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Structure and mechanism of formation of an important ion in doping control

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Abstract

An ion with *m*/*z* 143 serves as a biomarker that is often continuously monitored in urine samples undergoing screening by electron ionization gas chromatography/mass spectrometry (EI GC/MS) for banned anabolic agents. The ion is known to arise from trimethylsilyl (TMS)-derivatized synthetic 17-hydroxy, 17-methyl steroids. The purpose of this work was to characterize, in detail, the origin(s), structure(s), and mechanism(s) of formation of such ions with *m*/*z* 143. High resolution mass spectrometry (HRMS) data revealed the elemental composition of the D-ring derived *m*/*z* 143 ion to be $C_7H_{15}OSi$. Analysis of dihydrotestosterone (DHT) and its 2-methyl substituted analog dromostanolone by HRMS revealed that an elementally equivalent ion of *m*/*z* 143 could be derived from the A-ring of TMS-derivatized 3-keto-enol steroids demonstrating that an abnormally intense peak in the *m*/*z* 143 extracted ion chromatogram of urine samples undergoing screening for banned anabolic agents does not necessarily indicate the presence of a 17-hydroxy, 17-methyl steroid. To gain information on ion structure, breakdown curves for the most abundant product ions of the *m*/*z* 143 ion were generated using both native and perdeutero-TMS derivatives, providing structures for second, third, and fourth generation product ions. An EI-mass spectrum of [16,16,17-$^2H_3$]-DHT (DHT-d3) demonstrated that one of the C-16 hydrogen atoms is removed prior to the formation of an ion that is highly analogous to the ion with *m*/*z* 143 strongly suggesting, in accord with all other evidence, one particular fragmentation pathway and resulting product: a resonance stabilized 3-(*O*-trimethylsilyl)but-1-ene ion.
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Keywords: *m*/*z* 143; Steroids; Anti-doping; Ion structure

1. Introduction

One of the primary goals of anti-doping research is to define biomarkers of doping, i.e., chemical entities that, when detected at or above certain concentrations, are indicative of a doping offense by the provider of the urine or blood sample under analysis. Although these markers are generally the banned substances or their metabolites, classification as such is not a requisite condition for their establishment as a doping biomarker. In fact, due to the manner in which sporting federation rules are written, ideal biomarkers would be general in nature such that detection of a single marker substance would indicate an anabolic doping offense. Details of the offense could be sorted out after an initial positive report, but the facile and rapid acquisition of a positive result based on a general biomarker would greatly simplify the analytical process. This idyllic scenario may never become possible, but the dramatic simplification of sample screening procedures appears to be within the realm of modern technology.

For over a decade, the technique of choice for screening urine samples for banned anabolic agents has been gas chromatography coupled to electron ionization mass spectrometry (EI GC/MS) [1–3]. Analyses of urine extract samples by GC/MS are typically run in selected ion monitoring (SIM) mode with multiple ion windows to obtain required sensitivity limits [3], but ions produced with a *m*/*z* of 143 are continually monitored in every sample by many world anti-doping agency-accredited laboratories during the course of screening trimethylsilyl (TMS)-

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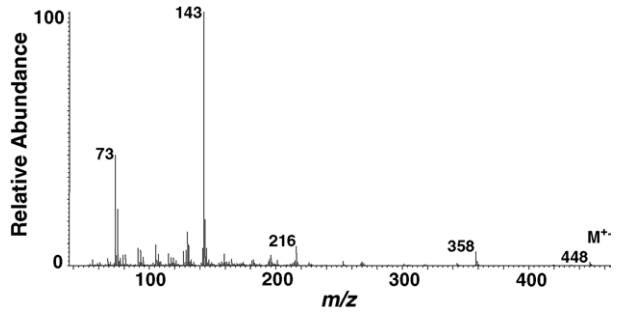
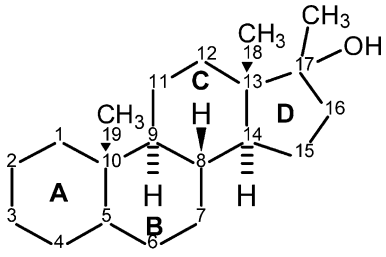


Fig. 1. EI-mass spectrum of TMS-derivatized epimetendiol (**7**)—a representative 17-hydroxy, 17-methyl synthetic steroid. The peak at *m*/*z* 448 represents the molecular ion (M^+). The origin, structure, and mechanism of formation of the ion represented by the base peak at *m*/*z* 143 are the focus of the work presented here.

derivatized urine sample extracts for banned anabolic substances (personal communication with Larry Bowers, United States Anti-Doping Agency Senior Managing Director of Technical and Information Resources).[1] A thorough investigation of the structure, molecular origin(s), and mechanism of formation of this ion, however, has not been reported even though work closely related to some aspects of that described here has been carried out [4,5]. It is understood that the ion consistently arises, frequently as the base mass spectral peak (Fig. 1), from the D-ring of 17-hydroxy, 17-methyl steroids (**1**) [5,6] a group of steroids that are predominately synthetic in origin. All the steroidal sources of this unique ion, however, have yet to be established. Thus, the ion at *m*/*z* 143 has served as a (imperfect) biomarker for the presence of synthetic steroids: abnormal and intense chromatographic peak(s) in the *m*/*z* 143 extracted ion chromatogram of a urine sample are typically subjected to further investigation. An example of the utility of this practice is illustrated in Fig. 2. A better understanding of the origin, structure, and mechanism of formation of this ion may provide a greater insight into its utility as a urinary biomarker of synthetic steroid use.



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2. Materials and methods

2.1. Materials

Ethylestrenol (4-estren-17α-ethyl-17β-ol), dromostanolone (5α-androstan-2α-methyl-17β-ol-3-one), mestanolone (5α-androstan-17α-methyl-17β-ol-3-one), and dihydrotestosterone (DHT) (5α-androstan-17β-ol-3-one) were obtained from Steraloids (Newport, RI, USA). Epimetendiol (17β-methyl-5β-androst-1-ene-3α,17α-diol), 3′-hydroxystanozolol (3′,17β-dihydroxy-17α-methyl-5α-androstan [3,2-c] pyrazole), 13β,17α-diethyl-3α-17β-dihydroxy-5α-gonane, and [16,16,17-$^2H_3$]-DHT (DHT-d3) were obtained from the National Analytical Reference Laboratory (New South Wales, Australia). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and bis(trimethylsilyl)acetamide (BSA) were purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). Ammonium iodide and ethanethiol were acquired from Sigma (St. Louis, MO, USA). Bis(trimethylsilyl)acetamide-d18 (BSA-d18) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Perfluorokerosene-H (PFK) was obtained from Lancaster Synthesis Inc. (Pelham, NH, USA).

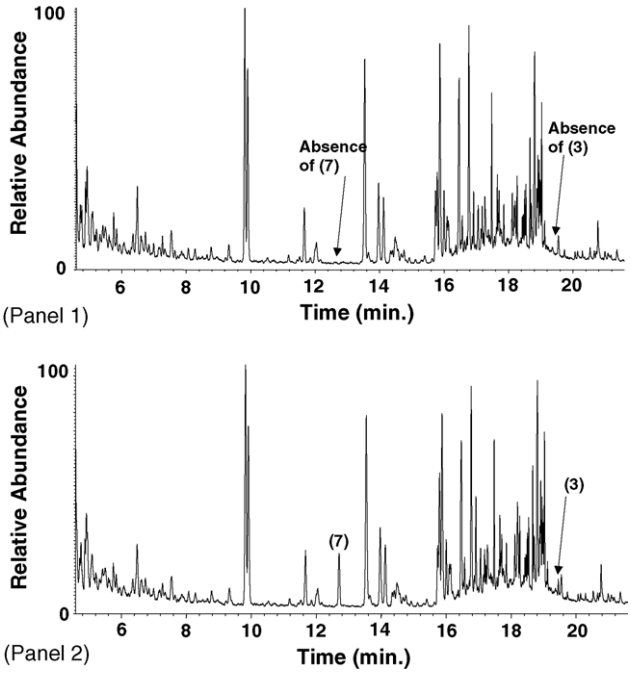


Fig. 2. Extracted ion chromatograms of *m*/*z* 143 from urine samples analyzed by GC/MS. Panel 1 shows a chromatogram from a blank urine sample. Panel 2 shows a chromatogram from a urine sample spiked with 20 ng/ml of epimetendiol (**7**) and 3′-hydroxystanozolol (**3**). Samples were prepared and extracted as described elsewhere [21], but derivatized with MSTFA/ammonium iodide/ethanethiol (1000:2:4, v/w/v), and analyzed by GC/MS as described in Section 2.

2.2. Sample preparation

All steroid samples derivatized with MSTFA were prepared at a final concentration of 300 ng/μl in MSTFA/NH_4I/ethanethiol (1000:2:4, v/w/v) by heating at 75 °C for 30 min. The NH_4I and ethanethiol ensure near-complete keto-enol trimethylsilylation (at the low risk of ethyl thio-incorporation into silylated steroid structures) [7,8], and therefore eliminates the need for methyl oxime derivatives. Following derivatiza-

[1] There are no published reports of this practice, however, it is a well known, well established practice within the anti-doping community.

tion samples were injected directly into the GC–MS. Samples derivatized with BSA and BSA-d18 were brought up to the same final concentration, but heated overnight at 60 °C. Keto-enol trimethylsilylation was generally not complete with this reagent and procedure, but this matter is inconsequential as differentially derivatized molecules are separated by GC.

2.3. Gas chromatography/mass spectrometry

Analysis of extracted urine samples by GC/MS was done on an Agilent GC-MSD instrument consisting of an Agilent 6890 GC coupled to an Agilent 5973 Inert MSD (G2579A performance turbo EI MSD model). One-microliter samples were injected in split mode (1:20) onto an injector kept at 280 °C. The GC was operated in constant flow mode with a starting carrier gas (He) linear velocity of 35 cm/s through a DB-1MS, 30 m × 0.25 mm I.D., 0.1 μm film capillary column. The initial oven temperature was set to 180 °C and without any initial hold time was ramped at 3.3 °C/min to 231 °C, followed by an immediate ramp to 310 °C and hold for 2 min, during which the oven was again heated to 325 °C and held for 1 min. The GC–MS transfer line was kept at 322 °C. The ion source was operated in EI mode at 230 °C, with a 70 eV filament. The quadrupole was operated at 150 °C and scanned from *m/z* 50–700 at a rate of 2.29 scans/s. The spectra from each scan were recorded individually, i.e., without averaging.

Analysis of samples by GC/MS/MS was carried out on a Varian 3400F GC (equipped with a Finnigan MAT A200S autosampler and a Varian 1077 split/splitless capillary injector) connected to a Finnigan MAT TSQ7000 triple quadrupole mass spectrometer. One-microliter injections of samples were made in splitless mode into an injector set at 270 °C and a pressure of 8 psi (~35 cm/s linear velocity) of helium. The GC oven, containing a DB-1MS, 30 m × 0.25 mm I.D., 0.25 μm film column, was ramped (after an initial 1 min hold) from 100 to 310 °C at 20 °C/min and held for 2 min. The ion source was operated in EI mode at 230 °C, with a 70 eV filament. Argon was used as the collision gas and was set at a pressure of 3.0 mTorr. Collision energies were varied as described. In MS/MS mode, the instrument was operated in product ion scan mode, scanning a mass range from 12 to 10 *m/z* units above the selected precursor ion at a rate of 2 scans/s. One precursor ion per run was isolated individually for collision-induced dissociation (CID) with a 1 *m/z* unit selection window.

Analyses of samples by direct insertion probe, high resolution mass spectrometry (HRMS) were performed on a Finnigan MAT 95 HRMS with Finnigan MAT ICIS II operating system by Dr. Elliot Rachlin at the Mass Spectrometry Facility housed within the Department of Chemistry at the University of Utah. Samples were heated, starting from 0 to 20 °C for 0.5 min, followed by additional heating from 40 °C/min to 280 °C, where the temperature was held for 10 min. Data were collected using electric sector scanning at a rate of 10 s/scan (with a 0.4 s interscan time) over a *m/z* range of 129–171. The single-scan spectrum with the greatest total ion current was reported. PFK was used as an internal calibrant gas. Following calibration, analytical values for PFK ions were always found to be within 0.2 ppm mass accuracy including a $C_4F_5^+$ ion with *m/z* 142.99201, which was baseline resolved from peaks of nominal mass 143 derived from TMS-derivatized steroids.

2.4. Quantum mechanical (density functional theory) calculations

Theoretical investigations using density functional theory (DFT) at the B3LYP/6-31G(d,p) level were carried out to provide first-principles information on the relative stability of any ion structure candidates. The B3LYP hybrid DFT method has been known to provide rather accurate energetic properties and vibrational frequencies of molecular species.[2] Candidate structures for the ion with *m/z* 143 were fully optimized and all possible resonance structures were taken into account. Normal mode analyses of these species were also performed to confirm their stability and to predict their infrared (IR) spectra for further possible identification. All calculations were done using the Gaussian03 program [9].

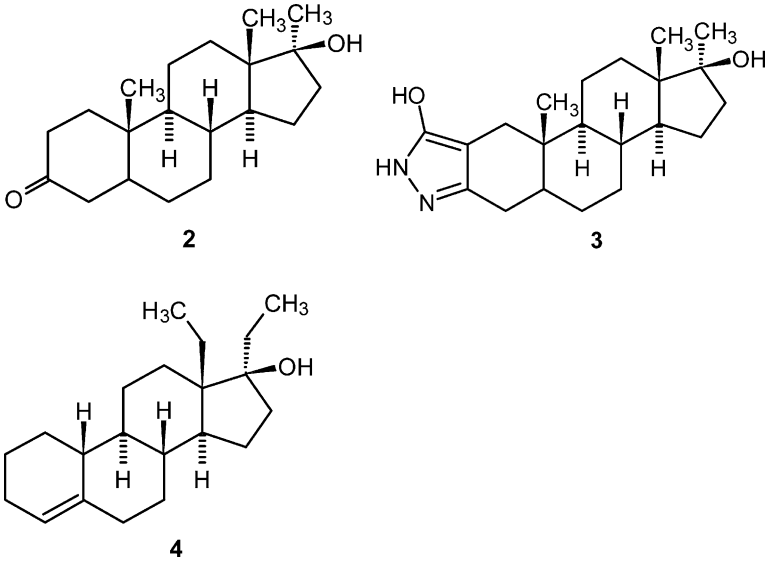
3. Results and discussion

3.1. HRMS

The TMS derivatives of two 17-hydroxy, 17-methyl steroid standards–mestanolone (**2**) and 3′-hydroxystanozolol (**3**) which produce *m/z* 143 ions when subjected to EI conditions were analyzed by direct insertion probe-EI-HRMS in order to establish the elemental composition of these ions. A related 17-hydroxy, 17-ethyl synthetic steroid, ethylestrenol (**4**), which produces an *m/z* 157 ion under EI conditions, was also analyzed by EI-HRMS. Given the known steroid elemental compositions, established valency rules, and a mass accuracy within 15 ppm (well within the range of the evaluated instrument accuracy), results indicated that the only possible elemental composition assignments are $C_7H_{15}OSi$ for the ion with *m/z* 143 and $C_8H_{17}OSi$ for the ion with *m/z* 157. Besides providing elemental composition, these assignments provide evidence for the suspected D-ring origin of the fragment ions in question, which is based on the predicted and observed 14 *m/z* unit difference between the 17-methyl and 17-ethyl steroids.

A calculation of the number of rings and double bonds in the *m/z* 143 and 157 ions (which also indicates whether the ion contains an odd or even number of electrons [10]), based on elemental composition, results in a number of 1.5. This indicates that there is one ring or double bond in the ion structure and that the ion under consideration is an even-electron ion.

[2] For example, the default optimization settings for calculating the energetic properties of water with Gaussian03 are accurate to within 0.001 kcal/mol [9].



3.2. Molecular origin

It was observed that dihydrotestosterone (**5**), an endogenous steroid, also produces an ion with *m*/*z* 143 even though the steroid does not contain 17-hydroxy and 17-methyl functional groups. DHT was also analyzed by HRMS (data not shown) and it was discovered that the ion with *m*/*z* 143 produced by DHT has the same elemental composition as that produced by mestanolone (**2**) and 3′-hydroxystanozolol (**3**). This means that some steroidal structural feature other than simultaneous 17-hydroxylation and 17-methylation is capable of producing a very similar or identical ion to the one with *m*/*z* 143 that arises from synthetic steroids. We hypothesized that this ion likely arose from the A-ring of DHT. To test this hypothesis HR mass spectra of dromostanolone (**6**), a synthetic steroid with a structure identical to DHT with the exception of the presence of a methyl group attached to carbon 2, were obtained. (The value of methylated analogs in the determination of EI-fragment origins for TMS-derivatized steroids is well founded [4].) The results showed the presence of a peak representing an ion with *m*/*z* 157 with the same elemental composition as the ion produced by ethylestrenol (**4**) supporting the A-ring origin hypothesis. Additional support for this hypothesis was gathered from the EI-mass spectrum of trimethylsilylated [16,16,17-$^2H_3$]-DHT (Fig. 3): the molecular ion was observed at *m*/*z* 437 (as opposed to *m*/*z* 434 for native DHT), but no shift was observed in the peak at *m*/*z* 143 suggesting that the ion with *m*/*z* 143 arises from somewhere in the molecule other than the D-ring. Apparently, under TMS-derivatized EI conditions, an ion with the same elemental composition is produced in abundance from the A-ring of 3-keto-enol steroids, as is produced from 17-hydroxy, 17-methyl steroids. Additional data (not shown) also demonstrates the generation of an ion (in low abundance, <25% RI) with *m*/*z* 143 from 3-hydroxy-4-ene steroids. Finally, Vouros and Harvey [11] have reported that an ion with the same elemental composition, but different structure than the ion of interest here, arises from a variety of 11-trimethylsiloxy steroids. These facts demonstrate that observation of an abnormally intense peak in the *m*/*z* 143 extracted ion chromatogram of urine samples undergoing screening for banned anabolic agents may not indicate the presence of a 17-hydroxy, 17-methyl steroid.

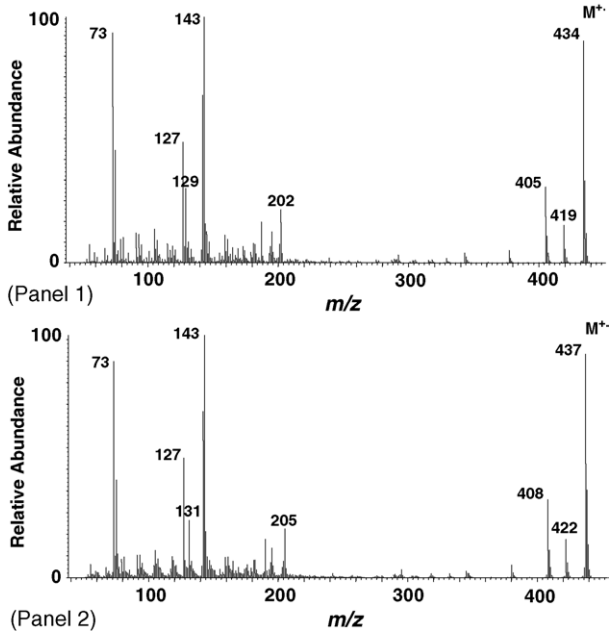
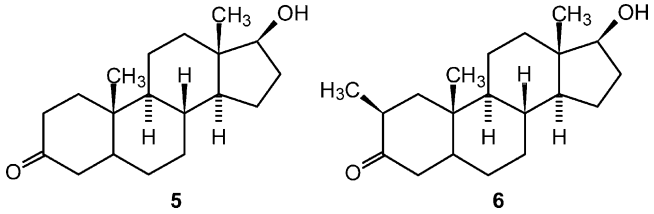


Fig. 3. EI-mass spectra of DHT (**5**) (Panel 1) and [16,16,17-$^2H_3$]-DHT (Panel 2). The significance of several important peaks is described in Sections 3.2 and 3.4.



3.3. MS/MS

To obtain detailed information on the structures of ions with *m*/*z* 143 and 157 described in Sections 3.1 and 3.2, fragmentation

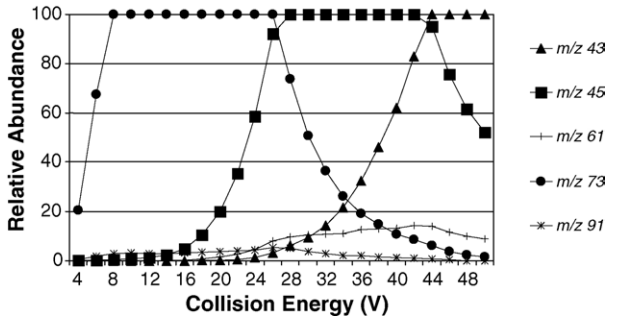
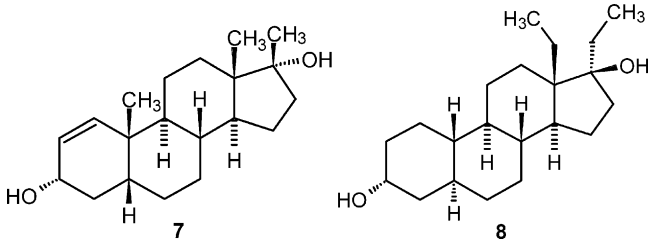


Fig. 4. Breakdown curve generated by CID and analysis by a second stage of mass spectrometry for the major product ions of the *m/z* 143 precursor ion derived from TMS-derivatized epimetendiol (**7**).

data at increasing collision energies were collected to construct breakdown curves [12] of the major product ions of D- and A-ring derived ions with *m/z* 143 and of D- and A-ring derived ions with *m/z* 157. Breakdown curves were obtained for epimetendiol (**7**) (gives a D-ring, and possibly some A-ring derived ions with *m/z* 143), 3′-hydroxystanozolol (**3**) (gives a D-ring derived ion with *m/z* 143), mestanolone (**2**) (gives D- and A-ring derived ions with *m/z* 143), DHT (**5**) (gives an A-ring derived ion with *m/z* 143), 13β,17α-diethyl-3α-17β-dihydroxy-5α-gonane (**8**) (gives a D-ring derived ion with *m/z* 157), ethylestrenol (**4**) (gives a D-ring derived ion with *m/z* 157), and dromostanolone (**6**) (gives an A-ring derived ion with *m/z* 157). For a given MS/MS-derived product ion, a breakdown curve consists of a plot of the relative abundance of the product ion versus collision energy. When breakdown curves for several product ions are displayed together, information on relative ease of formation and the identities of second, third, and subsequent generation product ions can be obtained [13]. Breakdown curves for all steroids analyzed were found to be nearly identical to that of epimetendiol (**7**) (Fig. 4) suggesting similar fragmentation pathways and structures for the D-ring and A-ring derived *m/z* 143 and *m/z* 157 ions. The breakdown curves indicate that the most easily formed product ion fragment of the ion with *m/z* 143 and the ion with *m/z* 157 is a trimethylsilyl ion (*m/z* 73) which gives rise to a methylsilyl ion (*m/z* 45) which dehydrogenates to produce an ion with *m/z* 43. (The methylsilyl ion has been documented to arise from the trimethylsilyl ion by others [14].) Alternate fragmentation pathways give rise to a trimethylsilyloxonium ion (*m/z* 91) and a methylsilyloxonium ion (*m/z* 61). In all cases, the ion with *m/z* 143 is one that easily produces, with less than 12 eV input energy, a trimethylsilyl ion upon CID.



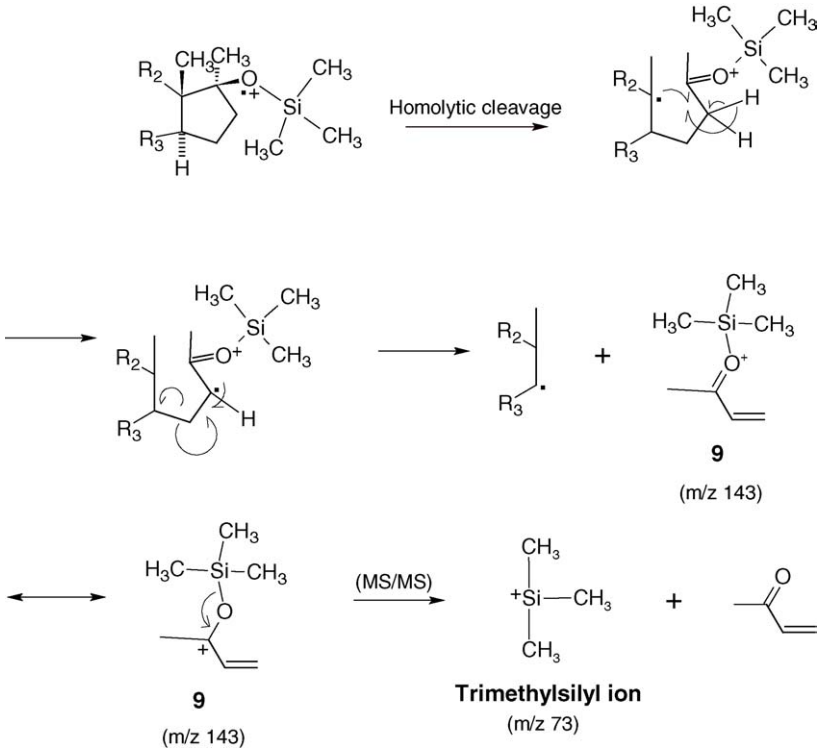
Upon derivatization of steroids **2–8** with BSA-d18 (which adds nine deuterium atoms per TMS group), the expected heavy analogs of the ions with *m/z* 143 and *m/z* 157 were detected at *m/z* 152 and 166, respectively, and had the expected relative abundance. (McCloskey et al. [15] have described the utility and interpretation of deuterium-labeled TMS derivatives in EI mass spectrometry.) Collision-induced dissociation studies on the deuterated ions with *m/z* 152 and *m/z* 166 were carried out, and analogs to products ions produced by the ions with *m/z* 143 and *m/z* 157 were observed with analogous abundances at *m/z* 82 (instead of *m/z* 73), *m/z* 100 (instead of *m/z* 91), and *m/z* 50 (instead of *m/z* 45) (data not shown). These specific *m/z* shifts support both the precursor- and the product-ion *m/z* assignments and structures.

The breakdown curves obtained here unambiguously demonstrate the production of trimethylsilyl and related ions upon CID of the ion with *m/z* 143. Many TMS-derivatized molecule fragments produce the set of product ions observed in the MS/MS spectra of the ion with *m/z* 143, but this cannot be assumed to occur without the proper evidence. Thus, by demonstrating the facile (very low CID energy) production of a trimethylsilyl ion, the data generated lend support to the structure assigned to the ion with *m/z* 143 (**9**, Scheme 1).

3.4. Deuterium isotope labeling studies

As can be seen from a comparison of the mass spectra of DHT-d3 and native DHT (Fig. 3), a peak at *m/z* 131 constitutes a 2 *m/z* unit increase over a peak at *m/z* 129 in the mass spectrum of native DHT. Based on previous studies on the EI-induced fragmentation of TMS-derivatized steroids [4], it is known that the ion with *m/z* 129 in D-ring saturated, 17-hydroxysteroids includes carbon atoms C-17 and C-16. Thus, an increase of 2 *m/z* units in the *m/z* 129 ion of the [16,16,17-$^2H_3$]-analog of DHT rather than an increase of 3 *m/z* units is significant because it indicates that one of the hydrogen atoms attached to C-16 or C-17 is abstracted during formation of the ion. Based on analogy to the well-characterized ethylene ketal EI-fragmentation pathway [16,17], Diekman and Djerassi [4] suggest an explanation for this 2 *m/z* unit shift in the form of a fragmentation pathway (mechanism) by which hydrogen from C-16 is abstracted and formation of an ion with *m/z* 129 occurs. All experimental data shown here, in agreement with these studies found in the literature, suggest that formation of the TMS-derivatized 17-hydroxy, 17-methyl steroid-derived ion with *m/z* 143 would form a highly analogous product (**9**) (by difference of an electronically uninvolved methyl group at C-17) via a highly analogous mechanism (Scheme 1). This mechanistic analogy is supported by Vouros and Harvey [18]. The ion structure (**9**) is identical to that initially suggested, but not experimentally proven, by Durbeck and Buker [5] after simple observation of an ion with *m/z* 143 in the spectra of TMS-derivatized 17-hydroxy, 17-methyl steroids.

Unfortunately, no [16,16-$^2H_2$]-labeled 17-hydroxy, 17-methyl steroid is commercially available and we lack the facility and funds to either synthesize one ourselves or to commission a custom synthesis. Also, no deuterium-labeling studies were carried out to fully assess the structure and mechanism of formation of the A-ring derived ion with *m/z* 143 because such analogs were not commercially available and because it is beyond the primary scope of the work presented here.



Scheme 1. Structure (**9**) and proposed mechanism of formation for the ion with *m*/*z* 143 derived from the D-ring of 17-hydroxy, 17-methyl steroids. The mechanism for facile formation of a trimethylsilyl ion (*m*/*z* 73) upon low energy CID of the *m*/*z* 143 ion is shown.

3.5. Stereochemistry

No major differences in abundance were observed for the *m*/*z* 143 ion produced by epimetendiol (**7**) as compared to that produced by 3′-hydroxystanozolol (**3**) nor were differences observed for the ion with *m*/*z* 143 produced by oxandrolone as compared to epioxandrolone, which are diastereomers (data not shown). Thus, stereochemistry appears not to play a significant role in formation of the *m*/*z* 143 ion.

3.6. Quantum mechanical calculations

Quantum mechanical calculations using density functional theory were used to establish the relative stabilities of proposed D-ring derived ion candidates that arose during the course of this investigation. As suggested by Gustaffson et al. [19] the production of highly stable end-point ions is a key driving force for fragmentation pathways. This is true for unimolecular fragmentations, where the products with lower free energy would have the lower activation energy, in accord with the Hammond postulate [20]. In terms of ground state free energy at 0 K (i.e., zero-point energy corrected potential energy), ion structure **9** was found to be the most thermodynamically stable species (by 28.5 kcal/mol) amongst other candidates formed by initial heterolytic fragmentation (not shown) that, ultimately, were found not to be in agreement with mass spectral evidence. This fact suggests that these types of calculations may be valuable in guiding the acquisition of experimental (mass spectral) evidence during the course of an investigation.

Mulliken population analyses for the established ion structure (**9**) indicates that the positive charge is mostly located on the Si atom (with an atomic partial charge greater than +0.8), whereas the partial charge of the oxygen atom is greater (more negative) than −0.4. These results point out that using chemical intuition and formal charge assignments to put the positive charge on a certain atom is not always accurate. The optimized structure, geometrical parameters, and calculated infrared absorbance spectrum of ion structure **9** were generated and are available upon request.

4. Conclusions

The results of this study verify that the ion with *m*/*z* 143 frequently observed in TMS-derivatized, EI-mass spectra of 17-hydroxy, 17-methyl steroids arise from the D-ring of these steroids. But, an ion with the same elemental composition ($C_7H_{15}OSi$) was also verified to arise from the A-ring of TMS-derivatized 3-keto-enol steroids such as DHT. Based on studies employing HRMS, GC/MS/MS, analog steroids, and deuterium-labeling, the D-ring derived ion with *m*/*z* 143 was determined to be a resonance stabilized, allylic ion, having a 3-(*O*-trimethylsilyl)but-1-ene structure (**9**). The mechanism of formation for this ion is proposed (Scheme 1).

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