Collision-Induced Dissociation Pathways of Anabolic Steroids by Electrospray Ionization Tandem Mass Spectrometry

Fuyu Guan, Lawrence R. Soma, and Yi Luo
Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, Pennsylvania, USA

Cornelius E. Uboh
Pennsylvania Equine Toxicology and Research Center, West Chester University, West Chester, Pennsylvania, USA

Scott Peterman
Thermo Electron Corporation, Somerset, New Jersey, USA

Anabolic steroids are structurally similar compounds, and their product-ion spectra obtained by tandem mass spectrometry under electrospray ionization conditions are quite difficult to interpret because of poly-ring structures and lack of a charge-retaining center in their chemical structures. In the present study, the fragmentation of nine anabolic steroids of interest to the racing industry was investigated by using triple quadrupole mass spectrometer, Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer, and a linear ion trap instrument. With the aid of an expert system software (Mass Frontier version 3.0), accurate mass measurements, and multiple stage tandem mass spectrometric (MS^n) experiments, fragmentation pathways were elucidated for boldenone, methandrostenolone, tetrahydrogestrinone (THG), trenbolone, normethandrolone and mibolerone. Small differences in the chemical structures of the steroids, such as an additional double-bond or a methyl group, result in significantly different fragmentation pathways. The fragmentation pathways proposed in this paper allow interpretation of major product ions of other anabolic steroids reported by other researchers in a recent publication [19]. The proposed fragmentation pathways are helpful for characterization of new steroids. The approach used in this study for elucidation of the fragmentation pathways is helpful in interpretation of complicated product-ion spectra of other compounds, drugs and their metabolites. (J Am Soc Mass Spectrom 2006, 17, 477–489) © 2006 American Society for Mass Spectrometry

Anabolic steroids are synthetic substances related to the male sex hormones (androgens), and are used for the treatment of metabolic disorders in man and animals. These agents can be illegally used to enhance performance in human and animal sports including horse racing [1–3]. The misuse of anabolic steroids is well documented; for example, the scandal of the "designer" drug tetrahydrogestrinone (THG) [4]. Anabolic steroids are prohibited for use by athletes in competition by the International Olympic Committee (IOC) [5], classified as controlled substances by the United States Drug Enforcement Agency, included under the Drugs of Misuse Act by the United Kingdom, and banned by the European Union for use in agricultural animals [6, 7]. Methods for qualitative and quantitative analysis of anabolic steroids based on gas chromatography integrated with mass spectrometry (GC-MS) or liquid chromatography coupled with mass spectrometry (LC-MS) have been reported [8–12]. Essential to the published LC-MS methods is selected reaction monitoring (SRM) based on tandem mass spectrometric (MS/MS) product ions. Interpretation of product-ion spectra of anabolic steroids and exploration of their collision-induced dissociation (CID) pathways are of importance to mass spectrometric characterization of new "designer" steroids, and helpful in understanding specificity of product ions of anabolic steroids for their detection, identification, and confirmation.

While interpretation of mass spectra obtained under electron ionization (EI) conditions is well understood and documented [13, 14], interpretation of product-ion spectra of $[M + H]^+$ ions acquired under atmospheric pressure ionization (API) MS/MS conditions is generating new

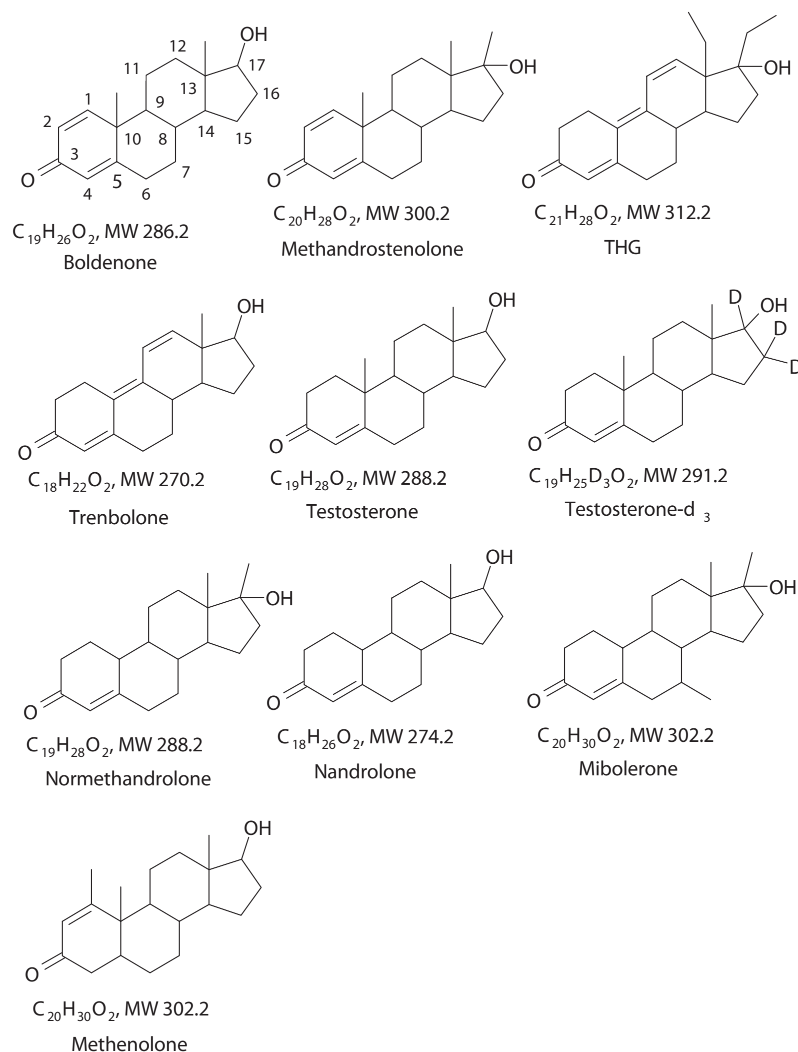
Published online February 17, 2006

Part of this article was presented at the 53rd ASMS Conference on Mass Spectrometry, June 5–9, 2005, San Antonio, TX, USA.

Address reprint requests to Dr. Cornelius E. Uboh, PA Toxicology and Research Center, 220 East Rosedale Ave., West Chester, PA 19382, USA. E-mail: ubohcorn@vet.upenn.edu

© 2006 American Society for Mass Spectrometry. Published by Elsevier Inc.
1044-0305/06/$32.00
doi:10.1016/j.jasms.2005.11.021

Received August 24, 2005
Revised November 29, 2005
Accepted November 29, 2005



Scheme 1. Chemical structures of anabolic steroids used in this study.

interest. Fragmentation pathways are reported for testosterone and testosterone hydroxyl analogs [15], oxosteroid Girard derivatives [16, 17], and steroid oximes [18] under ESI conditions. Recently, Thevis et al. reported electrospray ionization tandem mass spectrometric characterization of chemically modified steroids including 21 gestrinone and testosterone analogs [19]. In this paper, formation of major product ions of selected anabolic steroids under ESI (+) MS/MS conditions are interpreted and fragmentation pathways are proposed for those steroids of which fragmentation pathways have not been previously reported.

Experimental

Chemicals

Boldenone, methandrostenolone, trenbolone, testosterone, normethandrolone, nandrolone, mibolerone, and methenolone (Scheme **1**, where the numbers in boldenone structure show the numbering convention for anabolic steroids and D in testosterone-d_3 molecule is the abbreviation for deuterium) were purchased from Steraloids (Newport, RI), and testosterone-d_3 [77546-39-5] was obtained from Sigma (St. Louis, MO). Tetrahydrogestrinone (THG) was kindly donated by Dr. Thomas Tobin at Maxwell Gluck's Equine Center of University of Kentucky, Lexington, KY. Ammonium formate (certified), HPLC grade methanol and water were obtained from Fisher Scientific (Pittsburgh, PA). Formic acid was purchased from EM Science (Gibbstown, NJ).

Stock formate buffer comprising 1.0 mol/mL ammonium formate and 1.0 mol/mL of formic acid was prepared from the dry chemical powder and concentrated formic acid. The pH of the buffer was 3.4. Working formate buffer solution (2 mmol/L each of ammonium formate and formic acid) was prepared by dilution of the stock formate buffer with HPLC grade water.

Stock solution (1.0 mg/mL) of each analyte was prepared by dissolving the individual dry chemical powder in HPLC grade methanol, and stored at 4 °C. Working solution of each analyte at concentration of 1.0 μg/mL in methanol/formate buffer (2 mmol/L) (50/50, vol/vol) was prepared by dilution of the stock solution with the methanol/formate buffer mixture, and stored at 4 °C.

Mass Spectrometry

Thermo-Finnigan TSQ Quantum AM triple stage quadrupole mass spectrometer, Thermo-Finnigan LTQ FT Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry in which the Thermo-Finnigan LTQ linear ion trap mass spectrometer was used as an inlet system for the FT-ICR mass spectrometer, and Thermo-Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corp, San Jose, CA) were used in this study. All the instruments were equipped with ESI sources and operated in positive ion mode. TSQ Quantum AM was calibrated with polytyrosine-1,3,6 (Thermo Electron Corp.) that comprised Tyr, $(Tyr)_3$, and $(Tyr)_6$, by following the instructions of the instrument manual; the FT-ICR instrument and the LTQ linear ion trap were calibrated with calibration mixture of caffeine, L-methionyl-arginyl-phenylalanyl-alanine (MRFA), and Ultramark 1621 (Thermo Electron Corp.). TSQ Quantum AM instrument was operated at unit mass resolution (FWHM set to 0.7 for both Q1 and Q3). Resolution of the FT-ICR instrument was set to 50,000 (FWHM), and its mass accuracy was better than 2 ppm with external calibration, according to the manufacturer. For the LTQ instrument, isolation width of 1.5 was used for MS/MS and multiple stage tandem mass spectrometric (MS^n) experiments. The ion transfer capillary temperature was set at 225 °C for each mass spectrometer. The other ESI source parameters for each instrument were optimized for infusion flow rate of 10 μL/min. For CID experiments on the TSQ Quantum AM instrument, Argon was used as the collision gas, and collision energy applied in the form of electric potential was used to effect fragmentation. For CID experiments on both the LTQ and FT-ICR instruments, helium (dampening gas) was used as the collision gas, and normalized collision energy (expressed in percentage), which is a measure of the amplitude of the resonance excitation RF voltage applied to the endcaps of the linear ion trap, was used to bring about fragmentation. Data acquisition and analysis were accomplished with Xcalibur software versions 1.3 and 1.4 (Thermo Electron Corp.).

Product-ion spectrum of each anabolic steroid was acquired by syringe infusion of 1.0 μg/mL of the analyte at 10 μL/min into the ESI source of each mass spectrometer. A good quality spectrum was obtained by averaging the acquired data over a period of 1 min.

Generation of Possible Fragmentation Pathways

Possible fragmentation pathways for each anabolic steroid were generated by an expert-system software, Mass Frontier version 3.0 (Thermo Electron). Mass Frontier uses a mathematical approach for the simulation of unimolecular ion-dissociation reactions, but it does not have a function to assess the stability of product ions from thermodynamic data or rates of reaction. As a result, the software generated many possible fragmentation pathways for an $[M + H]^+$ ion, and many possible fragmentation routes for a pathway. Most of the possible product ions predicted by Mass Frontier for the $[M + H]^+$ ion were not visible in its experimental product-ion spectrum. Only a few of the predicted product ions were present in the experimental product-ion spectrum. A fragmentation pathway generating a product ion present in the experimental spectrum was manually picked, then reasonable and the most possible fragmentation routes in the pathway were chosen and manually finalized.

Results and Discussion

ESI (+) Product-Ion Spectra

The product-ion spectra of the anabolic steroids (Figure 1) acquired on the triple stage quadrupole instrument are different from each other despite only minor changes in chemical structure. For example, boldenone and testosterone have quite different product ion profiles but differ only by double-bond, as do testosterone and normethandrolone, trenbolone and THG. In short, the product-ion spectra of boldenone (Figure 1a), methandrostenolone (Figure 1b), testosterone (Figure 1f), and methenolone (Figure 1j) are featured by dominance of a few abundant product ions in each spectrum, indicating generation of these product ions from each of the relevant precursor ions by energetically favored fragmentation pathways. The product-ion spectra of the remaining steroids are crowded with many product ions, suggesting that the precursor $[M + H]^+$ ions have undergone many competitive fragmentation pathways.

Accurate Masses for Product Ions

To aid interpretation of the product-ion spectra, accurate mass measurements were conducted on product ions of the anabolic steroids using the FT-ICR instrument. The product- ion spectra of the anabolic steroids (Figure 2) acquired on the FT-ICR instrument look quite different from those acquired on the triple quadrupole instrument (Figure 1). This result is not surprising at all, since ion activation on the linear ion trap part of the FT-ICR is different from that on a triple stage quadrupole instrument. In CID on an ion trap, only precursor ions are activated, while in CID on a triple quadrupole, product ions can be activated by subsequent collisions and may undergo further fragmentation. As a result, observed from

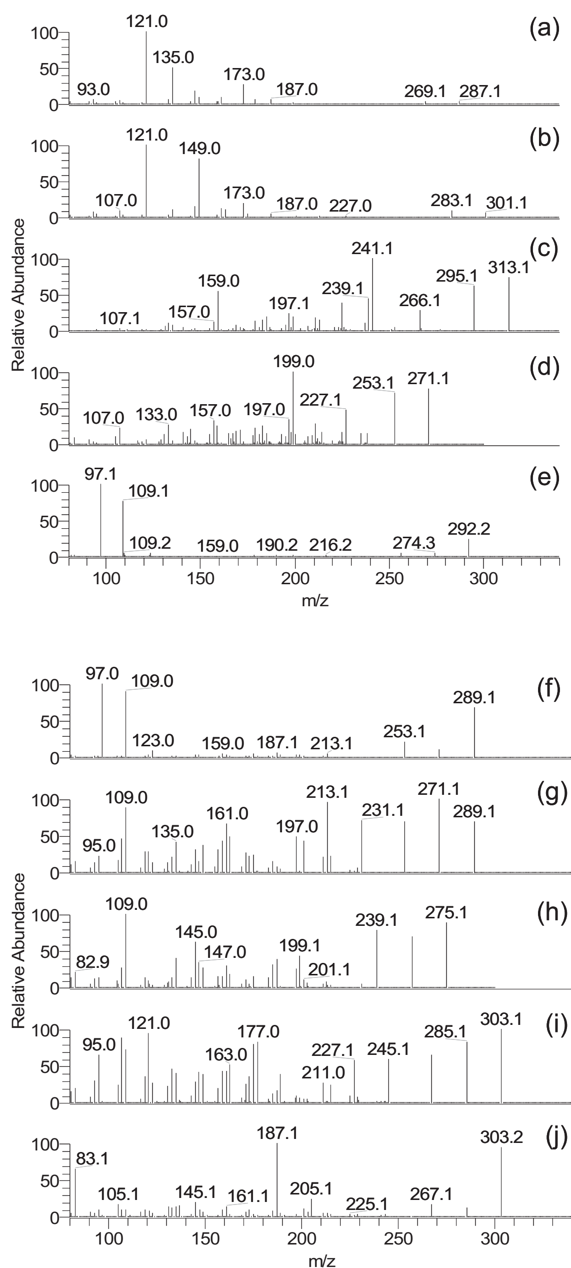


Figure 1. Product ion spectra of ten anabolic steroids acquired on the triple quadrupole instrument. Shown from top to bottom are the spectra of boldenone (**a**), methandrostenolone (**b**), THG (**c**), trenbolone (**d**), testosterone-d$_3$ (**e**), testosterone (**f**), normethandrolone (**g**), nandrolone (**h**), mibolerone (**i**), and methenolone (**j**). Collision energy was 15 eV for boldenone, methandrostenolone, THG, and methenolone; 20 eV for trenbolone and mibolerone; 16 eV for testosterone-d$_3$ and testosterone; 18 eV for normethandrolone; 17 eV for nandrolone. The major product ions of mibolerone not annotated are *m/z* 175, *m/z* 109, and *m/z* 107 (Figure 1**i**).

CID on an ion trap are usually the first generation of product ions at high mass end, while from CID on a triple quadrupole instrument, observed are final and stable product ions at low mass end. It should be pointed out that although some dominant product ions that were observed on the triple stage quadrupole instrument, such as the product ion of *m/z* 121 (Figure 1b) from methandrostenolone and the product ions of *m/z* 109 and *m/z* 97 from testosterone (Figure 1f), have low ion abundance in the corresponding product-ion spectrum acquired on the FT-ICR instrument (Figure 2b and 2e), yet high mass accuracy

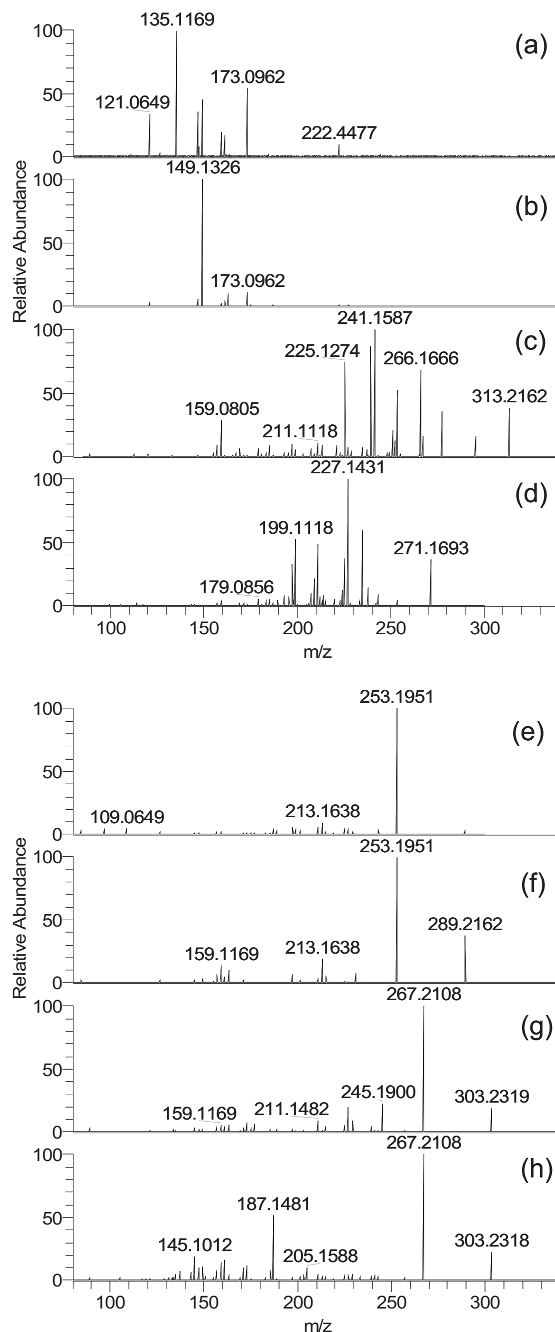


Figure 2. Accurate mass product-ion spectra of eight anabolic steroids acquired on the FT-MS instrument (with wideband activation). Shown from top to bottom are the spectra of boldenone (**a**), methandrostenolone (**b**), THG (**c**), trenbolone (**d**), testosterone (**e**), normethandrolone (**f**), mibolerone (**g**), and methenolone (**h**). Collision energy was 31% for boldenone, 30% for methandrostenolone, 32% for THG, 40% for trenbolone, 38% for testosterone, 35% for normethandrolone, 37% for mibolerone, and 34% for methenolone.

Table 1. Accurate masses for product ions of the anabolic steroids and their elemental compositions and number of rings plus double bonds (RDB)

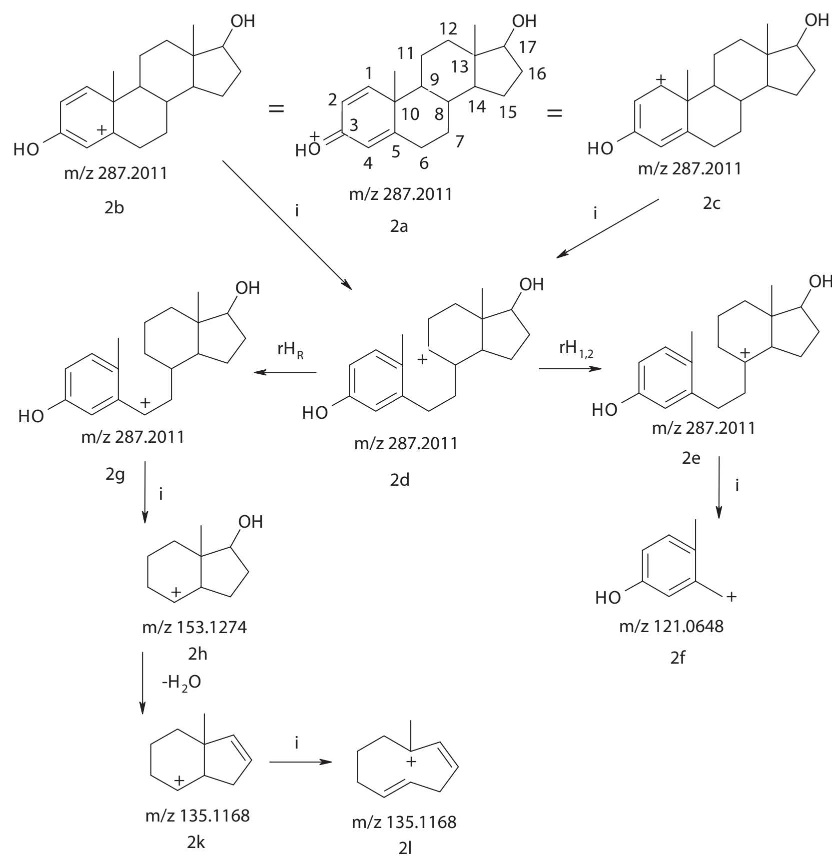
	m/z (Th) measured[a]	Elemental composition[b]	RDB calculated[c]	*m/z* (Th) predicted[d]	Δ *m/z* (Th)[e]	Δ *m/z* (ppm)[e]
			Product ions			
Boldenone	135.1169	$C_{10}H_{15}$	3.5	135.1168	0.0001	0.74
	121.0649	$C_8H_9O_1$	4.5	121.0648	0.0001	0.83
Methandrostenolone	149.1326	$C_{11}H_{17}$	3.5	149.1325	0.0001	0.67
	121.0649	$C_8H_9O_1$	4.5	121.0648	0.0001	0.83
THG	266.1666	$C_{19}H_{22}O_1$	9.0	266.1665	0.0001	0.38
	241.1587	$C_{17}H_{21}O_1$	7.5	241.1587	0.0000	0.00
	239.1431	$C_{17}H_{19}O_1$	8.5	239.1430	0.0001	0.42
	225.1274	$C_{16}H_{17}O_1$	8.5	225.1274	0.0000	0.00
	213.1274	$C_{15}H_{17}O_1$	7.5	213.1274	0.0000	0.00
	211.1118	$C_{15}H_{15}O_1$	8.5	211.1117	0.0001	0.47
	197.0961	$C_{14}H_{13}O_1$	8.5	197.0961	0.0000	0.00
	159.0805	$C_{11}H_{11}O_1$	6.5			
Trenbolone	227.1431	$C_{16}H_{19}O_1$	7.5	227.1430	0.0001	0.44
	199.1118	$C_{14}H_{15}O_1$	7.5	199.1117	0.0001	0.50
Testosterone	109.0649	$C_7H_9O_1$	3.5	109.0648	0.0001	0.92
	97.0649	$C_6H_9O_1$	2.5	97.0648	0.0001	1.03
Normethandrolone	231.1744	$C_{16}H_{23}O_1$	5.5	231.1743	0.0001	0.43
	213.1638	$C_{16}H_{21}$	6.5	213.1638	0.0000	0.00
				109.0648		
Mibolerone	245.1900	$C_{17}H_{25}O_1$	5.5	245.1905	−0.0005	−2.0
	177.1275	$C_{12}H_{17}O_1$	4.5	177.1279	−0.0004	−2.3
	121.1013	C_9H_{13}	3.5	121.1017	−0.0004	−3.3
	109.1013	C_8H_{13}	2.5	109.1017	−0.0004	−3.7
Methenolone	187.1481	$C_{14}H_{19}$	5.5			
			$[M + H]^+$ ions[f]			
Trenbolone	271.1693			271.16926	0.00004	0.15
THG	313.2162			313.21621	−0.00001	−0.03
Testosterone	289.2162			289.21621	−0.00001	−0.03
Normethandrolone	289.2162			289.21621	−0.00001	−0.03
Mibolerone	303.2319			303.23186	0.00004	0.13
Methenolone	303.2318			303.23186	−0.00006	−0.20

[a] *m/z* measured = experimental values obtained using the FT-ICR instrument.
[b] Elemental composition derived from the measured *m/z* with tolerance of 2 ppm (mass accuracy by the FT-ICR instrument is better than 2 ppm). Actually, when valence rules and candidate compositions encompassing $C_{0-21}H_{3-31}O_{0-2}$ (covering the maximal numbers of carbon, hydrogen and oxygen atoms in the precursor ions) are considered, the nearest alternative composition is 99 ppm apart.
[c] RDB calculated = the number of RDB calculated from the elemental composition.
[d] *m/z* predicted = the *m/z* value predicted for the product ion by the proposed fragmentation pathway or the *m/z* value derived from the elemental composition of the anabolic steroids.
[e] Δ *m/z* = *m/z* measured − *m/z* predicted.
[f] Accurate masses are listed for the $[M + H]^+$ ions with known elemental composition to show confidence in mass accuracies achieved in the present study.

was still achieved on those product ions (Table 1). To demonstrate the confidence in mass accuracy achieved on the FT-ICR instrument, the accurate *m/z* values of the $[M + H]^+$ ions of THG, trenbolone, testosterone, normethandrolone, mibolerone, and methenolone (Figure 2c–h) are listed in Table 1. The mass accuracies for the $[M + H]^+$ ions were within 0.2 ppm.

Approach to Proposing Fragmentation Pathways

Unlike fragmentation of odd-electron molecular ions ($M^{+\cdot}$) generated under EI conditions, which can be initiated by an unpaired electron, fragmentation of even-electron $[M + H]^+$ ions generated under ESI conditions is generally initiated by the positive charge. Interpretation of product-ion spectra of the anabolic steroids and elucidation of their fragmentation pathways are quite difficult because they do not contain a charge-retaining center such as a nitrogen atom in their molecules and there are several rings in their chemical structures. To overcome this difficulty, Mass Frontier was used in addition to accurate mass measurements and MS^n experiments, to aid in the elucidation of fragmentation pathways. The approach is briefly explained here: first, automatic generation of possible fragmentation pathways for an anabolic steroid by Mass Frontier; second, manual selection of an appropriate fragmentation pathway by comparing accurate *m/z* value of an experimental product ion of the steroid with that predicted by Mass Frontier; finally, manual finalization of the fragmentation routes in the appropriate pathway



Scheme 2. Fragmentation pathway of boldenone proposed for generation of the major product ions of *m/z* 121 and *m/z* 135.

using knowledge of even-electron (EE^+) ion fragmentation. For example, an experimental product ion of boldenone is of *m/z* 121.0649 (Figure 2a), while Mass Frontier predicted two possible product ions of *m/z* 121.0648 and 121.1012 for the same nominal mass. The predicted product ion of *m/z* 121.0648 was in agreement with the experimental product ion of *m/z* 121.0649, and thus, the possible pathways predicting the product ion of *m/z* 121.0648 were chosen. It should be pointed out that there were several predicted pathways leading to generation of the product ion of *m/z* 121.0648. Even for a particular pathway, there are several possible fragmentation routes for generation of the *m/z* 121.0648 ion. The fragmentation pathways and routes were further examined and chosen according to the following criteria: (1) inductive cleavage occurs at a carbon atom with the most branches or strains; (2) stability of transition-state carbonium ions follows the order CR_3^+ (tertiary) > CHR_2^+ (secondary) > CH_2R^+ (primary) [20]; (3) formation of a product ion is highly dependent on its stability, and the stability depends on whether a positive charge on the product ion is stabilized by resonance and/or inductive effects. Given the above criteria, the most likely pathway and routes for generation of the experimental product ion of *m/z* 121.0649 from boldenone were chosen and finalized (Scheme 2). In Scheme 2, "i" indicates inductive cleavage, $rH_{1,2}$ represents the rearrangement of a hydrogen atom to an adjacent carbon atom with concurrent α site rearrangement of the charge, and rH_R stands for the rearrangement of a hydrogen atom to a remote site with concurrent γ site rearrangement of the charge. Fragmentation pathway for generation of the product ion of *m/z* 135.1169 from boldenone was similarly proposed (Scheme 2). Using the same elucidation procedure, we proposed fragmentation pathways for the other anabolic steroids. It should be noted that the $[M + H - 18]^+$ and $[M + H - 36]^+$ ions in the product-ion spectra of the anabolic steroids (Figure 1) obviously resulted from loss of one or two H_2O molecules from the $[M + H]^+$ ions and thus, the corresponding fragmentation pathways are not presented in this paper.

Boldenone and Methandrostenolone

In the fragmentation pathway proposed for boldenone (Scheme 2), it is reasonable to assume that protonation occurs at the 3-carbonyl oxygen atom in the $[M + H]^+$ ion because the carbonyl group is in conjugation with two pairs of double bonds. The positive charge on the 3-carbonyl oxygen atom (Scheme **2a**) may transiently locate on either the 1- or 5-carbon atom (Scheme **2c** or **b**) because of resonance [13]. The charge on either position leads to cleavage of the bond between the 9,10-carbon

atoms because the 10-carbon atom is the most branched and strained; concurrent formation of the phenyl ring is energetically favored. The charge-induced β bond cleavage and concurrent formation of a double-bond, such as the fragmentation step from **2b** or **c** to **d** in Scheme **2** are reasonable [13, 21]. The γ-position charge-transfer from the 9-carbon atom (Scheme **2d**) to the 6-carbon atom (Scheme **2g**) is driven by stabilization of the charge at the 6-carbon atom (similar to benzyl cation). The transition-state species with the charge on the 9-carbon atom (Scheme **2d**) undergoes two different and competitive routes to produce the final major product ions of *m/z* 121.0648 (a stable ion similar to benzyl cation with its charge stabilized by resonance [13]) and of *m/z* 135.1168 (also stabilized by resonance). In the fragmentation pathway, formation of the phenyl ring in the transition-state species (Scheme **2d**) together with stability of the final product ions accounts for their dominance in the product-ion spectrum. The loss of an H_2O molecule from the boldenone $[M + H]^+$ ion may occur at any step in the fragmentation pathway, as verified by the product-ion spectra of boldenone $[M + H]^+$ and $[M + H - H_2O]^+$ ions acquired on the linear ion trap (not shown). The product-ion spectra showed that the product ions of *m/z* 121 and *m/z* 135 are generated from the $[M + H]^+$ ion and from the $[M + H - H_2O]^+$ ion as well. Besides, the product-ion spectra also showed that the product ion of *m/z* 251 from loss of two H_2O molecules from boldenone $[M + H]^+$ ion is almost not seen, suggesting that loss of the second H_2O molecule from the $[M + H]^+$ ion is negligible. In comparison, loss of the second H_2O molecule from the testosterone $[M + H]^+$ ion is abundant as seen by the ion of *m/z* 253 in the product-ion spectra of the $[M + H]^+$ ion and the $[M + H - H_2O]^+$ ion (not shown). These results support the proposed formation of the phenyl ring in the transition-state species (Scheme **2d**) since only the hydroxyl group attached to a phenyl ring is difficult to eliminate in low-energy CID.

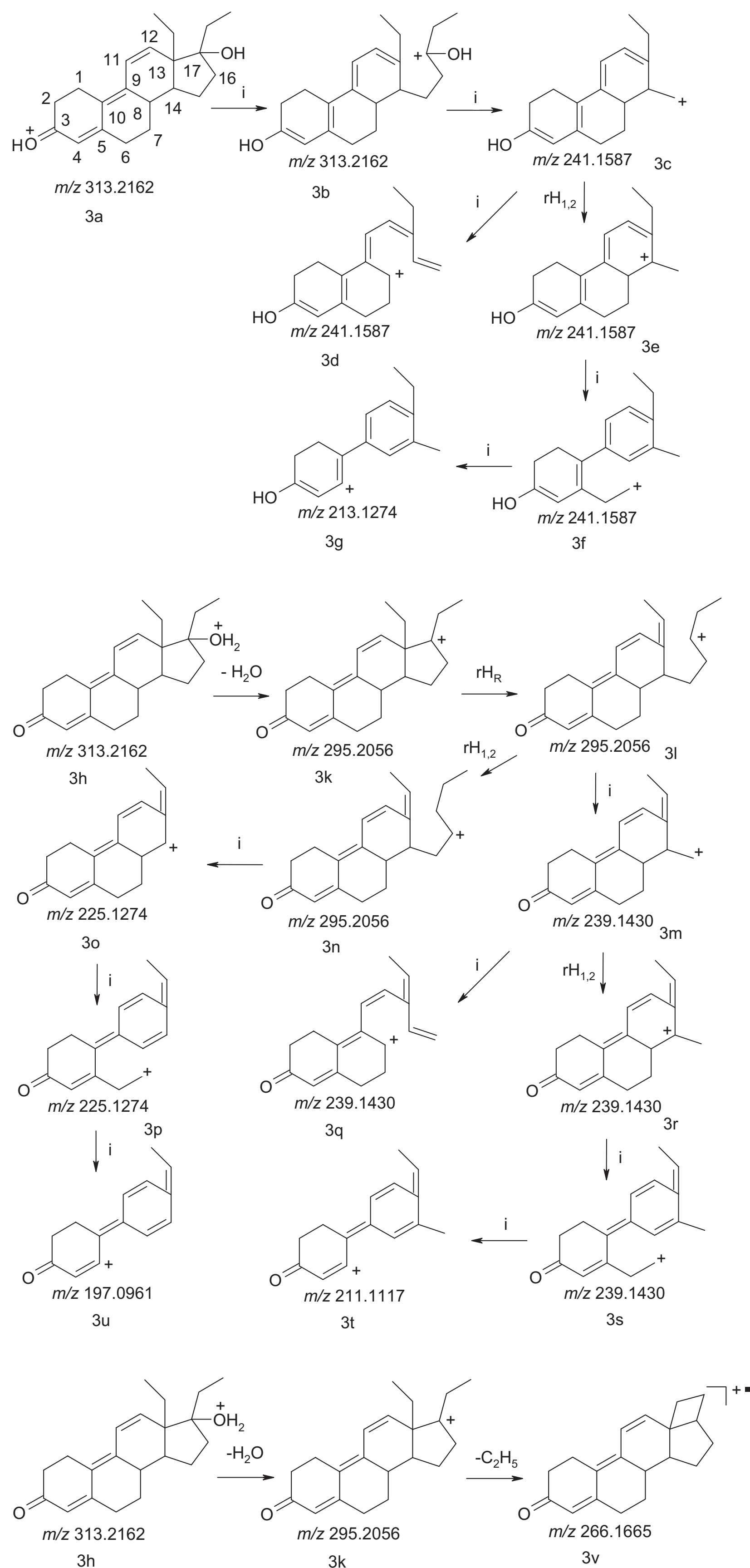
The proposed structures for the two major product ions of boldenone agree with the elemental composition and number of RDB (Table 1) derived from their experimental accurate masses. For example, the elemental composition for the predicted product ion of *m/z* 121.0648 (Scheme **2f**) is $C_8H_9O_1$, which is the same as that for the experimental product ion of *m/z* 121.0649 (Table 1). The RDB value for the predicted product ion of *m/z* 121.0648 is 4, and agrees with the calculated RDB value of 4.5 for the experimental product ion of *m/z* 121.0649 (the RDB of 4.5 ends in 0.5 and thus, indicates an even-electron ion; the true RDB value is 4 after subtraction of 0.5 [13]).

Methandrostenolone has similar chemical structure and, thus, has similar fragmentation pathways. The only difference between methandrostenolone and boldenone is the presence of additional 17-methyl group in the former, and this accounts for the product ion of *m/z* 149.1326 of methandrostenolone.

THG and Trenbolone

In the product-ion spectrum of THG acquired on the triple stage quadrupole instrument (Figure 1c), there were six major product ions of *m/z* 266, *m/z* 241, *m/z* 239, *m/z* 225 (not annotated), *m/z* 197, and *m/z* 159. The formation of the first five ions was interpreted by the fragmentation pathway proposed in this study (Scheme **3**). Protonation of THG primarily occurs at the 3-carbonyl oxygen atom since the carbonyl group is in conjugation with the conjugated double-bond system. The charge on the 3-carbonyl oxygen atom in the $[M + H]^+$ ion can be delocalized on the 12-carbon atom because the 3-carbonyl group is in conjugation with the 4,9,11-double-bond system. In addition, the 13-carbon atom is the most branched and strained. As a result, the bond between the 13,17-carbon atoms cleaves and a double-bond between the 12,13-carbon atoms forms, resulting in the transition-state species (Scheme **3b**). This species undergoes further fragmentation, yielding the final product ions of *m/z* 241 (Scheme **3d**) and *m/z* 213 (Scheme **3g**). The former (Scheme **3d**) is stable (similar to benzyl cation). The fragmentation routes proposed for generation of these two product ions agree with evidence from MS/MS and MS/MS/MS experiments of the THG $[M + H]^+$ ion on the linear ion trap, showing that the product ion of *m/z* 241 was generated from the $[M + H]^+$ ion but not from the $[M + H - H_2O]^+$ ion. Besides, the elemental composition and RDB value for the predicted product ions are in accordance with the experimental accurate masses (Table 1).

In addition to protonation at the 3-carbonyl oxygen atom in THG, protonation may also occur at the 17-hydroxyl oxygen atom. The $[M + H]^+$ ion with protonation at the 17-hydroxyl oxygen atom generates the product ions of *m/z* 266, *m/z* 239, *m/z* 225, and *m/z* 197 (Scheme **3**). In the fragmentation step from the transition-state species 3k to 3l, a hydrogen atom at the 13-methylene group is rearranged to the 17-carbon atom, the carbon atom of the 13-methylene group becomes positively charged, and then the charge causes cleavage of the bond between the 13,17-carbon atoms and formation of the 13-double-bond that is driven by stabilization of the conjugated double-bond system. The transition-state species (3l) undergoes different and competitive fragmentation routes to produce the final product ions of *m/z* 239, *m/z* 225, *m/z* 211 and *m/z* 197. The proposed fragmentation routes for generation of these product ions are supported by product-ion spectrum (not shown) of the $[M + H - H_2O]^+$ ion of THG showing generation of those four product ions from the $[M + H - H_2O]^+$ ion. Theoretically, the 3-carbonyl oxygen atom has higher proton affinity than the 17-hydroxyl oxygen atom because the former is conjugated with the 4,10,11-double-bond system and, thus, much higher population of $[M + H]^+$ ion with protonation at the 3-carbonyl oxygen atom than at the 17-hydroxyl oxygen atom is expected under ESI conditions. However, even though population of $[M + H]^+$ ion with



Scheme 3. Fragmentation pathway of THG proposed for generation of the major product ions of *m/z* 241, *m/z* 213, *m/z* 225, *m/z* 239, *m/z* 211, and *m/z* 266.

protonation at the 17-hydroxyl oxygen atom is quite low, product ions from this $[M + H]^+$ ion were experimentally observed, as described above. A possible explanation for this experimental result is that the ionizing proton is mobile upon collisional activation and thus, can protonate the 17-hydroxyl oxygen atom. Furthermore, the 17-ethyl group in THG molecule makes the 17-carbon atom highly strained and thus, fragmentation can be initiated at this carbon atom.

The product ion of *m/z* 266 from THG (Figure 1c) is interesting since its generation from the $[M + H]^+$ ion cannot be interpreted by Mass Frontier. It is a radical ion (an odd-electron ion) as indicated by the even number of its RDB value derived from its experimental accurate mass (Table 1). The fragmentation route for generation of this product ion had to be manually proposed (Scheme **3**). Based on the RDB value (Table 1), structure for the radical ion of *m/z* 266 is proposed (Scheme **3v**). On the fragmentation route from the transition-state species (Scheme **3k**) to the final product ion (Scheme **3v**), either the 13- or the 17-ethyl group may eliminate according to the results of Thevis et al. from deuterium-labeled THG [19]. Generation of the product ion of *m/z* 266 via loss of H_2O molecule and ethyl radical group from the $[M + H]^+$ ion is supported by the results from deuterium-labeled THG [19] and EI spectrum of THG [22].

The production of *m/z* 159 from THG (Figure 1c) cannot be interpreted by Mass Frontier, and it is not generated from further fragmentation of the product ion of *m/z* 266 as concluded from product-ion spectrum (not shown) of the ion of *m/z* 266 of THG.

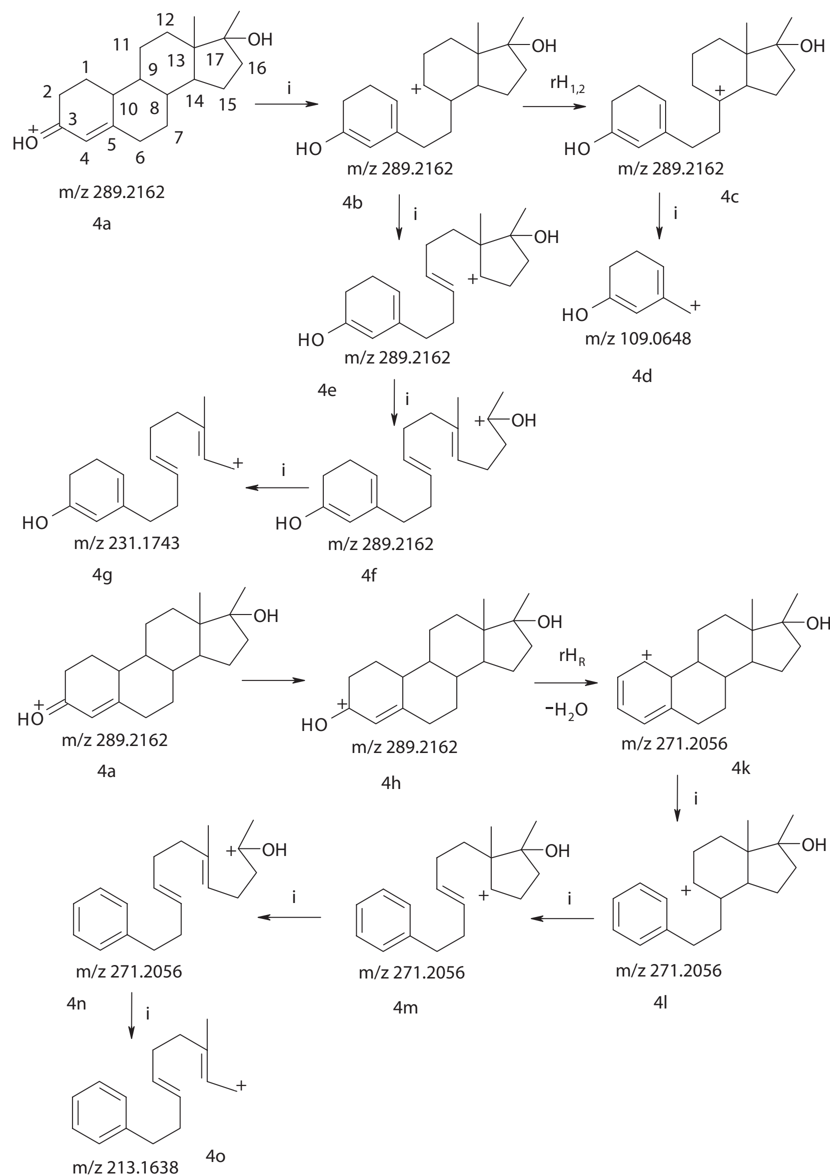
According to the fragmentation pathway proposed for THG in this study, the major product ions of *m/z* 241 and *m/z* 213 from gestrinone, dihydrogestrinone and d_4-THG, and the major product ions of *m/z* 227 and *m/z* 199 from altrenogest and propyltrenbolone reported [19] can be interpreted.

Trenbolone generated two major product ions of *m/z* 227 and *m/z* 199 on the triple stage quadrupole instrument (Figure 1d), and they can be interpreted by the fragmentation pathway proposed for THG (specifically, the fragmentation routes leading to generation of the product ions of *m/z* 241.1587 and *m/z* 213.1274 from THG, respectively, in Scheme **3**). The product ions of *m/z* 227 and *m/z* 199 were generated from the $[M + H]^+$ ion but not from the $[M + H - H_2O]^+$ ion, as concluded from the MS/MS and MS/MS/MS spectra of trenbolone $[M + H]^+$ ion acquired on the linear ion trap. In comparison with THG, no abundant product ion generated from trenbolone $[M + H]^+$ ion with protonation at the 17-hydroxyl oxygen atom was observed using the triple quadrupole instrument. This result can be explained with consideration of the slight difference between chemical structures of trenbolone and THG. In trenbolone, absence of the 17-ethyl group that is present in THG causes any transition-state species with the charge on the 17-carbon atom to be less stable. As a result, fragmentation of trenbolone via the $[M + H]^+$ ion with protonation at the 17-hydroxyl oxygen atom is not a favorable fragmentation route.

Testosterone, Normethandrolone, Nandrolone, and Mibolerone

Testosterone is similar to normethandrolone and nandrolone in chemical structure (Scheme **1**), but it has a unique product ion of *m/z* 97 (Figure 1f) that is absent in the product-ion spectra of normethandrolone and nandrolone (Figure 1g and h). In addition, testosterone-d_3 has the same product ions of *m/z* 97 and *m/z* 109 (Figure 1e) as does testosterone, indicating that these product ions do not contain the 16-CHD_2 and 17-CDOH region of the molecule. The MS/MS/MS spectrum (not shown) of testosterone indicates that the product ions of *m/z* 97 and *m/z* 109 originate from $[M + H]^+$ ion instead of $[M + H - H_2O]^+$ ion. Based on these evidences plus accurate masses of the product ions, fragmentation pathway of testosterone (not shown) was proposed for generation of the product ions of *m/z* 97 and *m/z* 109. Although the proposed fragmentation pathway is in agreement with the results (elemental composition and RDB) from accurate mass measurements of the product ions, it is not in accordance with the results from product-ion spectra of testosterone deuterium-labeled at various positions reported by Williams et al. [15]. The fragmentation pathway proposed by Williams et al. for generation of the product ion of *m/z* 109 from testosterone, involving the unusual rearrangement of a hydrogen atom from the 6-carbon atom to the 10-carbon atom [15], seems correct. This result indicates the importance of deuterium labeling in fragmentation pathway studies, and reveals failure of Mass Frontier to predict the right fragmentation pathway for testosterone. The failure of Mass Frontier in this case shows its limitation in predicting fragmentation pathways for compounds involving unusual hydrogen rearrangements (Mass Frontier version 4.0 uses a database of published fragmentation pathways in an attempt to overcome this limitation).

Normethandrolone, nandrolone, and mibolerone do not undergo the fragmentation pathway of testosterone for generation of the product ion of *m/z* 109, proposed by Williams et al. [15], because they lack a10-methyl group in their molecules that is present in testosterone molecule and necessary for that fragmentation pathway to occur [15]. The product ion of *m/z* 109 of normethandrolone, nandrolone, and mibolerone must be generated by other fragmentation pathways. The fragmentation route for generation of this product ion (*m/z* 109) from normethandrolone was proposed together with those for generation of the product ions of *m/z* 231 and *m/z* 213 (Scheme **4**). In the initial $[M + H]^+$ ion (Scheme **4a**), the charge on the 3-carbonyl oxygen atom can transiently locate on the 5-carbon atom, and by induction the charge on the 5-carbon atom leads to formation

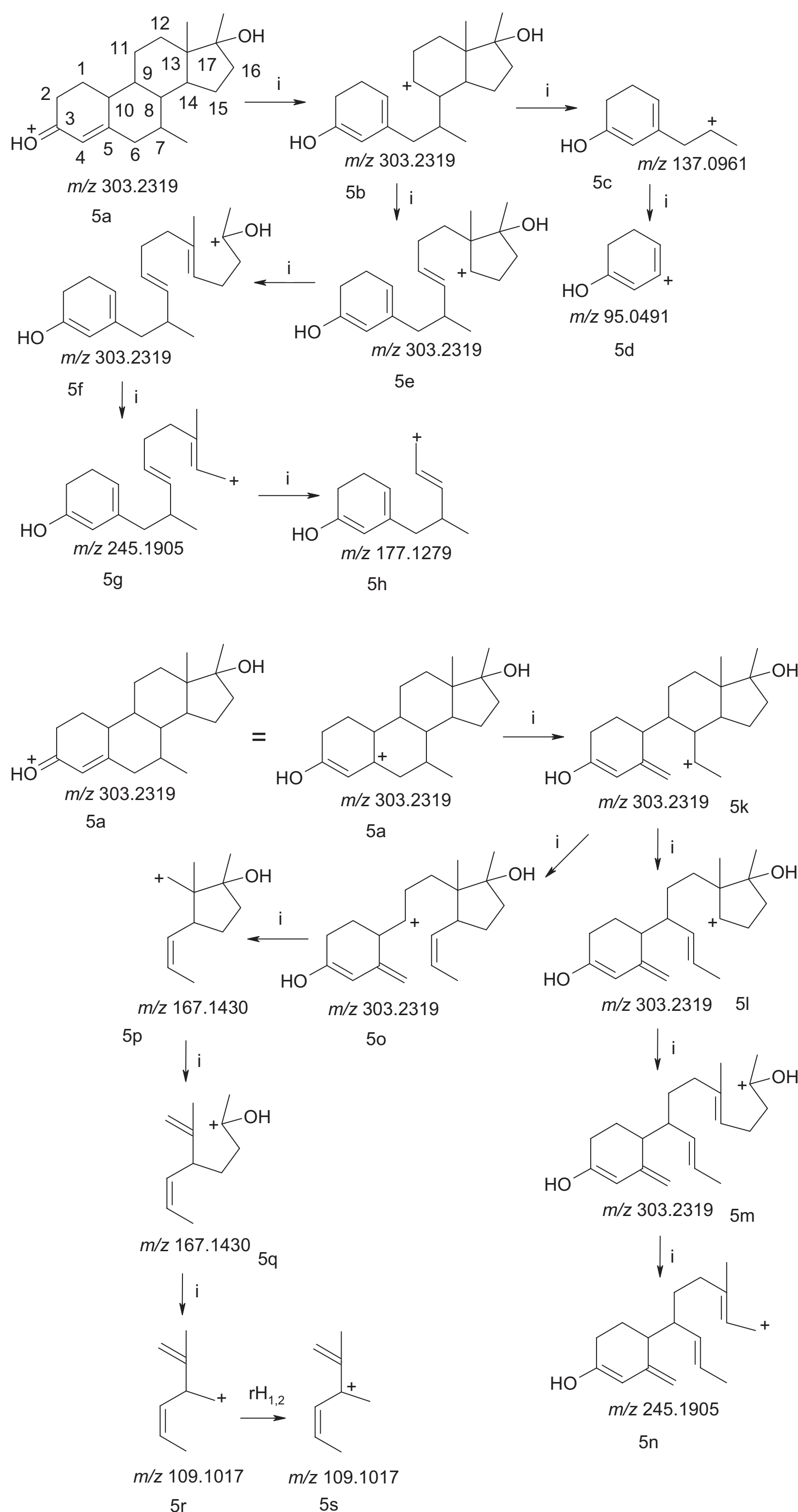


Scheme 4. Fragmentation pathway of normethandrolone proposed for generation of the major product ions of *m/z* 231, *m/z* 109, and *m/z* 213.

of the transition-state species (Scheme **4b**). It should be pointed out that the electron-donating 17-methyl group helps to stabilize the positive charge on the 17-carbon atom in the transition-state species (Scheme **4f** and **n**) and, thus, makes possible generation of the product ions of *m/z* 231 and *m/z* 213. The proposed fragmentation routes for generation of the product ions of *m/z* 213 and *m/z* 231 agree with the results from MS/MS, MS/MS/MS, and MS/MS/MS/MS spectra (not shown) of normethandrolone acquired on the LTQ instrument. The spectra indicate that the product ion of *m/z* 231 was generated from $[M + H]^+$ ion instead of $[M + H - H_2O]^+$ ion while the product ion of *m/z* 213 was produced from $[M + H - H_2O]^+$ ion but not from $[M + H - 2H_2O]^+$ ion. In addition, the elemental composition and RDB value for the predicted product ions of *m/z* 231 (Scheme **4g**) and *m/z* 213 (Scheme **4o**) are in accordance with those for the experimental product ions (Table 1).

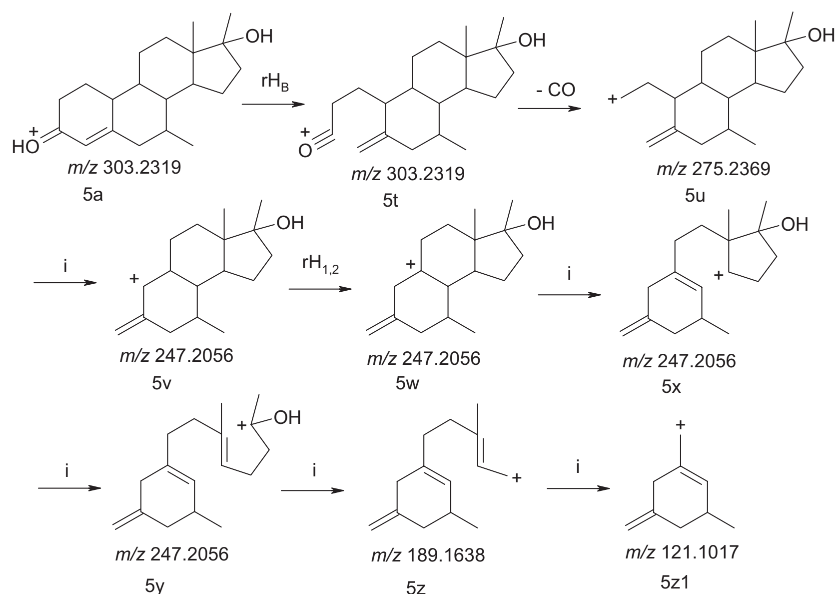
According to this fragmentation pathway for normethandrolone, the major product ions of *m/z* 231, *m/z* 213, and *m/z* 109 from norethisterone and the major product ions of *m/z* 245, *m/z* 227, and *m/z* 109 from norgestrel and norbolethone reported [19], can be interpreted.

Nandrolone has the same product ion of *m/z* 109 as does normethandrolone, and fragmentation pathway generating this product ion from nandrolone is the same as that from normethandrolone (Scheme **4**). Unlike normethandrolone, nandrolone does not have a 17-methyl group in its molecule and, thus, does not undergo the fragmentation of normethandrolone involving the transition-state 17-carbonium species



Scheme 5. Fragmentation pathway of mibolerone proposed for generation of the major product ions of *m/z* 95, *m/z* 177, *m/z* 109, *m/z* 245, and *m/z* 121.

(Scheme **4f** and **n**) because the electron-drawing 17-hydroxyl group in nandrolone causes any 17-carbonium species to be unstable. As a result, nandrolone does not have the product ions relevant to the product ions of *m/z* 231 and *m/z* 213 from normethandrolone.



Scheme 5. (Continued)

Mibolerone is similar to normethandrolone in chemical structure, but the additional 7-methyl group in its structure results in alternative fragmentation routes (Scheme **5**) from those for normethandrolone (Scheme **4**). In Scheme **5**, rH_B represents charge site hydrogen rearrangement. In mibolerone molecule, because of the existence of the electron-donating 7-methyl group, the transition-state species (Scheme **5c**) with the charge on the 7-carbon atom becomes competitive and thus, leads to generation of the product ion of *m/z* 95 (Scheme **5d**) that was experimentally observed. In addition, because of the existence of the electron-donating 7-methyl group, fragmentation routes via cleavage of the bond between the 6,7-carbon atoms come into play to generate the transition-state species (Scheme **5k**) and the final product ions of *m/z* 245 (Scheme **5n**) and *m/z* 109 (Scheme **5s**). It should be noted that the transition-state species (Scheme **5t**) is stable according to McLafferty and Turecek [13], and loss of a carbon monoxide molecule from an even-electron ion such as the transition-state species (Scheme **5t**) is even easier than loss of an H_2O molecule according to the Field's rule and their proton affinity values [13]. It is surprising that the minor difference between the chemical structures of mibolerone and normethandrolone results in quite different and distinct fragmentation pathways. The proposed fragmentation route leading to generation of the product ion of *m/z* 245 is supported by MS/MS and MS/MS/MS spectra (not shown) of mibolerone $[M + H]^+$ ion indicating that this product ion was generated from $[M + H]^+$ ion but not from $[M + H - H_2O]^+$ ion. The elemental composition and RDB value for the predicted product ions of *m/z* 245 (Scheme **5n**), *m/z* 177 (Scheme **5h**), *m/z* 121 (Scheme **5z1**), and *m/z* 109 (Scheme **5s**) agree with those for the experimental product ions (Table 1), respectively.

Methenolone

Methenolone is slightly different in chemical structure from "boldenone type" and "testosterone type" of steroids discussed above, and its product-ion spectrum is distinctly different from those of boldenone and testosterone types of steroids. The fragmentation pathway for generation of the major product ion of *m/z* 187 from methenolone was proposed but not presented because it was not in agreement with the results from product-ion spectra of 5α-androst-1-en-17β-ol-3-one (1-testosterone) and deuterium-labeled metenolone (methenolone) [19]. The fragmentation pathway for generation of the *m/z* 187 ion from 1-testosterone and methenolone proposed by Thevis et al. [19], involving methyl rearrangement and retro-Diels-Alder reaction, seems correct. The failure of Mass Frontier to predict the right fragmentation pathway for methenolone shows its limitation in predicting fragmentation pathways for compounds involving methyl rearrangements or special rearrangements such as retro-Diels-Alder reaction.

Conclusion

Although very similar in chemical structure, the anabolic steroids gave rise to quite different product-ion spectra unique for each steroid under ESI (+) MS/CID/MS conditions. These spectra were interpreted, and fragmentation pathways of the anabolic steroids were proposed with the aid of Mass Frontier, accurate mass measurements by the FT-ICR instrument and MS^n experiments with the linear ion trap. Mass Frontier is helpful in generating possible fragmentation pathways that would otherwise be very difficult to manually perform. However, the software cannot specify a definite fragmentation pathway for a steroid, and manual

selection of the reasonable pathway is, therefore, necessary. Furthermore, Mass Frontier (Version 3.0) has its limitation: it is not able to take into consideration unusual hydrogen rearrangement, methyl rearrangement, and special rearrangement such as retro-Diels-Alder reaction.

Small differences in chemical structures of the steroids, such as an additional double-bond, methyl, or ethyl group, result in significantly different fragmentation pathways. For example, the additional 1-double-bond in boldenone, compared with testosterone, makes the fragmentation pathway for boldenone distinctly different from that for testosterone. The methyl group at the C-10 position in testosterone has a remarkable effect on fragmentation, compared with its absence in normethandrolone, nandrolone, and mibolerone. The additional methyl group at the C-7 position in mibolerone, compared with normethandrolone, results in significant differences between fragmentation pathways for mibolerone and normethandrolone.

The proposed fragmentation pathways are useful in understanding fragmentation of structurally similar steroids as well as uniqueness of the formation of their product ions. The approach used in this study is helpful for future studies on fragmentation pathways of other compounds, drugs, and their metabolites.

Acknowledgments

This study was funded by the Pennsylvania Horse and Harness Racing Commissions to whom the authors are very thankful. Financial contribution was also made by the Pennsylvania Harness Horsemen Association at Pocono Downs. The authors are very grateful to Dr. Thomas Tobin and his group at the Maxwell Gluck's Equine Center of the University of Kentucky, Lexington, KY, for donating THG for this study.

References

1. Vogel, G. A. Race to the Starting Line. *Science* **2004**, *305*(5684), 632–635.
2. Kicman, A. T.; Gower D. B. Anabolic Steroids in Sport: Biochemical, Clinical, and Analytical Perspectives. *Ann. Clin. Biochem.* **2003**, *40* (Part 4), 321–356.
3. Parssinen, M.; Seppala, T. Steroid Use and Long-term Health Risks in Former Athletes. *Sports Med.* **2002**, *32*(2), 83–94.
4. Catlin, D. H.; Sekera, M. H.; Ahrens, B. D.; Starcevic, B.; Chang, Y. C.; Hatton, C. K. Tetrahydrogestrinone: Discovery, Synthesis, and Detection in Urine. *Rapid Commun. Mass Spectrom.* **2004**, *18*(12), 1245–1249.
5. World Anti-Doping Agency, the World Anti-Doping Code—the 2005 Prohibited List. www.wada-ama.org/rtecontent/document/list_2005.pdf. Accessed May 19, 2005.
6. Draisci, R.; Palleschi, L.; Ferretti, E.; Lucentini, L.; Quadri, F. D. Confirmatory Analysis of 17β-Boldenone, 17α-Boldenone and Androsta-1,4-Diene-3,17-Dione in Bovine Urine by Liquid Chromatography-Tandem Mass Spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2003,** *789,* 219–226.
7. Van Poucke, C.; Van Peteghem, C. Development and Validation of a Multi-Analyte Method for the Detection of Anabolic Steroids in Bovine Urine with Liquid Chromatography-Tandem Mass Spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2002,** *772,* 211–217.
8. Impens, S.; De Wasch, K.; De Brabander, H. Determination of Anabolic Steroids with Gas Chromatography-Ion Trap Mass Spectrometry Using Hydrogen as Carrier Gas. *Rapid Commun. Mass Spectrom.* **2001,** *15,* 2409–2414.
9. Yoon, J. M.; Lee, K. H. Gas Chromatographic and Mass Spectrometric Analysis of Conjugated Steroids in Urine. *J. Biosci.* **2001,** *26,* 627–634.
10. Reilly, C. A.; Crouch, D. J. Analysis of the Nutritional Supplement 1AD, Its Metabolites, and Related Endogenous Hormones in Biological Matrices Using Liquid Chromatography-Tandem Mass Spectrometry. *J. Anal. Toxicol.* **2004,** *28,* 1–10.
11. Buiarelli, F.; Cartoni, G. P.; Coccioli, F.; De Rossi, A.; Neri, B. Determination of Trenbolone and Its Metabolite in Bovine Fluids by Liquid Chromatography-Tandem Mass Spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2003,** *784,* 1–15.
12. Starcevic, B.; DiStefano, E.; Wang, C.; Catlin, D. H. Liquid Chromatography-Tandem Mass Spectrometry Assay for Human Serum Testosterone and Trideuterated Testosterone. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2003,** *792,* 197–204.
13. McLafferty, F. W.; Turecek, F. *Interpretation of Mass Spectra, 4th ed.*; University Science Book: Sausilito, CA, 1993, pp 27, 52, 55, 140, 343.
14. Brown, F. J.; Djerassi, C. Elucidation of the Course of the Electron Impact Induced Fragmentation of α,β-Unsaturated 3-Keto Steroids. *J. Am. Chem. Soc.* **1980**, *102*(2), 807–817.
15. Williams, T. M.; Kind, A. J.; Houghton, E.; Hill, D. W. Electrospray Collision-Induced Dissociation of Testosterone and Testosterone Hydroxyl Analogs. *J. Mass Spectrom.* **1999**, *34*(3), 206–216.
16. Griffiths, W. J.; Liu, S.; Alvelius, G.; Sjoevall, J. Derivatization for the Characterization of Neutral Oxosteroids by Electrospray and Matrix-Assisted Laser Desorption/Ionization Tandem Mass Spectrometry: The Girard P Derivative. *Rapid Commun. Mass Spectrom.* **2003,** *17,* 924–935.
17. Shackleton, C. H.; Chuang, H.; Kim, J.; de la Torre, X.; Segura, J. Electrospray Mass Spectrometry of Testosterone Esters: Potential for Use in Doping Control. *Steroids* **1997**, *62*(7), 523–529.
18. Liu, S.; Sjoevall, J.; Griffiths, W. J. Analysis of Oxosteroids by Nano-Electrospray Mass Spectrometry of Their Oximes. *Rapid Commun. Mass Spectrom.* **2000,** *14,* 390–400.
19. Thevis, M.; Bommerich U.; Opfermann, G. Schaenzer, W. Characterization of Chemically Modified Steroids for Doping Control Purposes by Electrospray Ionization Tandem Mass Spectrometry. *J. Mass Spectrom.* **2005,** *40,* 494–502.
20. Uggerud, E. Physical Organic Chemistry of the Gas Phase. Reactivity Trends for Organic Cations. In *Topics in Current Chemistry*; *Vol. CCXXV, Modern Mass Spectrometry*; Schalley, C. A., Ed.; Springer: New York City, NY, 2003; p 8.
21. Fleurat-Lessard, P.; Pointet, K.; Milliet, A. Contrasting Behavior of Tetracene and Perylene in Collision-Induced Dissociation: A Theoretical Interpretation. *J. Mass Spectrom.* **1999**, *34*(7), 768773.
22. Karpiesiuk, W.; Lehner, A. F.; Hughes, C. G.; Tobin, T. Preparation and Chromatographic Characterization of Tetrahydrogestrinone, a New "Designer" Anabolic Steroid. *Chromatographia* **2004,** *60,* 359–363.