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Studies on the *in vivo* metabolism of Methylstenbolone and detection of novel long term metabolites for doping control analysis

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/dta.2736

Abstract

The anabolic-androgenic steroid methylstenbolone (MSTEN; $2\alpha,17\alpha$ -dimethyl- 17β -hydroxy- 5α -androst-1-en-3-one) is available as a so-called designer steroid or nutritional supplement. It is occasionally detected in doping control samples, predominantly tested and confirmed as the glucuronic acid conjugate of MSTEN. The absence of other meaningful metabolites reported as target analytes for sports drug testing purposes can be explained with the advertised metabolic stability of MSTEN.

In 2013, a first investigation into the human metabolism of MSTEN was published, and 2 hydroxylated metabolites were identified as potential targets for initial testing procedures in doping controls. These metabolites were not observed in recent doping control samples that yielded adverse analytical findings for MSTEN, and in the light of additional data originating from a recent publication on the *in vivo* metabolism of MSTEN in the horse, revisiting the metabolic reactions in humans appeared warranted. Therefore, deuterated MSTEN together with hydrogen isotope ratio mass spectrometry (IRMS) in combination with high accuracy/high resolution mass spectrometry were employed. After oral administration of a single dose of 10 mg of doubly labeled MSTEN, urine samples were collected for 29 days.

Up to 40 different deuterated MSTEN metabolites were detected in post-administration samples, predominantly as glucuronic acid conjugates, and all were investigated regarding their potential to prolong the detection window for doping controls. Besides MSTEN excreted glucuronidated, three additional metabolites were still detectable at the end of the study on day 29. The most promising candidates for inclusion into routine sports drug testing methods ($2\alpha,17\alpha$ -dimethyl- 5α -androst-1-ene- $3\beta,17\beta$ -diol and $2\alpha,17\alpha$ -dimethyl- 5α -androst-1-ene- $3\alpha,17\beta$ -diol) were synthesized and characterized by NMR.

Keywords

Methylstenbolone, hydrogen isotope ratio mass spectrometry, high resolution-high accuracy mass spectrometry, human metabolism, doping control

Introduction

Anabolic-androgenic steroids like Methylstenbolone (MSTEN; $2\alpha,17\alpha$ -dimethyl- 17β -hydroxy- 5α -androst-1-en-3-one) are prohibited in sports according to the regulations of the

World Anti-Doping Agency (WADA).[1] Despite an increasing number of anti-doping legislations in numerous countries, a decrease of the trafficking of steroidal drugs (as well as other doping agents) is barely noticeable and they are readily available *via* the World Wide Web. For many years, anabolic agents have been among the most frequently detected banned substances in sport.[2]

In doping control analysis, the targeting of urinary metabolites of administered compounds is frequently the superior strategy over aiming at detecting the intact and unmodified compound itself. Therefore, comprehensive investigations of phase-I- and phase-II-metabolic reactions of doping agents have contributed to an improved detectability in numerous cases.

Regarding MSTEN, so far only one study reported on the human *in vivo* metabolism, and two hydroxylated urinary metabolites were described.[3] Both metabolites were detectable in urine for a longer time period (up to 7 days) than MSTEN itself. These results however did not reflect recent findings in doping control samples in which MSTEN glucuronide but not the reported hydroxylated metabolites were detected. Also, additional hydroxylated metabolites identified in *in vitro* and humanized mouse metabolism studies on MSTEN were not directly applicable to sports drug testing.[4] In a recent investigation focusing on the metabolism of MSTEN in the horse, several metabolites were described that were detectable for a longer time period than MSTEN itself.[5] Based on these results, revisiting the metabolism of MSTEN in humans seemed advisable in order to elucidate the found discrepancies between described and observed urinary metabolites and to facilitate an improved detection of this banned steroid in doping control analysis.

In order to generate a comprehensive profile of urinary MSTEN metabolites, a recently established approach utilizing hydrogen isotope ratio (HIR) determinations was applied.[6-9] Therefore, two-fold deuterated MSTEN was administered to one healthy male volunteer, and collected urine samples were processed and analyzed using gas chromatography/thermal conversion/isotope ratio mass spectrometry (GC/TC/IRMS) to detect urinary steroids comprising at least one deuterium atom. All identified metabolites were further analyzed by gas chromatography/high resolution-high accuracy/mass spectrometry (GC-HRMS) to validate their deuterium content and to elucidate structural characteristics.

Promising metabolite candidates were investigated regarding their potential to improve and prolong the traceability of MSTEN administrations. In order to corroborate the utility of newly identified metabolites for doping controls, an excretion study was conducted using unlabeled MSTEN, and urine samples were analyzed under authentic routine sports drug testing

conditions, complemented by the herein discovered, synthesized, and additionally NMR-characterized metabolites.

Experimental

Reagents and chemicals

Pyridine, glacial acetic acid, sodium hydroxide, methanol, sulfuric acid, acetonitrile (ACN), tert-butyl methyl ether (TBME) and cyclohexane were purchased from Merck (Darmstadt, Germany). Acetic anhydride was from Fluka/Sigma-Aldrich (Steinheim, Germany). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Chemische Fabrik Karl Bucher (Waldstetten, Germany). All reagents and chemicals were of analytical grade. β -glucuronidase from *Escherichia coli* was from Roche Diagnostics GmbH (Mannheim, Germany).

Deuterated MSTEN (4,4-²H₂-MSTEN, chemical purity > 98%) was synthesized and purified by high performance liquid chromatography (HPLC) in-house employing established protocols.[10,11] Non-deuterated MSTEN was purchased from Toronto Research Chemicals (North York, ON, Canada). Methasterone (2 α ,17 α -dimethyl-5 α -androstane-17 β -ol-3-one) was from Steraloids (Newport, RI, USA) and lithium aluminium hydride (LiAlH₄) from Sigma (Deisendorf, Germany).

Elimination studies

Deuterated MSTEN was administered orally (10 mg, dissolved in ethanol/water 30/70 v/v) to one healthy male volunteer (43 years old, 180 cm tall with a weight 84 kg) who declared not to have used any nutritional supplements or medication at least 4 weeks prior to this study. Three blank morning urine samples were collected the days before the administration, followed by the collection of all urine samples during the first 48 h. Additionally, all morning urines were sampled until day 6 post-administration, followed by one morning urine sample every other day until day 29.

Six months later, the same volunteer orally administered 20 mg of non-deuterated MSTEN, again dissolved in ethanol/water 30/70 v/v. Also here, three blank urine samples were collected before administration, and all morning urine specimens were collected for the next 27 days,

followed by collections of morning urine samples every other day until day 39. Also, an additional set of elimination study urine samples accounting for a post-administration time of 64 h was available, originating from an earlier research project. The samples were obtained post-administration of 20 mg of MSTEN by one healthy male volunteer (age: 59 years). All specimens were stored frozen at -20°C until analysis. All studies were approved by the Ethical Committee of the National Institute of Sports of Romania (Bucharest, Romania, approval #2283/2016) and written informed consent was provided by the volunteers.

Sample preparation for HIR determinations

The different steps in sample preparation for all specimens collected during the elimination study with deuterated MSTEN are described in detail elsewhere.[12-15] In brief: Twenty milliliters of urine are applied onto a preconditioned solid-phase extraction (SPE) cartridge (Chromabond[®] C18, Macherey-Nagel, Düren, Germany), washed with water and eluted with methanol. After evaporation, the dried residue is reconstituted in 2 mL of aqueous phosphate buffer (pH 7) and extracted with TBME to yield the unconjugated steroid fraction. To the aqueous residue, β -glucuronidase is added, and after 60 min at 50°C an aqueous potassium carbonate buffer (pH 10) is added to allow for partitioning of the formerly glucuronidated steroids into TBME. The aqueous layer is subsequently acidified and applied onto another SPE cartridge followed by acidic solvolysis, employing sulfuric acid in ethyl acetate. Finally, the formerly sulfoconjugated steroids are extracted.

The fraction of unconjugated steroids is immediately forwarded to HIR determinations while the formerly glucuronidated and sulfated steroids are subjected to high performance liquid chromatographic clean-up. This is accomplished using an Agilent 1100 HPLC system (Waldbronn, Germany) equipped with a XBridge[™] Shield RP18 5 mm (4.6 x 250 mm) column, protected by a XBridge[™] Shield RP18 5 mm (4.6 x 20 mm) guard column from Waters (Eschborn, Germany). Solvents used for gradient elution are A: water and B: ACN. The gradient starts from 20 % ACN, increases to 100 % ACN within 25 min and is held for 5 min followed by re-equilibration for 5 min at 20 % ACN. The flow is set to 1 mL/min and the injection volume is 100 μL . Fractions are collected using a FOXY R1 automatic fraction collector (Axel Semrau, Sprockhövel, Germany) from 4.00 to 10.00, from 10.01 to 13.00, from 13.01 to 16.00, from 16.01 to 18.00, from 18.01 to 20.00, from 20.01 to 24.00 and from 24.01 to 30.00 min.

All fractions are evaporated and trimethylsilylated using MSTFA in ethyl acetate (20/80, v/v) at 70°C for 45 min.[16] After evaporation, samples are transferred into auto-sampler vials using 200 µL of ethyl acetate, dried in a desiccator, and reconstituted as necessary for IRMS or HRMS determinations.

Gas chromatography/isotope ratio mass spectrometry setup

The instrumentation used for all HIR determinations was a Delta V Plus IRMS (Thermo Fisher Scientific, Bremen, Germany) coupled to a Trace GC 1310 equipped with a TriPlusRSH Autosampler via the GC IsoLink CNH and the ConFlow IV (all Thermo). The thermal conversion ceramic tubing (320 mm length, 0.5 mm inner diameter, 1.5 mm outer diameter from IVA Analysentechnik, Meerbusch, Germany) was operated at 1450°C for pyrolysis. The IRMS was hyphenated to an ISQ mass spectrometer (Thermo) operated in positive electron ionization mode recording total ion chromatograms from m/z 50 to 800.

The GC column used was a HP-Ultra 1 column (length 17 m, i.d. 0.2 mm, film thickness 0.11µm) from Agilent (Waldbronn, Germany). The temperature program started at 100°C held for 2 min, then with 30°C/min to 184°C, then with 6°C/min to the final temperature of 310°C which was held for 3.5 min. Injections were done in splitless mode at 300°C using the programmed flow option starting with 4 mL/min of helium (purity 5.0), held for 1.5 min and then changed with a speed of 10 mL/min to 1.2 mL/min for the analytical run. The injection volume was 4 µL of ethyl acetate. Data was acquired using either Isodat 3.0 or Xcalibur 2.2 (both Thermo).

Gas chromatography/high resolution mass spectrometry

In order to presumably identify the structure and the deuterium content of detected metabolites, all HIR samples were diluted by a factor of 5 to 20 and re-injected on an Agilent 7200 Accurate-Mass Q-TOF system coupled to an Agilent 7890A gas chromatograph (Santa Clara, CA). The GC column and parameters were equivalent to those described above, the injection volume was reduced to 2 µL and the Pulsed Splitless option was chosen with an initial pressure of 40 psi. Data covering a scan range from m/z 50 to 800 with a scan speed of 333 ms/spectrum was acquired and evaluated using MassHunter software (version B.06, Agilent). The instrument was mass calibrated prior to each sequence in order to achieve mass accuracy in the range of ± 5 ppm.

Additional studies to improve the identification and detectability of those metabolites important for doping control analysis were conducted on a Q Exactive GC Orbitrap GC-MS/MS system (Thermo) under routine doping control conditions. In a first step, selected samples were re-prepared as described above employing the HPLC clean-up but subsequently derivatized using MSTFA/NH₄I/ethanethiol 1000:2:3 (v:w:v).[17] Finally, sample preparation was conducted according to routine analytical conditions to demonstrate the feasibility to directly implement the most promising target analytes.[18,19] Also here, the GC was equipped with a HP-Ultra 1 analytical column, but the temperature program was slightly different. The initial temperature of 180°C was increased with 3°C/min to 240°C and then with 40°C/min to 320°C and held for 2 min. Injections were performed in split mode at 300°C with a split and purge flow of 5 mL/min each. Constant flow was set to 1 mL/min and transfer line temperatures were at 280 and 300°C. Data was acquired in Full MS mode covering a scan range of *m/z* 50 to 700 or in Parallel Reaction Monitoring using an isolation window of *m/z* 1.3. Resolution was set to 60.000 and data was evaluated using Xcalibur (Version 4.0, Thermo). Daily mass calibration of the instrument yielded mass accuracy in the range of ± 2 ppm.

Synthesis of the new long-term metabolite

Ten milligram of MSTEN were dissolved in 2 mL of tetrahydrofuran (THF). Then, 200 µL of a solution of lithium aluminum hydride (1 M in THF) was added dropwise. The mixture was vortexed and after 10 min at room temperature, 5 mL of water were added and reaction products were liquid-liquid-extracted using twice 5 mL of TBME. After evaporation, the dried residue was reconstituted in 2 mL of ACN/water (50/50) and forwarded to HPLC clean-up on the above mentioned system. The employed gradient was modified to start with 60 % ACN, increasing to 80 % ACN in 10 min, then to 100 % ACN in 0.1 min, where it was held for 5 min prior to re-equilibration for 5 min at starting conditions. The injection volume was reduced to 50 µL to prevent column overload. Besides the fraction containing MSTEN (collection window from 8.21 to 9.00 min), 2 fractions containing metabolites were collected (from 7.10 min to 7.70 min and from 7.71 min to 8.20 min) and forwarded to nuclear magnetic resonance (NMR) spectroscopy.

Nuclear magnetic resonance spectroscopy

Prior to NMR analysis, the reduced MSTEN products were subjected to another LC fractionation step using an Agilent (Waldbronn, Germany) 1100 Series HPLC equipped with a Macherey-Nagel (Düren, Germany) Nucleodur C18 Gravity column (250 x 4.0 mm, 5 µm particle size). The eluents used were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Gradient elution was conducted starting at 20% B for 1 min, increasing to 95% B within 25 min, maintaining 95% B for 10 min followed by re-equilibration at 0% B. The flow rate of 1 mL/min was directed to an Agilent 1260 Fraction Collector (Waldbronn, Germany). Time-based fractions of 6 s were collected. The assessment of the fractions by LC/HRMS, were performed on a Q Exactive mass spectrometer, (Thermo, Bremen, Germany). The substance of interest was detected at a retention time at 17.50 min and 5 fraction á 6 s have been collected. The fractions was dried in a vacuum concentrator, and the sample was dissolved and transferred into the NMR tube using 50 µL of deuterated chloroform (CDCl₃). To address the analytical questions, ¹³C, ¹H, ¹H,¹H-COSY, ¹³C-¹H-HSQC, ¹³C-¹H-HMBC, and NOESY experiments were conducted utilizing a BRUKER AVANCE NEO 800 MHz NMR spectrometer (Bruker, Karlsruhe, Germany).

Results and discussion

Urinary MSTEN metabolites detected by HIR

The employed untargeted metabolite identification approach allows for the sensitive detection of those analytes that retain at least one of the drug's deuterium atoms during metabolic conversion, as long as the metabolites' recovery from urine is ensured by the applied sample preparation and derivatization technique. Furthermore, the physico-chemical properties of the analyte needs to be compatible with gas chromatographic analyses. In Figure 1, the GC/TC/IRMS results of HPLC fractions collected before and after the administration of deuterated MSTEN are shown. Due to the very low natural abundance of deuterium compared to hydrogen, the IRMS is very sensitive to excess deuterium if the instrument is operated under conditions dedicated to the determination of natural HIR.

Especially at the beginning of the excretion study, glucuroconjugated MSTEN represents one of the most abundant metabolites together with several hydroxylated metabolites excreted into

urine as unconjugated or glucuronidated metabolic products. After approximately 5 days, peaks are still visible in the HIR chromatograms close to the detection limit.

As visible in Figure 1 (lower part, zoom area), the isotope ratio of pregnanediol (PD) seems to be influenced by the administration of MSTEN, too. This is however not attributed to the metabolic conversion of MSTEN to PD but to an artefact of the measurement. During the thermal conversion process, deuterium atoms can be absorbed by the heated aluminum tubing and released when the subsequently eluting compound exiting the GC column is pyrolyzed. Therefore, confirmation of the deuterium content of signals identified in the HIR chromatogram by GC-HRMS under comparable chromatographic conditions is particularly important in order to identify and exclude such analytical artefacts. In the present study, over 40 metabolites were confirmed to contain at least 1 deuterium atom.

MSTEN metabolites suitable for doping control analysis

As the applied derivatization technique (MSTFA in ethyl acetate) during the first step of metabolite detection was not in accordance with the usually applied routine doping control methods, samples were re-prepared in agreement with routine protocols.

At first, besides changing the derivatization technique, all sample preparation steps were kept including the HPLC fractionation-based target analyte purification. The partitioning of unconjugated steroids was omitted as no long term metabolites were expected to exist in this fraction. All metabolites found at this stage of the study are summarized in Table 1.

In agreement with literature data on human and especially horse metabolism, the majority of MSTEN metabolites was found to be hydroxylated (24 out of 40 metabolites.[3,5] Regarding phase-II-metabolism, 25 metabolites were excreted glucuronidated and only 6 metabolites were found simultaneously as both glucurono- and sulfo-conjugated analytes.

Comparing the order of elution and mass spectra, the herein observed metabolites 14 and 18 were assigned to urinary metabolites of MSTEN reported by Calvacanti *et al.*[3] Unfortunately, applying a similar approach concerning the numerous metabolites identified in the horse proved considerably more complex as the gas chromatographic conditions differed substantially and the majority of metabolites was discussed based on liquid chromatography-mass spectrometry-derived data.[5] With a distinct loss of 103 Da under EI-MS conditions, one metabolite (number 5 in Table 1) showed similar features as the metabolite M1c tentatively characterized as 20-hydroxymethylstenbolone by Choi *et al.* [5] At close scrutiny of the corresponding mass spectrum of metabolite 5, however, a structure of 20-

hydroxymethylstenbolone was not confirmed in the present study as depicted in Figure 2. For both the deuterated and the non-deuterated metabolite, the characteristic fragment at m/z 143.0886 representing a diagnostic D-ring fragment composed of the carbons C-15-17 and C-20 plus the O-TMS moiety is still present, which precludes an additional hydroxyl group at C-20.[20] A C-18-hydroxylated long term metabolite as described for metandienone also showing the characteristic loss of 103 Da is not possible here either, as a 18-nor-steroid does not match the found elemental composition of $C_{21}H_{32}O_3$. [21] Taking into account the abundant A-ring fragments at m/z 168.0962 and 195.1196 (respectively at m/z 169.1026 and 196.1261 in the deuterated analogs) and especially the fragment at m/z 282.1465 (deuterated at m/z 283.1525) still comprising 2 TMS-moieties, the hydroxyl group is more likely to be located at C-19 or C-21 (*i.e.* the methyl group attached to C-2). Especially for 19-hydroxylated steroids, the loss of m/z 103 is well established.[22,23] With the data at hand, it is not possible to unequivocally identify the position of hydroxylation and further research will be required to clarify this aspect. The metabolite was visible up to 18 days post-administration application, but three other metabolites outperformed this result.

Methylstenbolone

The administered compound itself is mentioned to be particularly resistant concerning metabolic reactions, and taking into account the results of this study the introduction of an additional methyl group into the A-ring of the steroid indeed appears to affect several phase-I-metabolic routes usually observed in steroid metabolism.[24] After preparing 10 mL of urine and HPLC purification, the deuterated glucurono- and sulfoconjugated MSTEN (metabolites 22 and 39, respectively, Table 1) was visible until the end of the study, *i.e.* for 29 days after a single oral application. In Figure 3, the extracted ion chromatograms for m/z 461.3253 (\pm 10 ppm) are depicted. This finding is in good agreement with results obtained for routine doping control samples yielding AAFs based on the sole presence of (glucurono-conjugated) MSTEN. The discrepancy between the herein accomplished detection window and earlier published data might be explained when analyzing excretion study urine sample data collected after the administration of non-deuterated MSTEN. Here, samples were prepared strictly according to routine methods, *i.e.* without additional HPLC purification step. Employing high resolution mass spectrometry together with parallel reaction monitoring (PRM), MSTEN was detectable for 24 and 25 days as glucuronic acid conjugate and sulfate, respectively, after application of a single oral dose of the drug. Switching to low resolution triple quadrupole mass spectrometry however did not allow for mass-resolving co-elutions close to the retention time of MSTEN,

impeding the unambiguous identification of the target analyte at low urinary concentrations. Depending on the applied precursor/product ion pair, MSTEN was only visible for 10 to 16 days after application, regardless whether the glucuronide or the sulfate was investigated.

Novel reduced long term metabolites

The HPLC fraction containing MSTEN also furnished two additional peaks of different retention times and different relative intensities but highly comparable mass spectra. These analytes were detected, alongside MSTEN, until the end of the elimination study. The more abundant compound was referred to as Epi-NEW_LTM (Table 1, metabolites 23 and 40) and the less abundant metabolite was termed NEW_LTM (Table 1, metabolites 21 and 38), both detected with a difference in retention time of 1.3 min. The high resolution mass spectrum of Epi-NEW_LTM is shown in Figure 4. The accurate mass of m/z 464.3457 corresponds to the elemental composition of $C_{27}H_{48}^2H_2O_2Si_2$ (mass error = 2.7 ppm) and besides the consecutive losses of a methyl radical (15 u) and the TMSOH (90 u), a characteristic loss of $C_3H_6^2H$ (44 u) is observed. In the publication of Choi *et al.*, methasterone is described as one metabolite of MSTEN in the equine.[5] An initial comparison of the mass spectra obtained for methasterone and the new long-term metabolites yielded similar fragments, but the comparison of methasterone certified reference material and both metabolites resulted in differing retention times under the described chromatographic conditions.

Further investigations on the new metabolites encompassing derivatization with a mixture of MSTFA/ethyl acetate corroborated the presence of two hydroxyl functions. Both metabolites were obtained from MSTEN as a result of chemical reduction using $LiAlH_4$, suggesting the formation of $2\alpha,17\alpha$ -dimethyl- 5α -androst-1-ene- $3\alpha,17\beta$ -diol (NEW_LTM) and $2\alpha,17\alpha$ -dimethyl- 5α -androst-1-ene- $3\beta,17\beta$ -diol (Epi-NEW_LTM). This hypothesis was confirmed by NMR analysis of the synthesized products; in particular the NOESY contacts of the protons located at the carbons C-3, C-5, C-9 and C-14 on one side of the steroidal skeleton as well as the NOESY contacts between the protons at C-19, C-8 and C-18 on the other side of the ring structures corroborates the stereochemical configuration of the metabolites (Figure 5 and supplemental information). Interestingly, both metabolites were predominantly found in the urinary fraction of glucuronic acid conjugates. This is particularly noteworthy as steroids exhibiting a 3β -OH-functionality preferentially undergo sulfoconjugation. A potential explanation would be the directing of phase-II-metabolism towards position C-17 due the additional methyl group in position C-2. Another unexpected aspect concerns the detected relative urinary concentrations of the two metabolites. Throughout the entire elimination study,

the 3 β -epimer (Epi-NEW_LTM) is excreted at higher concentrations than the 3 α -epimer (NEW_LTM). In contrast to this, the endogenously produced androsterone (3 α -hydroxy-5 α -androstane-17-one) always prevails over epiandrosterone (3 β -hydroxy-5 α -androstane-17-one) in human urine and androsterone is excreted predominantly as glucuronide while epiandrosterone is exclusively found in the fraction of sulfoconjugated steroids.

In the second excretion study employing non-deuterated MSTEN, both metabolites proved to be suitable for sports drug testing. Regarding Epi-NEW_LTM, peaks are visible for 20 and 23 days (glucuronidated and sulfoconjugated, respectively) and NEW_LTM for 27 and 12 days (glucuronidated and sulfoconjugated, respectively). The results obtained for the glucuronidated NEW_LTM are depicted in Figure 6. Despite being excreted at lower urinary concentrations compared to Epi-NEW_LTM (found between 40 % and 70 % of the higher concentrated epimer), the detectability of this metabolite was found to be superior due to lower interferences originating from the biological matrix.

This finding was also corroborated by the third excretion study performed by a different individual, demonstrating good detectability of both NEW_LTM and Epi-NEW_LTM until the end of the study (64 h after administration of non-deuterated MSTEN).

Novel oxygenated long term metabolite

Among the numerous hydroxylated metabolites found in this study, only one candidate showed the potential to prolong the detection time (Table 1, metabolite 15, OXO_LTM) for MSTEN administrations. This metabolite, observed only as glucuronide, was also visible until the end of the study employing deuterated MSTEN. The high resolution mass spectrum of the per-TMS derivative (Figure 7, upper part) directly suggested a location of the hydroxyl function at a position other than C-16 as characteristic fragment ions reported for this metabolite in humans such as m/z 218 and m/z 231 were missing.[25] Besides usual fragment ions derived from demethylation or loss of TMSOH, abundant ions at m/z 314.2041 (C₂₀H₂₈²HOSi), 244.1265 (C₁₅H₁₈²HOSi), and especially 221.1332 (C₁₃H₁₉²HOSi) were found. As all these fragments still contain one deuterium atom and only one TMS-group is present, it is plausible that the steroidal A- and B-rings are included in these structures without being affected by hydroxylation. Further investigations employing MSTFA-derivatization of the hydroxyl functions only suggest the presence of a 16-oxo group. The mass spectra (Figure 7, lower part) of the mono-TMS derivative showed highly characteristic fragment ions at m/z 170.1090, 157.1024, 143.0512, and 130.0782, which were described in the literature as specific for 16-

oxo-17-methyl-steroids.[25] A peculiar fragment ion was found at m/z 319.2388, whose molecular formula of $C_{20}H_{31}^2H_2OSi$ was tentatively attributed to the consecutive eliminations of a methyl radical (15 u), carbon monoxide (28 u) presumably from the steroidal A-ring, and ketene (42 u) from the D-ring. Performing Q-TOF MS/MS experiments using the fragment at m/z 319.2388 as precursor ion demonstrated the absence of an oxygen atom in the fragments derived from the A-ring of the steroid, supporting the above-mentioned hypothesis regarding structural features of m/z 319.2388. The fragmentation of this metabolite was not further investigated as no reference material was synthesized. Synthesis was postponed after the results of the excretion study employing non-deuterated MSTEN showed a significant co-elution overlapping with the new metabolite, limiting its utility in routine doping controls.

Conclusion

The combination of hydrogen isotope ratio mass spectrometry with high resolution/high accuracy mass spectrometry was successfully applied to the investigation of MSTEN metabolism. Even though MSTEN appears to be comparably resistant to metabolic enzymes, up to 40 metabolites were identified. The metabolic inertness was reflected by the fact that deuterated MSTEN itself was detected as intact (whilst conjugated) drug until the end of the elimination study. Two novel long-term metabolites NEW_LTM and Epi-NEW_LTW exhibited similar detection windows (27 and 20 days respectively) for sports drug testing purposes, and especially NEW_LTM was found to represent a promising complement for doping controls. As Epi-NEW_LTM showed comparable detection windows, it should also be monitored taking into account the probability of matrix interferences on the one or the other target compound. The third new long-term metabolite OXO_LTM was found to be less suitable for doping control analysis as it was largely indistinguishable from an endogenous substance eluting at the same retention time under the chosen chromatographic conditions.

Acknowledgments

This project was partly conducted with support of the Manfred-Donike Institute for Doping Analysis (Cologne, Germany) and the Federal Ministry of the Interior, Building and Community (Berlin, Germany).

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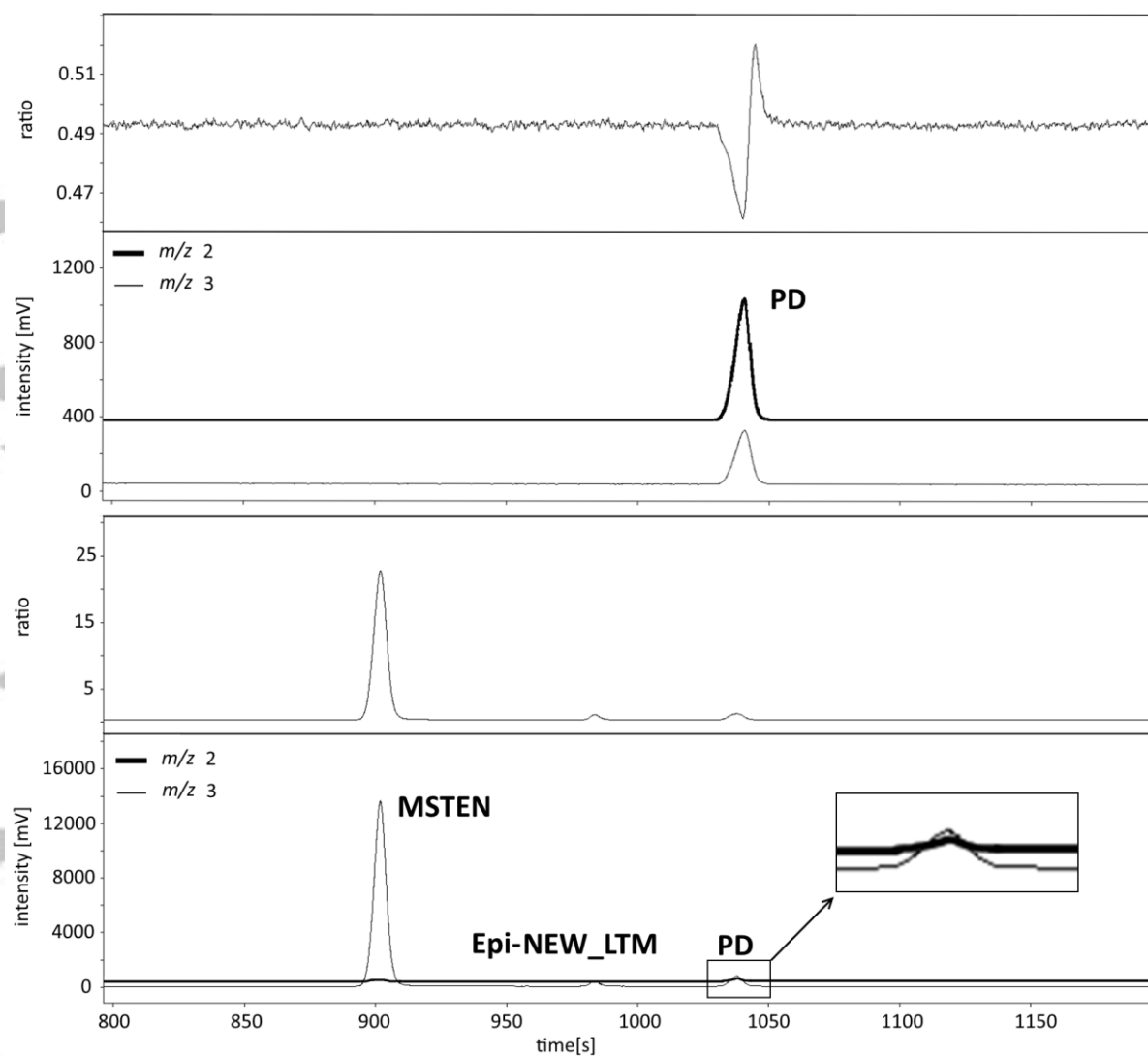
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Table 1: Confirmed deuterated metabolites found in urine after administration of 10 mg of deuterated MSTEN. G refers to metabolites excreted glucuronidated or unconjugated and S to sulfated metabolites. Metabolites marked with an asterisk were found in both fractions. The retention times are given for the routine set up employed on the GC-Orbitrap.

Consecutive number	HPLC fraction	Retention time	Accurate mass [m/z]	Elemental composition	Mass error [ppm]
1	G_I	13.4	326.2062	C ₁₇ H ₃₀ D ₂ O ₂ Si ₂	0.3
2		19.5	563.3414	C ₃₀ H ₅₃ D O ₄ Si ₃	4.9
3	G_II	16.0	549.3608	C ₃₀ H ₅₅ D O ₃ Si ₃	2.5
4		17.3	449.3235	C ₂₆ H ₄₅ D ₂ O ₂ Si ₂	0.1
5		17.4	549.3607	C ₃₀ H ₅₅ D O ₃ Si ₃	2.3
6		17.6	449.3236	C ₂₆ H ₄₅ D ₂ O ₂ Si ₂	0.3
7		18.3	549.3611	C ₃₀ H ₅₅ D O ₃ Si ₃	2.9
8*	G_III	12.1	395.2794	C ₁₈ H ₄₃ D ₂ O ₃ Si ₃	-0.7
9		13.6	475.2487	C ₂₅ H ₃₉ D ₂ O ₃ Si ₃	0.7
10		16.1	339.2136	C ₁₈ H ₃₁ D ₂ O ₂ Si ₂	-0.8
11*		17.1	550.3665	C ₃₀ H ₅₄ D ₂ O ₃ Si ₃	1.5
12*		17.9	552.3831	C ₃₀ H ₅₆ D ₂ O ₃ Si ₃	3.1
13		18.1	549.3613	C ₃₀ H ₅₅ D O ₃ Si ₃	3.3
14		18.9	549.3611	C ₃₀ H ₅₅ D O ₃ Si ₃	2.9
15	19.9	547.3449	C ₃₀ H ₅₃ D O ₃ Si ₃	2.1	
16	G_IV	16.5	549.3609	C ₃₀ H ₅₅ D O ₃ Si ₃	2.7
17		17.0	549.3608	C ₃₀ H ₅₅ D O ₃ Si ₃	2.4
18		19.4	552.3848	C ₃₀ H ₅₆ D ₂ O ₃ Si ₃	6.2
19		21.6	551.3752	C ₃₀ H ₅₇ D O ₃ Si ₃	0.2
20	G_V	10.7	461.325	C ₂₇ H ₄₇ D O ₂ Si ₂	-0.1
21*		12.9	464.3464	C ₂₇ H ₄₈ D ₂ O ₂ Si ₂	-1.1
22*		14.0	461.3253	C ₂₇ H ₄₇ D O ₂ Si ₂	0.5
23*		14.2	464.3464	C ₂₇ H ₄₈ D ₂ O ₂ Si ₂	-1.2
24		14.3	387.2698	C ₂₄ H ₃₇ D O ₂ Si	-0.1
25		19.5	463.3392	C ₂₇ H ₄₉ D O ₂ Si ₂	-3.2
26*	S_III	12.0	395.2797	C ₁₈ H ₄₃ D ₂ O ₃ Si ₃	0.1
27		14.0	217.1917	C ₁₆ H ₂₁ D ₂	-1.5
28		14.3	320.2496	C ₂₀ H ₃₂ D ₂ O Si	-0.9
29		15.4	339.2136	C ₁₈ H ₃₁ D ₂ O ₂ Si ₂	-0.9
30		16.8	552.3832	C ₃₀ H ₅₆ D ₂ O ₃ Si ₃	3.3
31*		17.1	550.3674	C ₃₀ H ₅₄ D ₂ O ₃ Si ₃	0.2
32	17.1	554.3988	C ₃₀ H ₅₈ D ₂ O ₃ Si ₃	3.2	

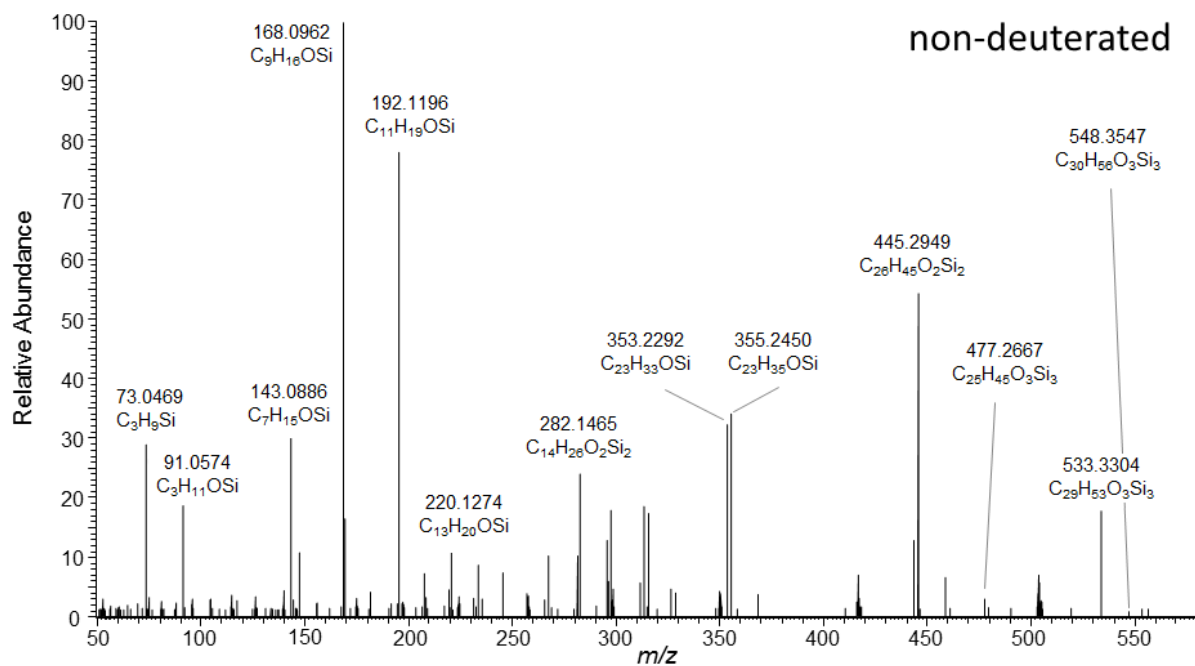
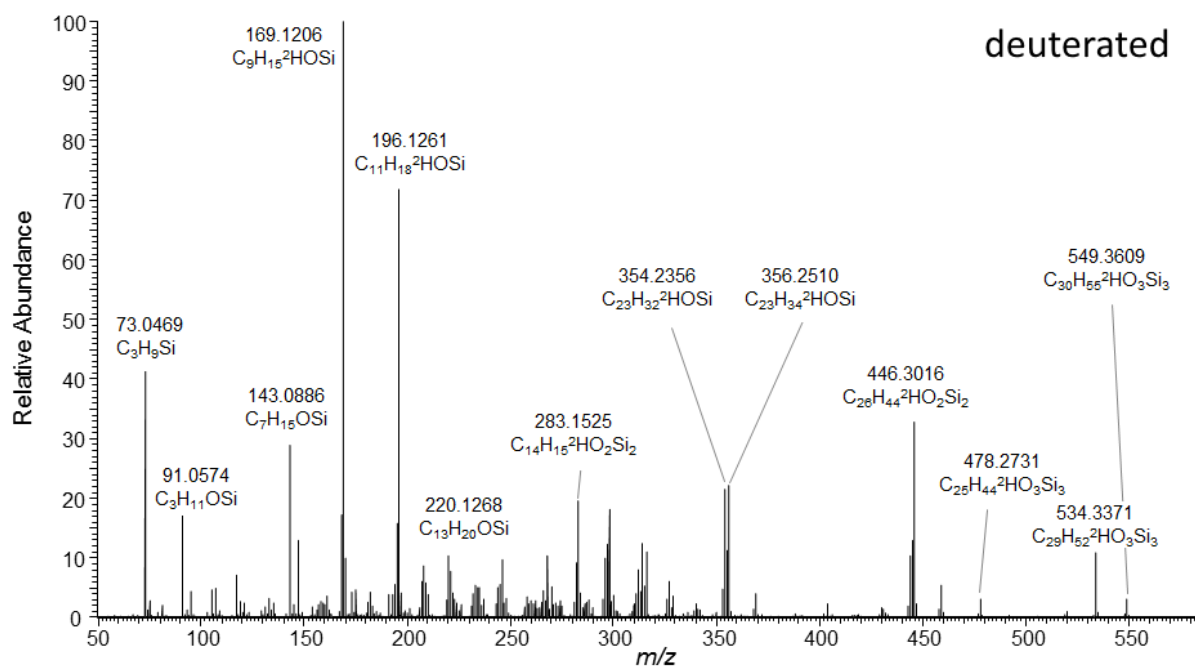
33*		17.8	552.3831	C ₃₀ H ₅₆ D ₂ O ₃ Si ₃	3.2
34		18.8	548.3549	C ₃₀ H ₅₂ D ₂ O ₃ Si ₃	3.2
35		19.9	546.339	C ₃₀ H ₅₀ D ₂ O ₃ Si ₃	2.6
36		20.4	546.3389	C ₃₀ H ₅₀ D ₂ O ₃ Si ₃	2.6
37		21.0	546.3388	C ₃₀ H ₅₀ D ₂ O ₃ Si ₃	2.3
38*	S_V	12.9	464.3466	C ₂₇ H ₄₈ D ₂ O ₂ Si ₂	-0.8
39*		14.0	461.3253	C ₂₇ H ₄₇ D O ₂ Si ₂	0.5
40*		14.2	464.3464	C ₂₇ H ₄₈ D ₂ O ₂ Si ₂	-1.2

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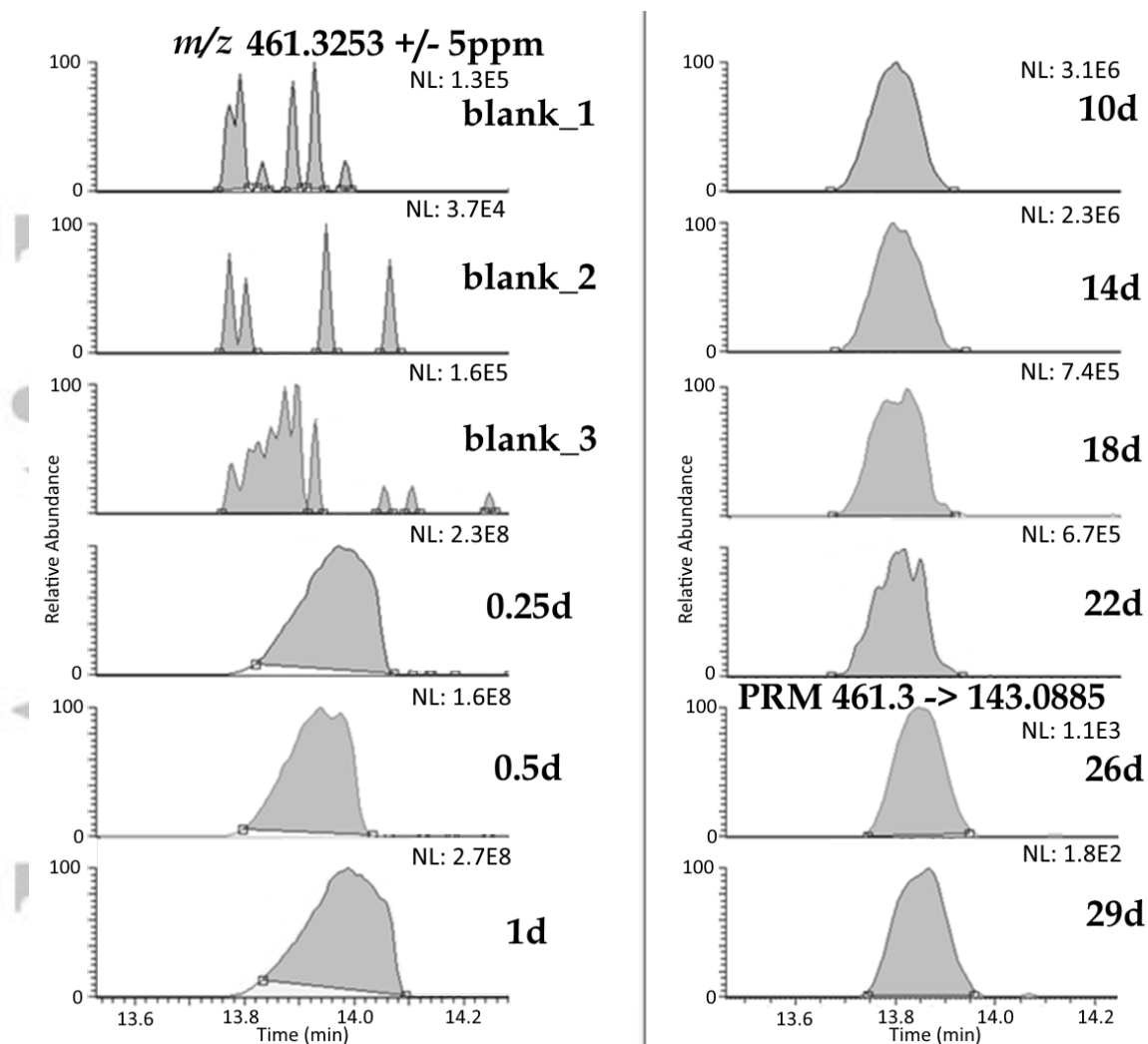


HIR chromatograms of HPLC fraction 5 before application (upper part) and 2 days after application of 10 mg deuterated methylstenbolone (MSTEN) in the lower part. Also visible are the endogenous pregnanediol (PD) and one novel long-term metabolite (Epi-NEW_LTM). Further information in the text.

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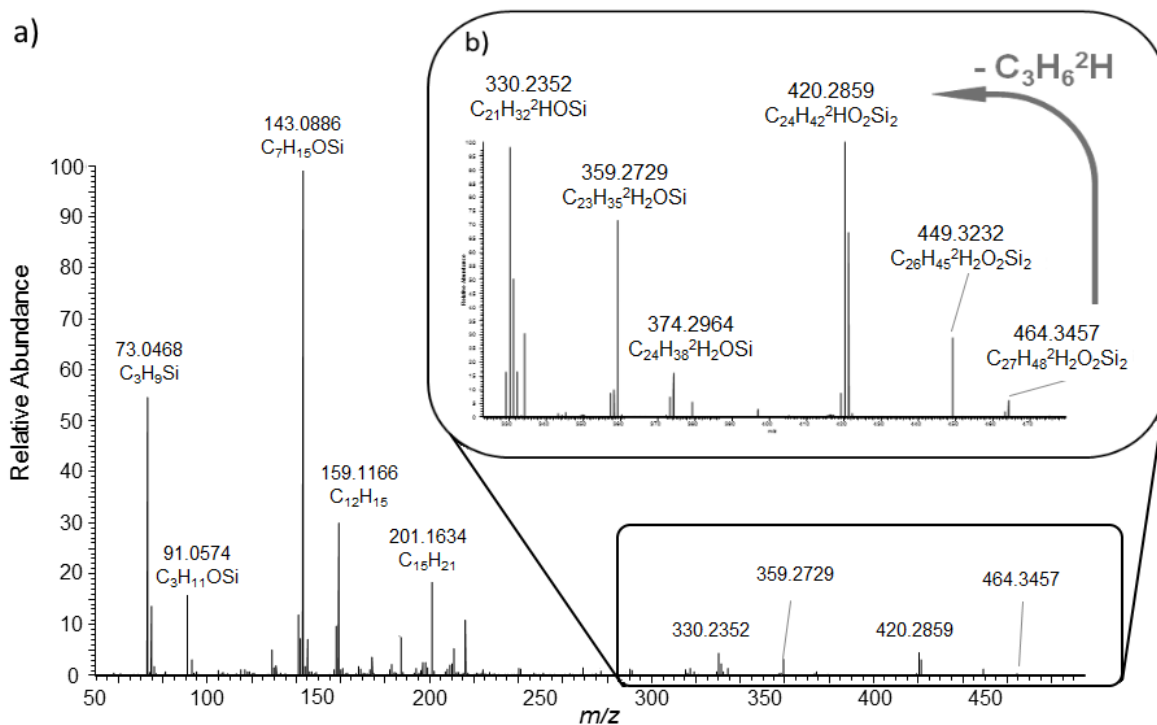


High resolution-parallel reaction monitoring mass spectra of metabolite 5. In the upper part, the deuterated metabolite using the precursor ion at m/z 549.3 is shown, in the lower part the non-deuterated metabolite with precursor ion at m/z 548.3, the deuterium label is present in the A-ring.



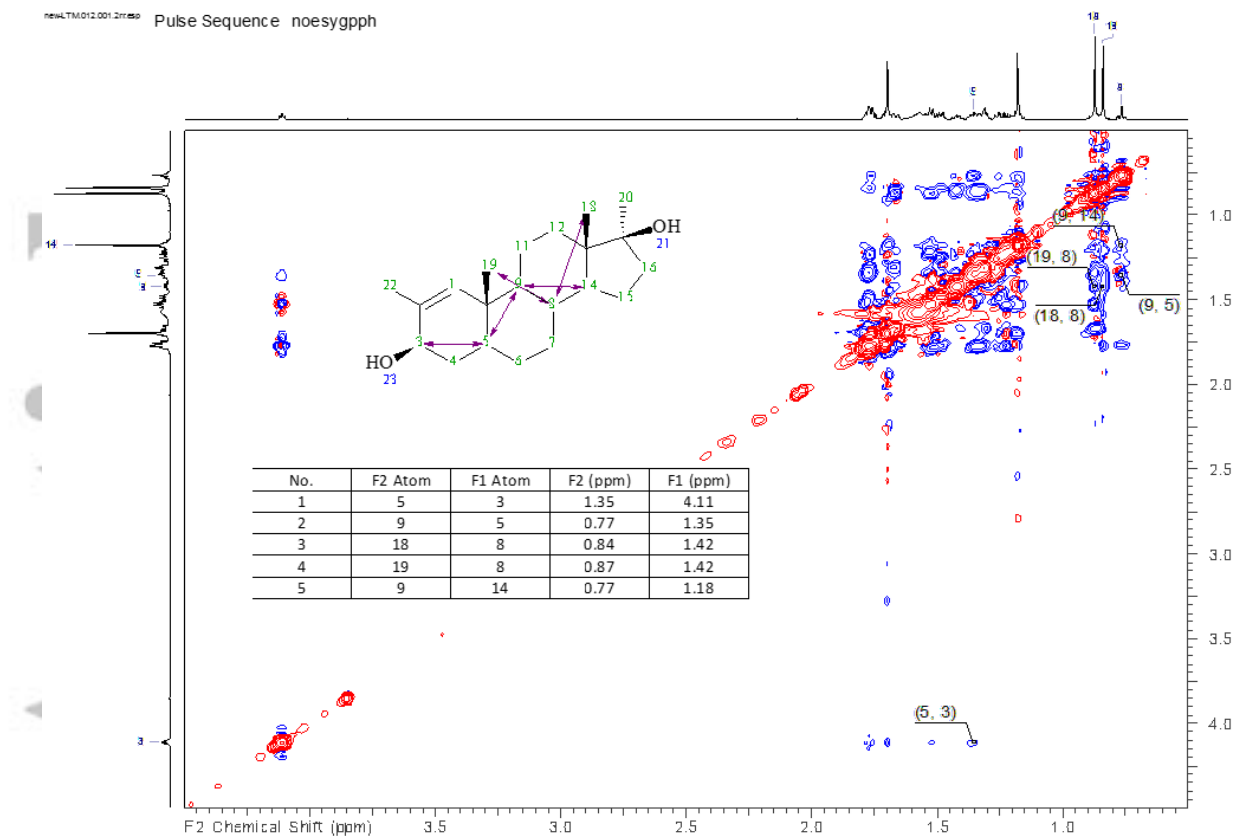
Extracted ion chromatograms for the bis-TMS derivative of mono-deuterated MSTEN. In the lower right part the parallel reaction monitoring (NCE 20 eV) employing m/z 461.3 as precursor is shown for the last 3 collected sample to demonstrate the enhanced detection capability by this mass spectrometric approach.

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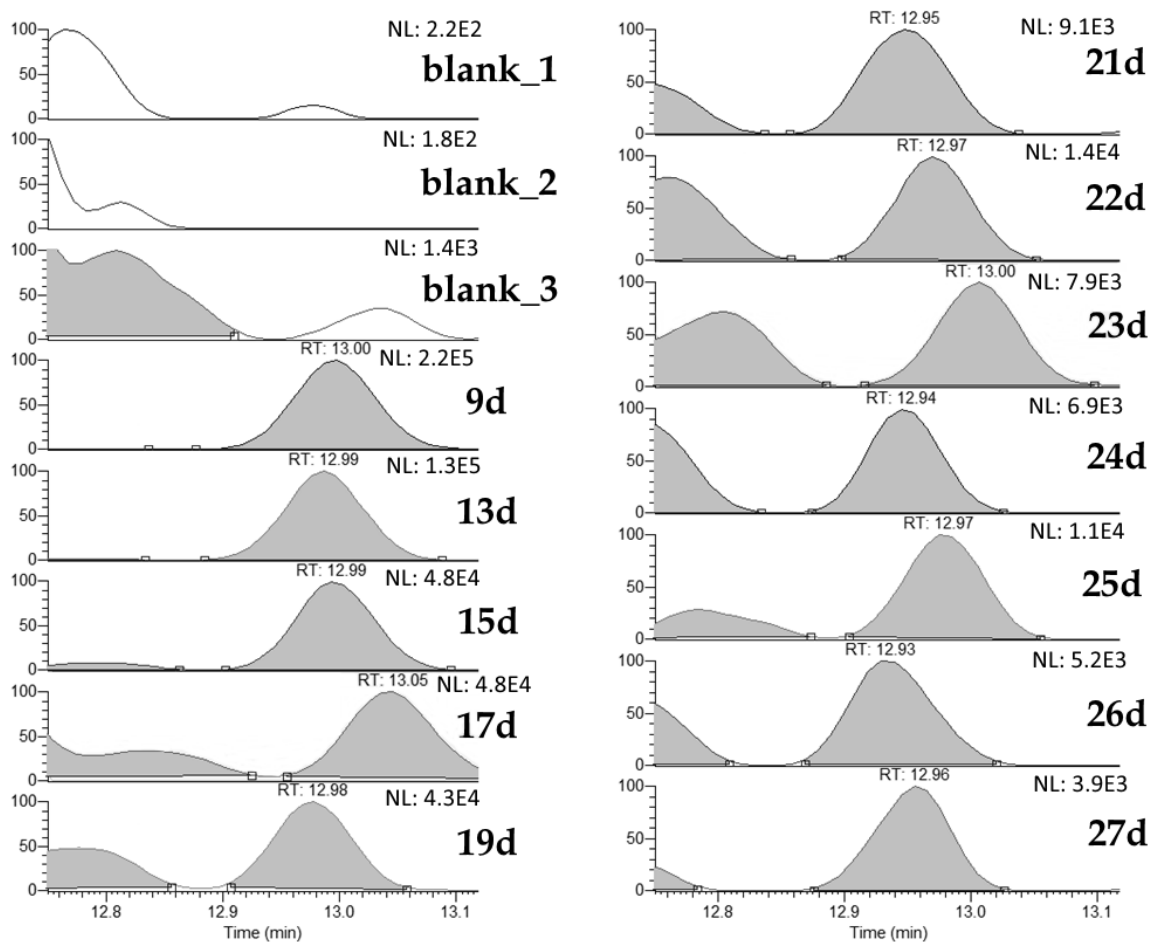
a) Full scan high resolution mass spectra obtained for the Epi-NEW_LTM, b) magnification of the range m/z 320 to 480, the grey arrow indicates the characteristic loss of 44 Da. Given elemental compositions were calculated within a mass error of ± 5 ppm. Further information in the text.

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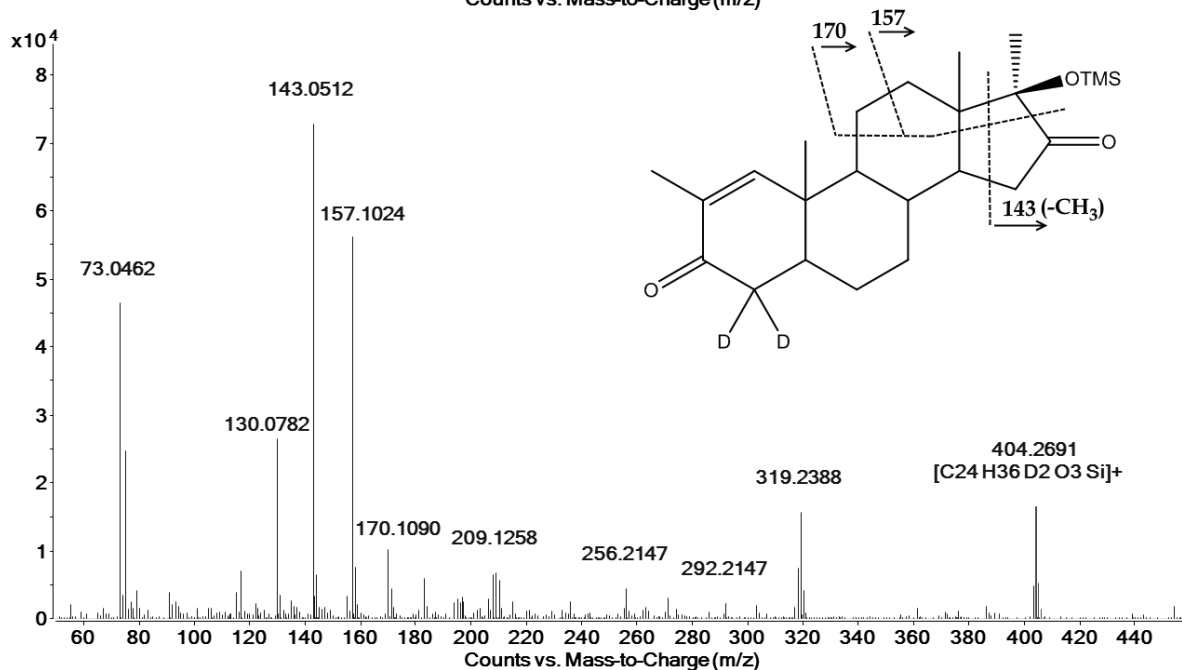
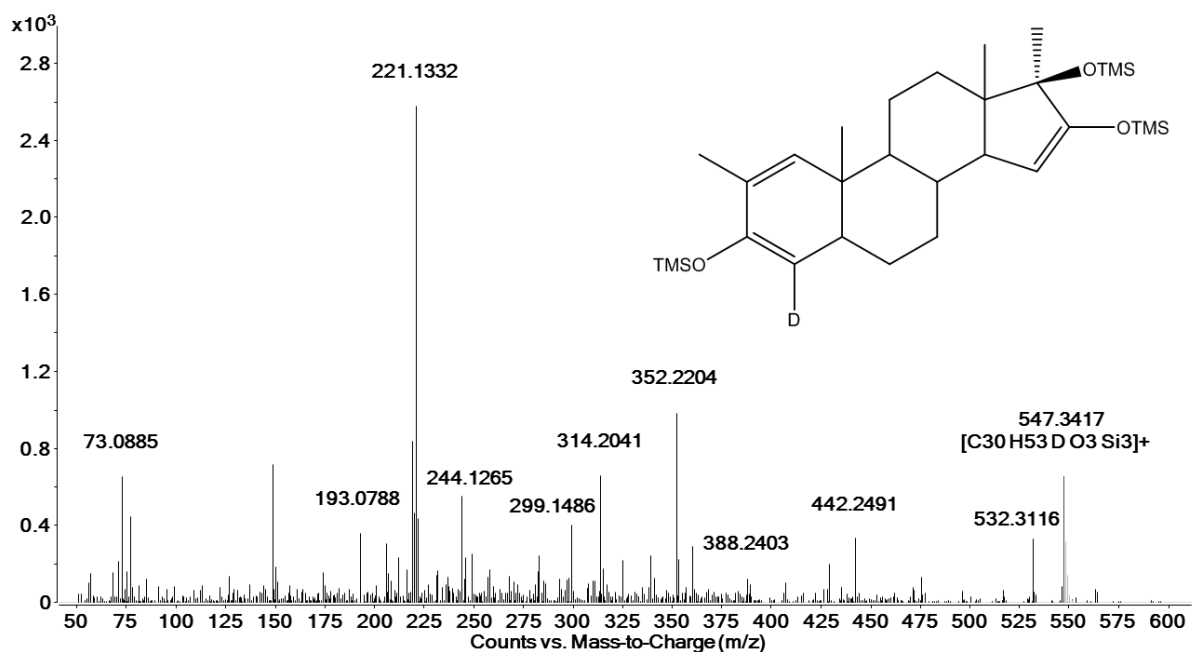
NOESY experiment on the Epi-NEW_LTM demonstrating the interaction of protons located on C-3, C-5, C-9, and C-14 as well as C-19, C-8, and C-18, respectively.

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Extracted ion chromatograms obtained employing parallel reaction monitoring (NCE 20 eV) of m/z 462.3 with the transition on m/z 143.0885 \pm 5ppm of the novel NEW_LTM.

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Full scan high resolution mass spectra obtained for OXO_LTM. Given elemental compositions were calculated within a mass error of ± 5 ppm. In the upper part the per-TMS derivative is shown, in the lower part the MSTFA derivative which enabled a preliminary structural elucidation. Further information in the text.