused compounds and their metabolic products.

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