RESEARCH ARTICLE

Structure Elucidation of the Diagnostic Product Ion at m/z 97 Derived from Androst-4-en-3-One-Based Steroids by ESI-CID and IRMPD Spectroscopy

Mario Thevis,¹ Simon Beuck,¹ Sebastian Höppner,¹ Andreas Thomas,¹ Joseph Held,² Mathias Schäfer,² Jos Oomens,^{3,4} Wilhelm Schänzer¹

¹Institute of Biochemistry–Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany

²Department of Chemistry, University of Cologne, Cologne, Germany

³FOM-Institute for Plasma Physics Rijnhuizen, Nieuwegein, The Netherlands

⁴University of Amsterdam, Amsterdam, The Netherlands

Abstract

Structure elucidation of steroids by mass spectrometry has been of great importance to various analytical arenas and numerous studies were conducted to provide evidence for the composition and origin of (tandem) mass spectrometry-derived product ions used to characterize and identify steroidal substances. The common product ion at m/z 97 generated from androst-4-ene-3-one analogs has been subject of various studies, including stable isotope-labeling and (high resolution/high accuracy) tandem mass spectrometry, but its gas-phase structure has never been confirmed. Using high resolution/high accuracy mass spectrometry and low resolution tandem mass spectrometry, density functional theory (DFT) calculation, and infrared multiple photon dissociation (IRMPD) spectroscopy employing a free electron laser, the structure of m/z 97 derived from testosterone was assigned to protonated 3-methyl-2-cyclopenten-1-one. This ion was identified in a set of six cyclic $C_6H_9O^+$ isomers as computed at the B3LYP/6-311++G(2d,2p) level of theory (protonated 3-methyl-2-cyclopenten-1-one, 2-methyl-2-cyclopenten-1-one and 2-cyclohexen-1-one). Product ions of m/z 97 obtained from MS² and MS³ experiments of protonated 3-methyl-2-cyclopenten-1-one, 2-methyl-2-cyclopenten-1-one, 2-cyclohexen-1-one, and testosterone corroborated the suggested gas-phase ion structure, which was eventually substantiated by IRMPD spectroscopy yielding a spectrum that convincingly matched the predicted counterpart. Finally, the dissociation pathway of the protonated molecule of testosterone to m/z 97 was revisited and an alternative pathway was suggested that considers the exclusion of C-10 along with the inclusion of C-5, which was experimentally demonstrated with stable isotope labeling.

Key words: Mass spectrometry, Anabolic steroids, Structure elucidation, Fragmentation

Dedicated to Professor Dr. Herbert Budzikiewicz.

Electronic supplementary material The online version of this article (doi:10.1007/s13361-011-0308-4) contains supplementary material, which is available to authorized users.

Correspondence to: Mario Thevis; e-mail: m.thevis@biochem.dshs-koeln.de

Introduction

Steroidal hormones have been of great importance in the diagnosis and treatment of a multitude of health conditions, and so-called steroid profiles have been subject

Received: 20 September 2011

Revised: 7 November 2011

Accepted: 24 November 2011

Published online: 16 December 2011

of research and routinely applied clinical analyses for several decades. While the seminal studies by Shackleton and co-workers in 1968 were conducted by means of thin-layer chromatography and direct reflectance densitometry, requiring a full week of manpower to analyze six urine specimens [1], the use of column chromatography [2–6] and, eventually, gas chromatography/mass spectrometry (GC-MS) [7–9] as well as liquid chromatography/(tandem) mass spectrometry [LC-MS/(MS)] [10] provided the speed, sensitivity, and robustness to establish test methods for blood and urine steroid analysis for clinical, forensic, and doping control purposes [11, 12]. Due to the importance of unambiguous identification and determination of the target compounds, exhaustive mass spectrometric studies were conducted concerning structure elucidation and characterization of dissociation patterns of steroidal compounds using electron ionization [13] and electrospray ionization (ESI) with collision-induced dissociation (CID) [14–18].

Being the primordial matter of all androgens and synthetic analogs, testosterone (Figure 1) was one of the first and most comprehensively studied steroids using ESI-MS/(MS). Stable isotope labeling at various positions of the steroidal skeleton with ^2H and ^{13}C and high resolution/high accuracy tandem mass spectrometry were employed to determine the origin of the most abundant product ions of the 3-keto-4-ene-based steroid, which were observed at m/z 97 and 109 (Figure 1). The composition of both ions from A- and B-ring-derived carbons, hydrogens, and oxygen was established in one of the earlier studies [14] as indicated for m/z 97 in Figure 1. Their diagnostic character for steroid structure assignment was outlined in subsequent studies [15, 18], and a possible fragmentation route for the product ion at m/z 97 as well as gas-phase ion structure was suggested [18]. However, a systematic study corroborating the gas-phase ion structure, particularly of m/z 97 (the generation of which requires several consecutive or simultaneous bond cleavages

and rearrangements), was missing. In this communication, high resolution/high accuracy tandem mass spectrometry, density functional theory (DFT) computation, and infrared multiple photon dissociation (IRMPD) spectroscopy employing a free electron laser were used to elucidate the structure of the diagnostic ion of testosterone at m/z 97, observed with most 3-keto-4-ene-derived steroidal analytes.

Experimental

Chemicals and Reference Material

Testosterone (>98%), 2-cyclohexen-1-one ($\geq 95\%$), 3-methyl-2-cyclopenten-1-one (97%), 2-methyl-2-cyclopenten-1-one (98%), formic acid ($\geq 98\%$), propionic acid $^{13}\text{C}_3$ (99%), 3-buten-2-one (99%), thionyl chloride (99%), and glutaric acid (99%) were purchased from Sigma-Aldrich (Deisendorf, Germany). Acetonitrile and water (ULC/MS grade) were from Biosolve, (Valkenswaard, The Netherlands).

Stock and Working Solutions

Stock solutions of testosterone, 2-cyclohexen-1-one, 3-methyl-2-cyclopenten-1-one, and 2-methyl-2-cyclopenten-1-one were prepared at 1 mg/mL in acetonitrile. Working solutions were prepared at 1 $\mu\text{g/mL}$ in water/acetonitrile (1:1, vol:vol) containing 0.1% formic acid.

Synthesis of $^{13}\text{C}_2$ -Labeled 8 α -Methyl-3,4,8,8 α -Tetrahydro-1,6(2H,7H)-Naphthalenedione

Stable isotope-labeled 8 α -methyl-3,4,8,8 α -tetrahydro-1,6(2H,7H)-naphthalenedione was accomplished using established methods [19, 20]. In brief, 2,6- $^{13}\text{C}_2$ -labeled 2-methyl-1,3-cyclohexadione was prepared from glutaric acid and $^{13}\text{C}_3$ -propionic acid. By a Michael addition of the labeled 2-

Figure 1. ESI product ion mass spectrum of the protonated molecule $[M + H]^+$ at m/z 289 of testosterone (recorded on an AB SCIEX TripleTOF 5600 with a collision offset voltage of 30 V)

methyl-1,3-cyclohexadione with 3-buten-2-one followed by cyclization under acidic conditions, the introduction of isotopic labels at distinct positions (8a and 11) of 8a-methyl-3,4,8,8a-tetrahydro-1,6(2*H*,7*H*)-naphthalenedione was achieved, resembling major features of the A/B-ring of testosterone and related substances.

Electrospray Ionization (Tandem) Mass Spectrometry [ESI-MS(/MS)]

High resolution/high accuracy ESI-MS(/MS) was conducted using an AB SCIEX TripleTOF 5600 instrument (Darmstadt, Germany) equipped with a DuoSpray ion source operated at 25 °C and 5000 V. The mass spectrometer was calibrated using the manufacturer's protocol allowing for mass accuracies < 3 ppm for the period of analysis. A minimum of 50 spectra was averaged to calculate the accurate masses of target ions. The declustering potential was set to 50 V and collision offset voltages were optimized to retain approximately 30% of relative abundance of the precursor ion. Nitrogen was used as collision and curtain gas supplied by a CMC nitrogen generator (CMC Instruments, Eschborn, Germany). Working solutions of the analytes were introduced via a syringe pump at a flow rate of 10 $\mu\text{L}/\text{min}$.

Analyses requiring MS³ experiments were conducted on an AB SCIEX 5500 QTrap mass spectrometer employing a Turbo V ion source at 25 °C and 5000 V. Also here, the working solutions were infused by means of a syringe pump at 10 $\mu\text{L}/\text{min}$. Collision and excitation energies were adjusted to provide comprehensive information on product ions as well as maintaining at least 10% (relative abundance) of the respective precursor ion. Collision gas was nitrogen, provided by the same CMC N₂-generator as mentioned above.

Infrared Multiple Photon Dissociation (IRMPD) Experiments

A 4.7 T Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer described in detail elsewhere was used for the IRMPD experiments [21–23]. Tunable radiation for the photodissociation experiments was generated by the free electron laser for infrared experiments (FELIX) [24]. For the present experiments, spectra were recorded over the wavelength range from 800 to 1800 cm^{-1} . Pulse energies were around 40 mJ per macropulse of 5 μs duration, decreasing to about 20 mJ toward the blue edge of the scan range. The full-width-at-half-maximum (FWHM) bandwidth of the radiation was typically 0.5% of the central wavelength. The m/z 97 product ion was produced from protonated testosterone (m/z 289) by CID in the transfer region of the Micromass Z-Spray electrospray source using a nozzle-skimmer potential difference of about 60 V. A solution of testosterone in methanol ($c=10^{-3}$ M) was introduced via a syringe pump at a flow rate of 15 $\mu\text{L}/\text{min}$ and the electrospray needle was generally held at a voltage of +3.2

kV. Ions were accumulated in a hexapole trap for about 4 s before being injected into the ICR cell via an octopole ion guide. Electrostatic switching of the dc bias of the octopole captures the ions in the ICR cell without the use of a gas pulse, avoiding collisional heating [25]. The ion at m/z 97 was irradiated for 3 s, corresponding to interaction with 15 macropulses of FELIX. The fragmentation reactions induced by IRMPD of the m/z 97 ion were observed to be analogous to those found upon low energy CID (see Figure 3a). The IRMPD spectrum was recorded by monitoring the most abundant product ions at m/z 67, 77, and 79 and the depletion of the precursor ion (m/z 97) over the 800–1800 cm^{-1} range. The IRMPD yield was determined from the precursor ion (I_P) and the product ion ($\Sigma I_{\text{product ions}}$) intensities after laser irradiation at each frequency:

$$\text{IRMPD yield} = \Sigma I_{\text{product ions}} / (I_P + \Sigma I_{\text{product ions}}) \quad (1)$$

The yield was normalized linearly for fluctuations in the laser power [26]. Deviations in relative band intensities may nonetheless occur due to non-linear effects in the excitation process and/or due to extensive depletion of the ion cloud on strong absorption bands (saturation) [27].

Computational Methods

Only cyclic candidate structures of the product ion at m/z 97 were used for computational analysis as stable isotope labelling and tandem mass spectrometry experiments suggested the exclusion of linear C₆H₉O⁺ isomers. DFT computations yielded the set of gas-phase ion structures of m/z 97 shown in Figure 2. Geometry optimized structures reported are derived from the resulting low energy local minima. All electronic structure calculations were carried out with the Gaussian 03 program package (rev. E.01; Wallingford, CT) using the B3LYP density functional [28] with the 6-311++G(2d,2p) basis set for all atoms. Harmonic frequency calculations verified that all structures corresponded to local minima on the potential energy surface (PES) and provided zero-point energies (ZPE).

Relative energies of the three pairs of strongly related isomeric structures differing in the orientation of the proton at the oxygen are reported. The resulting converged local geometries were used to obtain harmonic frequencies. The well-established overestimation of these frequencies by theory was corrected using the standard scaling approach [29, 30] with a scaling factor of 0.98, which was found to be best suited for the level of theory applied [26, 31]. Spectra were generated by using calculated IR intensities broadened by a Lorentzian of width 10 cm^{-1} . The computed spectra were compared with the experimental IRMPD spectrum of m/z 97 generated by in-source CID of the protonated testosterone molecular ion at m/z 289 in order to make a structural assignment.

Figure 2. Candidate structures of O-protonated gas-phase ions for m/z 97 identified by theory. Relative energies found by DFT identify the conformers (a), (c), and (e) shown in the middle column to be the minimum structures of the three pairs of isomers

Results and Discussion

Understanding the collisionally activated dissociation processes of gas-phase ions and subsequent formations of product ions is of particular importance in forensic and doping control analysis in order to unambiguously characterize target compounds and their ‘designer’ derivatives by mass spectrometry-based approaches. Steroidal agents have been of growing concern due to the enormous variety of analogs being presumably and reportedly misused, which share (among the 3-keto-4-ene derived steroids) the product ion at m/z 97. Detailed studies employing different stable isotope labels within the testosterone molecule as well as ESI using D_2O demonstrated the incorporation of C-1–C-4, and C-19, inclusive of all corresponding hydrogen atoms [14, 15]. In addition, the presence of the oxygen at C-3 was confirmed by high-resolution MS analysis substantiating the sum formula of $C_6H_9O^+$ of m/z 97, the inclusion of C-10 and the exclusion of C-5 was assumed, and a

tentative dissociation pathway leading to the product ion was suggested [18]. The need, however, to rearrange/cleave three C–C bonds without involving adjacently located hydrogen atoms (or at least without transferring those from the charge-retaining moiety to the leaving group) represents a complex phenomenon, which the current study aims to shed light on by elucidating the gas-phase ion structure of the final product with a mass-to-charge ratio of 97.

Electrospray Ionization (Tandem) Mass Spectrometry [ESI-MS(/MS)]

Low and high resolution MS^n experiments were conducted using testosterone as a representative of steroids with a 3-keto-4-ene nucleus to generate the authentic ion at m/z 97 (Figure 1). Further fragmentation of m/z 97 using the linear ion trap device of the QTrap system yielded the MS^3 spectrum depicted in Figure 3a, and

Figure 3. Product ion spectra obtained from precursors at m/z 97 resulting from (a) CID of $[M + H]^+$ at m/z 289 of testosterone (MS³), (b) $[M + H]^+$ of 3-methyl-2-cyclopenten-1-one (MS²), (c) $[M + H]^+$ of 2-methyl-2-cyclopenten-1-one (MS²), and (d) $[M + H]^+$ of 2-cyclohexen-1-one (MS²). All spectra were recorded under identical CID conditions

accurate masses of resulting product ions were obtained from pseudo-MS³ experiments conducted by nozzle-skimmer dissociation and subsequent MS/MS QqTOF analysis (Table 1). In addition to the testosterone product ion at m/z 97, the protonated molecules of 3-methyl-2-cyclopenten-1-one, 2-methyl-2-cyclopenten-1-one, and 2-cyclohexen-1-one were subjected to identical conditions of collision-induced dissociation (CID), the product ion spectra of which are shown in Figure 3b–d, respectively.

The dissociation of m/z 97 derived from testosterone (Figure 3a) yields a product ion pattern identical to that obtained from the protonated 3-methyl-2-cyclopenten-1-one (Figure 3b) in quality (presence of ions) and quantity (relative abundances). The release of a methyl radical (15 u) is observed producing m/z 82 with $C_5H_6O^+$ presumably constituting the radical cation of cyclopentenol, which supports the proposed presence of the methyl group (C-19) in the precursor ion. Further intense product ions are generated at m/z 79 and 77 by the consecutive losses of water (18 u) and hydrogen (2 u) from the precursor ion species (m/z 97), which are suggested to represent the cation of 5-methyl-cyclopenta-1,3-diene (m/z 79) and the consecutively formed benzene cation (m/z 77) as corroborated by accurate mass determination and MSⁿ experiments (Table 1). Moreover, the elimination of carbon monoxide (28 u) from m/z 97 was found to yield the low-abundance product ion at m/z 69, possibly forming the cation of methyl-cyclobutane that further releases hydrogen (2 u) to yield m/z 67. Another minor product ion is observed at m/z 53, which is suggested to be composed of the carbon atoms 1, 2, 10, and 19, giving rise to the cation of buta-1,3-diene. Finally, two product ions were detected in QqTOF analyses at m/z 55 and 53 (data not shown) with accurate masses defining the elemental compositions of $C_3H_5O^+$ and $C_3H_3O^+$, respectively, which are suggested to comprise C-1–C-3 and produce protonated propenal and propynal. A summary of the proposed dissociation pathway is presented in Scheme 1. All of these product ions derived from testosterone's m/z 97 are also found in spectra recorded from the protonated molecules of 2-methyl-2-cyclopenten-1-one and 2-cyclohexen-1-one (Figure 3c and d, respectively) with small but significant differences in relative ion abundances, particularly concerning the product ions at m/z 67, 69, 79, and 82. These differences indicate the diversity of the gas-phase ions and corroborate the assumption that the ion

Scheme 1. Proposed dissociation pathway of m/z 97

Figure 4. IRMPD-spectrum of the product ion at m/z 97 formed by ESI-in-source-CID of the testosterone molecular ion at m/z 289 compared with the computed spectra of potential candidate structures (see Figure 2) of protonated 3-methyl-2-cyclopenten-1-one (2a and 2b), 2-methyl-2-cyclopenten-1-one (2c and 2d) and of 2-cyclohexen-1-one (2e and 2f)

m/z 97 due to the considerable similarities of the computed spectra demonstrating only minor differences.

Considerations on the Generation of m/z 97

With the multi-disciplinary confirmation of the gas-phase ion structure of m/z 97 as protonated 3-methyl-2-cyclopenten-1-one, revisiting the suggested formation of m/z 97 is possible. On the basis of stable isotope labeling and accurate mass

measurements, the presence of C-1–C-4, C-19, and the oxygen at C-3 including all corresponding hydrogen atoms was demonstrated [14, 15]. However, evidence supporting the assumption that C-10 is incorporated while C-5 is not has never been provided. Complementary to the postulated dissociation route and ion formation pathway leading to the 3-hydroxy-1-methylcyclopent-2-en-1-ylum ion [18] an alternative fragmentation leading to m/z 97 is conceivable with C-10 being excluded and C-5 included in the final product ion. The

suggestion arises from considerations regarding the driving force(s) that enable the rearrangements leading to this characteristic and abundant ion such as charge delocalization and stabilization (resulting in energetically favored gas-phase ion structures).

Following protonation of the 3-keto function (Scheme 2a, **a**), the location of the charge at C-5 (Scheme 2a, **b**) is energetically equivalent enabling (or triggering) the migration of C-19 towards C-5 in a pinacol-like (Wagner-Meerwein) rearrangement as reported for steroidal compounds in solution many decades ago [32, 33]. The simultaneous shift of the C-5–C-6 bond to C-6–C-10 allows the retention of the charge in the established conjugated π -electron system and is suggested to generate a structure as proposed in Scheme 2a, **c**. The subsequent rearrangement of the C–C bond between C-1 and C-10 (generating a C-1–C-5 linkage) accompanied by the disintegration of C-10 (by transferring the electrons constituting the C-5–C-10 bond to C-10) results in the resonance structure of the observed product ion at m/z 97 (Scheme 2a, **d**). The corroboration of this suggested fragmentation pathway of androst-4-en-3-one-based steroids, however, required specific labeling of C-5 or C-10, which was accomplished for the model compound 8a-methyl-3,4,8,8a-tetrahydro-1,6(2*H*,7*H*)-naphthalenedione (Figure 5). Here, the methyl residue and its adjacent carbon atom (C-8a) were isotopically labeled, and the product ion at m/z 97 of the unlabeled analyte (Figure 5a) shifts to m/z 98 and 99 (Figure 5b) in approximately identical abundances. The intense presence of m/z 98.0682 ($^{12}\text{C}_5^{13}\text{CH}_3\text{O}$, error: 0.7 ppm) verifies the above mentioned hypothesis that the elimination of C-10 occurs during the formation of this product ion as the C-19

methyl residue was shown to remain in the product ion at m/z 97 in earlier deuterium labeling studies [14].

If the methyl residue does not migrate in the course of precursor ion dissociation allowing for the exclusion of C-10 in the resulting product ion, both labels must remain in m/z 97 causing a shift to m/z 99. As shown in Figure 5b, the ion at m/z 99.0715 ($^{12}\text{C}_4^{13}\text{C}_2\text{H}_5\text{O}$, error: 0.3 ppm) is in fact generated besides m/z 98 from the protonated precursor ion in equally high intensity, supporting the earlier described pathway and composition of m/z 97 [18]. Since this pathway was reported as tentative, the DFT data obtained from the present study were used to provide additional information and, based on these, an alternative route is depicted in Scheme 2b. Identical to the fragmentation suggested for m/z 98 (Scheme 2a), initial protonation and subsequent rearrangement is proposed to generate the ion Structure **b**. The subsequent rearrangement of the C-9–C-10 bond forming a C-9–C-5 linkage is suggested, accompanied by the simultaneous generation of a C-4–C-10 link under participation of the C-3=C-4 double bond (Scheme 2b, **e**). DFT geometry optimization calculations excluded the option to maintain the double bond structure between C-3 and C-4 and localize the charge at C-10 but postulated the energetically favored structure comprising a C-4–C-10 linkage. The elimination of the steroidal B-, C-, and D-ring structures including C-5 finally yields the 3-hydroxy-1-methylcyclopent-2-en-1-ylum ion (**f**) as a resonance structure of protonated 3-methyl-2-cyclopenten-1-one, the characterized product ion at m/z 97 (and its isotope-labeled analog).

Overall, the final structure(s) of m/z 97 proposed here are well in agreement with the ion structure suggested by Pozo et al. [18] and the fact that the dissociation route towards this

Scheme 2. Suggested dissociation routes of testosterone to yield the product ion at m/z 97. Isotopically labeled carbons in the model substance 8a-methyl-3,4,8,8a-tetrahydro-1,6(2*H*,7*H*)-naphthalenedione are indicated with an asterisk

Figure 5. ESI product ion mass spectra of $[M + H]^+$ of **(a)** m/z 179 of 8a-methyl-3,4,8,8a-tetrahydro-1,6(2H,7H)-naphthalenedione, and **(b)** m/z 181 of $^{13}C_2$ -8a-methyl-3,4,8,8a-tetrahydro-1,6(2H,7H)-naphthalenedione, recorded with a collision offset voltage of 20 V

characteristic and peculiar product ion of steroids bearing a 3-keto-4-ene structure is a complex phenomenon is reinforced. The new and complementary information obtained from the present study suggests the presence of (at least) two different pathways leading to m/z 97 from the protonated precursor ion of testosterone. While one of these dissociation routes necessitates the migration of C-19 (presumably to C-5), the other includes the rearrangement of those carbon bonds considered in the earlier proposal but under different mechanistic aspects, particularly concerning the formation of intermediates as supported by/deduced from DFT computational data.

Conclusions

Modern tools of analytical chemistry allow detailed insights into gas-phase dissociation processes, provide corroborating information on dissociation pathways and rearrangement

mechanisms, and enable the characterization of product ion structures. In the present study, the elucidation of structure and genesis of the diagnostic product ion at m/z 97 commonly produced by 3-keto-4-ene-based steroidal compounds, such as testosterone and its derivatives, was investigated using high resolution/high accuracy (tandem) mass spectrometry, MS^n experiments, stable isotope labeling, DFT computation, and IRMPD-spectroscopy. The combined information obtained from various experimental and in silico studies, the considerable abundance of the product ion combined with the unusual composition including C-1–C-4, C-19, and either C-5 or C-10 necessitated comprehensive and complementary data sets that eventually revealed the existence of at least two dissociation routes that both seem to lead to protonated 3-methyl-2-cyclopenten-1-one, presumably as (3-methylcyclopent-2-en-1-ylidene)oxonium ion, a product ion also known from early mass spectrometry studies on

steroids using electron ionization (EI) but there evidently resulting from the steroidal D-ring [34].

Acknowledgments

The authors acknowledge support for this study by the Federal Ministry of the Interior of the Federal Republic of Germany and the Manfred-Donike Institute for Doping Analysis (Cologne, Germany). M.S. thanks Dr. Britta Redlich and Dr. Giel Berden (FOM Institute) for kind and skilful assistance, the German Science Foundation (DFG) and the European Community for funding (FP7/2007-2013; grant no. 226716).

References

- Shackleton, C.H., Charro-Salgado, A.L., Mitchell, F.L.: Urinary neutral steroid profile analysis in adults and infants. *Clin. Chim. Acta* **21**, 105 (1968)
- Shackleton, C.H., Honour, J.W.: Simultaneous estimation of urinary steroids by semi-automated gas chromatography. Investigation of neonatal infants and children with abnormal steroid synthesis. *Clin. Chim. Acta* **69**, 267 (1976)
- Weykamp, C.W., Penders, T.J., Schmidt, N.A., Borburgh, A.J., van de Calseyde, J.F., Wolthers, B.J.: Steroid profile for urine: Reference values. *Clin. Chem.* **35**, 2281 (1989)
- Grondal, S., Eriksson, B., Hagenas, L., Werner, S., Curstedt, T.: Steroid profile in urine: A useful tool in the diagnosis and follow-up of adrenocortical carcinoma. *Acta Endocrinol. (Copenhagen)* **122**, 656 (1990)
- Malunowicz, E.M., Ginalska-Malinowska, M., Romer, T.E., Ruszczyńska-Wolska, A., Dura, M.: Heterogeneity of urinary steroid profiles in children with adrenocortical tumors. *Hormone Res.* **44**, 182 (1995)
- Vestergaard, P.: The analysis of urinary hormonal steroids. *Lipids* **15**, 710 (1980)
- Honour, J.W., Brook, C.G.: Clinical indications for the use of urinary steroid profiles in neonates and children. *Ann. Clin. Biochem.* **34**(Pt 1), 45 (1997)
- Kikuchi, E., Yanaihara, H., Nakashima, J., Homma, K., Ohigashi, T., Asakura, H., Tachibana, M., Shibata, H., Saruta, T., Murai, M.: Urinary steroid profile in adrenocortical tumors. *Biomed. Pharmacother.* **54** (Suppl 1), 194s (2000)
- Homma, K., Hasegawa, T., Nagai, T., Adachi, M., Horikawa, R., Fujiwara, I., Tajima, T., Takeda, R., Fukami, M., Ogata, T.: Urine steroid hormone profile analysis in cytochrome P450 oxidoreductase deficiency: Implication for the backdoor pathway to dihydrotestosterone. *J. Clin. Endocrinol. Metab.* **91**, 2643 (2006)
- Janzen, N., Peter, M., Sander, S., Steuerwald, U., Terhardt, M., Holtkamp, U., Sander, J.: Newborn screening for congenital adrenal hyperplasia: Additional steroid profile using liquid chromatography-tandem mass spectrometry. *J. Clin. Endocrinol. Metab.* **92**, 2581 (2007)
- Makin, H.L.J., Gower, D.B. (eds.): Steroid Analysis. Springer, Heidelberg - London - New York (2010)
- Thevis, M.: Mass Spectrometry in Sports Drug Testing—Characterization of Prohibited Substances and Doping Control Analytical Assays. Wiley, Hoboken (2010)
- Budzikiewicz, H., Djerassi, C., Williams, D.H.: Structure elucidation of natural products by mass spectrometry - Vol. II: Steroids, terpenoids, sugars, and miscellaneous classes. Holden-Day, Inc, San Francisco (1964)
- Williams, T.M., Kind, A.J., Houghton, E., Hill, D.W.: Electrospray collision-induced dissociation of testosterone and testosterone hydroxy analogs. *J. Mass Spectrom.* **34**, 206 (1999)
- Thevis, M., Opfermann, G., Bommerich, U., Schänzer, W.: Characterization of chemically modified steroids for doping control purposes by electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **40**, 494 (2005)
- Thevis, M., Schänzer, W.: Mass spectrometric analysis of androstan-17 β -ol-3-one and androstadiene-17 β -ol-3-one isomers. *J. Am. Soc. Mass Spectrom.* **16**, 1660 (2005)
- Thevis, M., Schänzer, W.: Mass spectrometry in sports drug testing: Structure characterization and analytical assays. *Mass Spectrom. Rev.* **26**, 79 (2007)
- Pozo, O.J., Van Eenoo, P., Deventer, K., Grimalt, S., Sancho, J.V., Hernandez, F., Delbeke, F.T.: Collision-induced dissociation of 3-keto anabolic steroids and related compounds after electrospray ionization. Considerations for structural elucidation. *Rapid Commun. Mass Spectrom.* **22**, 4009 (2008)
- Eder, U.G., Sauer, U., Weichert, R.: Total synthesis of optically active steroids. 6. New type of asymmetric cyclization to optically active steroid CD partial structures. *Angew. Chem. Int. Ed.* **10**, 496 (1971)
- Schick, H., Lehmann, G., Hilgetag, G.: Notiz zur Synthese von 2-Methyl- und 2-Äthyl-cyclopentandion-(1,3). *Chem. Berichte* **102**, 3238 (1969)
- Dreiocker, F., Oomens, J., Meijer, A.J.H.M., Pickup, B.T., Jackson, R.F.W., Schäfer, M.: Structure elucidation of dimethylformamide-solvated alkylzinc cations in the gas phase. *J. Org. Chem.* **75**, 1203 (2010)
- Polfer, N.C., Oomens, J.: Reaction products in mass spectrometry elucidated with infrared spectroscopy. *[Berichte Der Bunsen-Gesellschaft] Phys. Chem. Chem. Phys.* **9**, 3804 (2007)
- Valle, J.J., Eyler, J.R., Oomens, J., Moore, D.T., van der Meer, A.F.G., von Helden, G., Meijer, G., Hendrickson, C.L., Marshall, A.G., Blakney, G.T.: Free electron laser-Fourier transform ion cyclotron resonance mass spectrometry facility for obtaining infrared multiphoton dissociation spectra of gaseous ions. *Rev. Sci. Instrum.* **76**, 023103 (2005)
- Oeppts, D., Van der Meer, A.F.G., Van Amersfoort, P.W.: The free-electron-laser user facility Felix. *Infrared Phys. Technol.* **36**, 297 (1995)
- Polfer, N.C., Oomens, J., Moore, D.T., von Helden, G., Meijer, G., Dunbar, R.C.: Infrared spectroscopy of phenylalanine Ag(I) and Zn(II) complexes in the gas phase. *J. Am. Chem. Soc.* **128**, 517 (2006)
- Drayss, M.K., Blunk, D., Oomens, J., Gao, B., Wyttenbach, T., Bowers, M.T., Schäfer, M.: Systematic Study of the Structures of Potassiated Tertiary Amino Acids: Salt Bridge Structures Dominate. *J. Phys. Chem.* **113**, 9543 (2009)
- Massah, A.R., Dreiocker, F., Jackson, R.F.W., Pickup, B.T., Oomens, J., Meijer, A.J.H.M., Schäfer, M.: Gas-phase study of new organozinc reagents by IRMPD-spectroscopy, computational modelling and tandem-MS. *[Berichte Der Bunsen-Gesellschaft] Phys. Chem. Chem. Phys.* **13**, 13255 (2011)
- Becke, A.D.: Density-functional thermochemistry. 3. The role of exact exchange. *J. Chem. Phys.* **98**, 5648 (1993)
- Irikura, K.K., Johnson, R.D., Kacker, R.N.: Uncertainties in scaling factors for ab initio vibrational frequencies. *J. Phys. Chem.* **109**, 8430 (2005)
- Foresman, J.B., Frisch, A.E.: Exploring Chemistry with Electronic Structure Methods. Gaussian, Inc., Pittsburgh (1996)
- Polfer, N.C., Oomens, J., Dunbar, R.C.: IRMPD spectroscopy of metal-ion/tryptophan complexes. *[Berichte Der Bunsen-Gesellschaft] Phys. Chem. Chem. Phys.* **8**, 2744 (2006)
- Lettré, H., Müller, M.: Einige Umsetzungen am Cholestantriol. *[Berichte der Deutschen Chemischen Gesellschaft]* **70**, 1947 (1937)
- Wendler, N.L.: Rearrangements in Steroids. In: de Mayo, P. (ed.) Molecular Rearrangements Part 1, p. 1020. Wiley-Interscience, New York (1963)
- Budzikiewicz, H.: Steroids. In: Waller, G.R. (ed.) Biochemical Applications of Mass Spectrometry, p. 251. John Wiley and Sons, New York (1972)