Mass Spectrometry of Drug Derivatives: A Contribution to the Characterization of Fragmentation Reactions by Labelling with Stable Isotopes

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Abbreviations

AAS	Anabolic Androgenic Steroids
BSA	N, O-bis(trimethylsilyl)acetamide
EI	Electron ionization
GC/C-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GC/MS	Gas chromatography-mass spectrometry
GC/MS/MS	Gas chromatography-tandem mass spectrometry
GC/QTOFMS	Gas chromatography-quadrupole-time-of-flight mass spectrometry
HRMS	High-resolution mass spectrometry
LC/MS(/MS)	Liquid chromatography-(tandem) mass spectrometry
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MTFA	N-methyltrifluoroacetamide
NH_4I	Ammonium iodide
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drug
$TiCl_4$	Titanium tetrachloride
TMCS	Trimethylchlorosilane
TMIS	Iodotrimethylsilane
TMS	Trimethylsilyl
TMSIM	Trimethylsilylimidazole
TMSOH	Trimethylsilanol
WADA	World Anti-Doping Agency

1 Introduction and aim of the project

The unequivocal structure elucidation of unknown analytes, such as unapproved designer drugs or new metabolites of presumably well-characterized compounds, is a challenge many researchers in life sciences are facing. Generally, nuclear magnetic resonance (NMR) and mass spectrometric (MS) techniques are used for structure confirmation. MS is suitable even for detecting low amounts of analyte in the pico-, femto- or attomole range[1]. One of the most relevant substance classes in anti-doping analysis are performance enhancing anabolic androgenic steroids (AAS), which led the ranking list with 44% of all reported findings in 2018 according to the World Anti-Doping Agency's (WADA) testing figures[2]. AAS bear health-related risks for athletes as well as for consumers in recreational sports due to their various side-effects[3] and their availability in "nutritional supplements" sold over the internet[4].

For screening of AAS in anti-doping laboratories gas chromatography mass spectrometry (GC/MS) is the method of choice after derivatization with silylating agents to improve chromatographic detection[5-8]. In this way, higher sensitivity and selectivity can be achieved[1,9,10]. The fragmentation patterns observed in the mass spectra correlate to the respective steroid structure[11]. Thus, in finding and characterizing diagnostic ions in the mass spectra of known steroids, the correct interpretation of data obtained for related unknown analytes is facilitated and methods of detection, for example the assignment of specific precursor ions in tandem mass spectrometry (MS/MS), can be updated.

Stable isotope labelling with deuterium (^{2}H) and ^{18}oxygen (^{18}O) is a practical and versatile approach for structure elucidation in mass spectrometry. The observed mass shifts in the spectra help to substantiate proposals for fragment ion generation[12].

The work presented herein can be divided into two main steps: the first step was to start from deuterium labelling methods for steroids first described in the 1960s[13,14] and 1970s[15,16] and develop them further to meet the requirements of today's laboratory techniques. Additionally, a new ^{18}O-labelling method via an imine intermediate was developed. In a second step, the practical applicability of the obtained isotopically labelled derivatives and their potential benefits for structure elucidation of GC/MS fragment ions were tested using modern instruments such as high-resolution and tandem mass spectrometers.

The new ^{18}O-labelling method was performed on the analgesic drug ketoprofen and related benzophenones as well as tested for its usefulness for the structural characterization of other substance classes, especially hydroxy steroids.

This work is expected to simplify fragment ion interpretation and indirectly contribute to preventive healthcare by possibly improving and complementing screening methods and mass spectral libraries for steroids.

2 Background

2.1 Stable isotopes in mass spectrometry

All isotopes of a single element share the same number of protons but differ in the number of neutrons and consequently their atomic mass number. In contrast to radioactive isotopes, stable isotopes are not prone to radioactive decay and are relatively safe to use, for example even in nutritional studies[17]. Deuterium (^{2}Hydrogen), ^{18}Oxygen, ^{13}Carbon and ^{15}Nitrogen are the most relevant stable isotopes employed in the life sciences[17,18]. Their natural abundances and radioactive counterparts with the respective half-lives[17] are summarized in Table 1. Since their discovery in the 1930s the use of stable isotopes in research is closely linked to the development and improvements of mass spectrometric techniques, for example improvements in sensitivity, mass accuracy and data output[18,19]. Stable isotopes have typically been employed for metabolic studies[20-22], newborn screening[23], doping control[24], environmental trace analysis[25], geology[26], ecology[27], and more recently in metabolomics[28,29] and proteomics[18,30]. A vast number of scientific papers on stable isotope labelling has been published to date including comprehensive reviews covering different aspects of the topic[18,19,31]. Besides the site-specific labelling of one or several compounds of interest, the use of stable isotope-labelled internal standards for quantitation is well-established in various research fields[23,32]. An advantage of such internal standards is the fact that they show very similar chromatographic and mass spectral behavior as the target analyte[33].

stable isotope	abundance	radioactive isotope	half-life
^{1}H	99.98%	^{3}H	12.23 years
^{2}H	0.02%		
^{12}C	98.9%	^{14}C	5730 years
^{13}C	1.1%	^{11}C	20.4 minutes
^{14}N	99.6%	^{13}N	9.9 minutes
^{15}N	0.4%		
^{16}O	99.76%	^{14}O	70 seconds
^{17}O	0.04%	^{15}O	122 seconds
^{18}O	0.20%		

Table 1: Stable and radioactive isotopes of hydrogen, carbon, nitrogen, and oxygen[17]

2.1.1 Isotopic labelling with deuterium

According to a detailed review series that listed stable isotope studies with ^{2}H, ^{13}C, ^{15}N, ^{18}O and ^{34}S conducted between 1971 and 1978[34-38], investigations on deuterium made up about 50% of the total number of articles included in the reviews[38]. Because today many deuterated reagents and so-called deuterated building blocks with high isotopic purity are commercially available[18], the introduction of deuterium labels into different compounds is comparatively uncomplicated and inexpensive[39]. In steroid research, ^{13}C-labelling is more prominent in metabolic studies, whereas ^{2}H-labelled steroids are routinely used as internal standards in quantitative approaches[39]. Typical synthetic procedures[39,40] employed for the incorporation of deuterium in the steroid backbone are acid or base-catalyzed exchange reactions[41-45], homogenous or heterogenous catalytic deuteration with deuterium gas over palladium/charcoal, platinum oxide or a rhodium catalyst[46-50], reduction with lithium aluminum deuteride[51] or sodium borodeuteride[40] in deuterated solvents and Grignard reactions with deuterium-labelled methyl magnesium bromide ($C^{2}H_{3}MgBr$)[39]. In case of GC/MS studies, isotopic labelling can additionally be achieved with deuterated derivatization reagents, such as [$^{2}H_{9}$]MSTFA[29], which requires less starting material and is especially helpful for the characterization of fragment ions, that contain trimethylsilyl (TMS) groups.

2.1.2 Isotopic labelling with ^{18}oxygen

^{18}O-labelling was mainly used for observational environmental research (hydrological, geochemical, and atmospheric studies) in the 1970s and evolved to be the method of choice to study reaction mechanisms and enzyme action[37], for example hydrolysis, esterification, and oxidation[18]. The most important compounds for enrichment with ^{18}O are water, phosphate, and sulfate[18]. Isotopic labelling of steroids with ^{18}O usually relied on an acid-catalyzed exchange method first described by Lawson *et al.*[52] and was infrequently performed until today[12,53-56]. The main drawbacks of the approach are the comparatively large amounts of starting material (for example 10 mg steroid standard and 100 µl $H_{2}{}^{18}O$)[56] and the incomplete conversion into the ^{18}O-labelled derivative due to the underlying equilibrium reaction mechanism.

Recently, ^{18}O-labelling was rediscovered for proteomics, mainly due to its simplicity and high versatility[30,57]. For protein quantification, enzymatic labelling with ^{18}O-water can be performed during protein digestion, which results in labelling at the peptide C-terminus. To prevent mixtures of non-labelled, single labelled and doubly labelled peptides, caution must be exercised regarding the correct pH, labelling time and conditions[30].

2.2 Investigated compounds

2.2.1 Ketoprofen and related Benzophenones

Ketoprofen is a nonsteroidal anti-inflammatory drug (NSAID) marketed worldwide for its analgesic and antipyretic efficacy[58]. Photosensitivity reactions are reported side effects[59] and are linked to the benzophenone structure, which is a common feature ketoprofen shares with many of its impurities[60].

2.2.2 Anabolic androgenic steroids (AAS)

There are five general groups of steroid hormones in the human body: estrogens, progestogens, androgens, glucocorticoids, and mineralocorticoids. They are based on a common structural backbone called the "steroid backbone". Due to variations in the structures, selectivity for the unique molecular target can be achieved[4]. Chemically modified drugs derived from endogenous steroids represent one of the most widely used class of therapeutic agents. These exogenous steroids are primarily used in birth control, hormone-replacement therapy, inflammatory conditions, and cancer treatment[61].

AAS are endogenous or synthetic substances related to the male sex hormones (androgens), which promote growth of skeletal muscle (anabolic effect) and the development of male sexual characteristics (androgenic effects)[62]. AAS are misused to improve athletic performance and are the most frequently detected doping substances in sports[2]. The most common adverse effects of AAS are reduced fertility and gynecomastia in men and masculinization in women and children[3,63]. High doses of AAS can lead to serious and irreversible organ damage[3].

Steroid research using mass spectrometry originated in the 1950s with a focus on fundamental research until about 1970. The main goal was to widen the therapeutic use of steroids using analytical methods that were still limited compared to today. Nowadays, analytical challenges for the detection of the misuse of anabolic agents are, for example, the administration of unapproved and/or new structurally slightly modified designer drugs, the increasing use of endogenous substances, micro-dosing, and genetic polymorphisms that lead to different metabolic patterns in the tested individuals[64-66]. Additionally, nutritional supplements contaminated with endogenous or exogenous AAS are problematic since their intake can cause inadvertent doping[67].

Industrial research in this field, on the other hand, is only carried out to a limited extent. Further development of methods and targeted basic research is therefore mainly relevant for anti-doping laboratories, illegal application in animal feed, toxicological forensics, and the control of the illegal market.

Since the 1970s, steroids have been analyzed with GC/MS, which allows effective separation that is especially relevant for complex biological mixtures. GC/MS is widely used, has relatively low cost, provides high sensitivity and selectivity, and has steadily growing, extensive spectra libraries[68]. High-resolution mass

spectrometers provide additional information on the molecular structure of substances and their metabolites. Due to the very high mass resolution, the elemental composition of molecules and their fragment ions can be calculated. For structure identification, especially in forensic investigations or in doping analysis, GC/MS measurements of steroid derivatives after trimethylsilylation was established. Despite the additional possibilities offered by liquid chromatography (tandem) mass spectrometry (LC/MS(/MS)), GC/MS(/MS) is used for the analysis in doping laboratories worldwide, particularly due to its exceptional separation performance[69]. During electron ionization (EI), molecules in the gas phase are bombarded with high-energy electrons. This produces ionic molecule radicals, which are highly reactive and therefore decompose into more stable fragment ions. The obtained typical fragmentation patterns provide important information about the structure of the analyte, since the reactions that occur during decay are highly dependent on the presence of functional groups and on the overall structure of the molecule[70].

2.3 Derivatization in gas chromatography

Derivatization in gas chromatography improves the detection of analytes in terms of sensitivity and robustness[5]. This is achieved with the exchange of chemical groups (e.g., hydroxy groups) by other groups (e.g., TMS groups) that influence the physical and chemical features of the analyte, for example, thermal stability, volatility, polarity and ionization efficiency[5]. Prior to GC/MS analysis, steroid hydroxy- and oxo-functions are usually derivatized to TMS ether and enol-TMS ether using different silylating agents and catalysts. Through silylation chromatographic as well as mass spectral characteristics of steroids are improved[5]. The same holds true for ketoprofen and its related benzophenones, in which case the acidic function can be derivatized with the silylating agent trimethylsilyl chloride (TMCS)[9] without the need of a catalyst.

2.3.1 MSTFA

MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide) is a very effective derivatization agent used to react with hydroxy as well as other functional groups. The derivatization mixture most commonly used in steroid research today consists of MSTFA/ammonium iodide (NH_4I)/ethanethiol (1000:2:3 (v/w/v)), from which iodotrimethylsilan (TMIS) as the actual silylating agent is generated *in situ*[9]. In this way not only hydroxy-groups are turned into TMS-ether but also oxo-groups can be converted into enol-TMS ethers after enolization (Figure 1, p. 7). The reducing agent ethanethiol is added to prevent the formation of iodine and its possible reaction with the steroid nucleus[9].

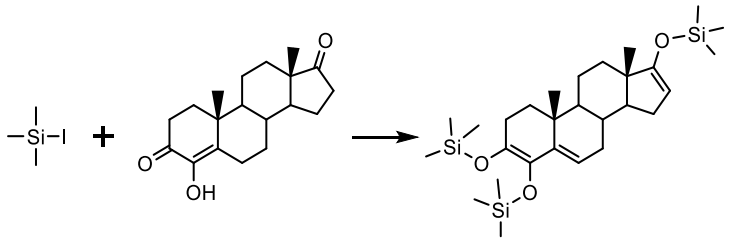

Figure 1: Silylation reaction of TMIS exemplified by formestane resulting in the 3,5-dienol TMS ether derivative

2.3.2 BSA

BSA (N, O-bis(trimethylsilyl)acetamide) is a well-known silylating reagent for steroids[71] and can also be used for the protection of alcohols, phenols, carboxylic acids, amino acids and amines[9,72]. It is rarely considered today for the derivatization of steroids due to the availability of more convenient alternatives. BSA provides a high silylation potential towards hydroxy groups[71] and transfers only one of its TMS groups onto the reaction partner (Figure 2). Byproducts like N-trimethylsilyl acetamide or acetamide are sufficiently volatile to be removed from the reaction mixture[73,74].

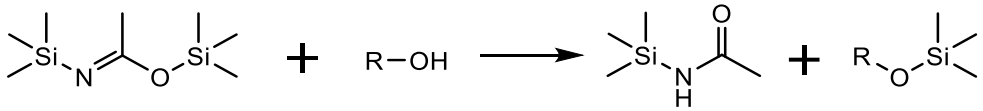

Figure 2: Trimethylsilylation of hydroxy group with BSA, R= rest of the compound

In order to enhance its silylation power, different catalysts for BSA are described in literature such as TMCS (1-20%), potassium acetate, trifluoracetic acid, MSTFA/I_2 (100:1, v/w), and piperidine as well as different solvents (pyridine, dimethylformamide, acetonitrile)[9,74].

The reaction of steroidal oxo-groups to TMS-enol ether with BSA only takes place sporadically and in an incomplete manner. In steroid analyses BSA is therefore usually used in fixed combinations with other silylating agents such as trimethylsiylimidazole (TMSIM) or TMCS[9]. Commercially available mixtures are, for example BSA/TMSIM/TMCS 3:3:2 or BSA with 5% TMCS.

Because BSA is only a mediocre silylating agent compared to MSTFA, the obtained chromatograms for the derivatized steroids often show multiple peaks for mono-, bis-, and tris-TMS derivatives per compound instead of only one for the pertrimethylsilylated derivative. Additionally, a higher percentage of different derivatization isomers (2,4-dienol and 3,5-dienol ethers) are observed. Because of these limitations, it is advisable to derivatize fewer steroid standards per GC/MS run with BSA compared to steroids derivatized with MSTFA, especially when the molecular masses are identical and the respective retention times unknown.

Nonetheless, depending on the purpose of the experiment, the generation of more than one derivative per steroid can also be an advantage. Several hydroxylated androstenedione metabolites (2α/β-, 4-, and 6α/β-hydroxyandrosten-4-ene-3,17-dione) for example, were coeluting as tris-TMS derivatives using a standard chromatographic method and could successfully be separated as mono-TMS derivatives[75].

3 Publications

3.1 Manuscript No. 1

Reconsidering mass spectrometric fragmentation in electron ionization mass spectrometry – new insights from recent instrumentation and isotopic labelling exemplified by ketoprofen and related compounds

Jaber Assaf, Annette Sophie Kollmeier, Christian Müller, Maria Kristina Parr

Rapid Communications in Mass Spectrometry 2019;33:215-228

https://doi.org/10.1002/rcm.8313

Rationale: In various fields of chemical analyses, structurally unknown analytes are considered. Proper structure confirmation may be challenged by the low amounts of analytes that are available, e.g., in early-stage drug development, in metabolism studies, in toxicology or in environmental analyses. In these cases, mass spectrometric techniques are often used to build up structure proposals for these unknowns. Fragmentation reactions in mass spectrometry are known to follow definite pathways that may help to assign structural elements by fragment ion recognition. This work illustrates an investigation of fragmentation reactions for gas chromatography/electron ionization mass spectrometric characterization of benzophenone derivatives using the analgesic drug ketoprofen and seven of its related compounds as model compounds.

Methods: Deuteration and ^{18}O-labelling experiments along with high-resolution accurate mass and tandem mass spectrometry (MS/MS) were used to further elucidate fragmentation pathways and to substantiate rationales for structure assignments. Low-energy ionization was investigated to increase confidence in the identity of the molecular ion.

Results: The high-resolution mass analyses yielded unexpected differences that led to reconsideration of the proposals. Site-specific isotopic labelling helped to directly trace back fragment ions to their respective structural elements. The proposed fragmentation pathways were substantiated by MS/MS experiments.

Conclusions: The described method may offer a perspective to increase the level of confidence in unknown analyses, where reference material is not (yet) available.

3.2 Manuscript No. 2

Mass spectral fragmentation analyses of isotopically labelled hydroxy steroids using low-resolution gas chromatography/mass spectrometry: A practical approach

Annette Sophie Kollmeier, Maria Kristina Parr

Rapid Communications in Mass Spectrometry 2020;34:e8769

https://doi.org/10.1002/rcm.8769

Rationale: Gas chromatography coupled to electron ionization mass spectrometry (GC/EI-MS) is used for routine screening of anabolic steroids in many laboratories after the conversion of polar groups into trimethylsilyl (TMS) derivatives. The aim of this work is to elucidate the origin and formation of common and subclass-specific fragments in mass spectra of TMS-derivatized steroids. Especially in the context of metabolite identification or analysis of designer drugs, isotopic labelling is helpful to better understand fragment ion generation, identify unknown compounds and update established screening methods.

Methods: Stable isotope labelling procedures for the introduction of [$^{2}H_{9}$]-TMS or ^{18}O were established to generate perdeuterotrimethylsilylated, mixed deuterated and ^{18}O-labelled derivatives for 13 different hydroxy steroids. Fragmentation proposals were substantiated by comparison of the abundances of isotopically labelled and unlabelled fragment ions in unit mass resolution GC/MS. Specific fragmentations were also investigated by high resolution MS (GC/ quadrupole time-of-flight MS, GC/QTOFMS).

Results: Methyl radical cleavage occurs primarily from the TMS groups in saturated androstanes and from the steroid nucleus in the case of enol-TMS of oxo or α,β-unsaturated steroid ketones. Loss of trimethylsilanol (TMSOH) is dependent on steric factors, degree of saturation of the steroid backbone and the availability of a hydrogen atom and TMSO group in the 1,3-diaxial position. For the formation of the $[M - 105]^{+}$ fragment ion, methyl radical cleavage predominates from the angular methyl groups in position C-18 or C-19 and is independent of the site of TMSOH loss. The common $[M - 15 - 76]^{+}$ fragment ion was found in low abundance and identified as $[M - CH_3 - (CH_3)_2SiH\text{-}OH]^{+}$. For the different steroid subclasses further diagnostic fragment ions were discussed and structure proposals postulated.

Conclusion: Stable isotope labelling of oxo groups as well as derivatization with deuterated TMS groups enables the detection of structure related fragment ion generation in unit mass resolution GC/EI-MS. This may in turn allow to propose isomeric assignments that are otherwise almost impossible using MS only.

3.3 Manuscript No. 3

In-depth gas chromatography/tandem mass spectrometry fragmentation analysis of formestane and evaluation of mass spectral discrimination of isomeric 3-keto-4-ene hydroxy steroids

Annette Sophie Kollmeier, Xavier de la Torre, Christian Müller, Francesco Botrè, Maria Kristina Parr

Rapid Communications in Mass Spectrometry 2020;34:e8937

https://doi.org/10.1002/rcm.8937

Rationale: The aromatase inhibitor formestane (4-hydroxyandrost-4-ene-3,17-dione) is included in the World Anti-Doping Agency's List of Prohibited Substances in Sport. However, it also occurs endogenously as do its 2-, 6- and 11-hydroxy isomers. The aim of this study is to distinguish the different isomers using GC/EI-MS for enhanced confidence in detection and selectivity for determination.

Methods: Established derivatization protocols to introduce [$^{2}H_{9}$]-TMS were followed to generate perdeuterotrimethylsilylated and mixed deuterated derivatives for 9 different hydroxysteroids, all with 3-keto-4-ene structure. Formestane was additionally labelled with $H_2{}^{18}O$ to obtain derivatives doubly labelled with [$^{2}H_{9}$]-TMS and ^{18}O. GC/MS mass spectra of labelled and unlabelled TMS-derivatives were compared. Proposals for generation of fragment ions were substantiated by high-resolution MS (GC/QTOFMS) and MS/MS experiments.

Results: Subclass specific fragment ions include *m/z* 319 for the 6-hydroxy and *m/z* 219 for the 11-hydroxy compounds. *m/z* 415, 356, 341, 313, 269 and 267 were indicative for the 2- and 4-hydroxy compounds. For their discrimination, the transition *m/z* 503→269 was selective for formestane. In 2-, 4- and 6-hydroxy steroids losses of a TMSO radical takes place as cleavage of a TMS originated methyl radical and a neutral loss of $(CH_3)_2SiO$. Further common fragments were elucidated as well.

Conclusion: With the help of stable isotope labelling, the structure of postulated diagnostic fragment ions for the different steroidal subclasses were elucidated. ^{18}O-labelling of the other compounds will be addressed in future studies to substantiate the obtained findings. To increase method sensitivity MS^3 may be suitable in future bioanalytical applications requiring 2- and 4-hydroxy discrimination.

4 Discussion

The presented stable isotope labelling procedures with [$^{2}H_{9}$]-TMS and ^{18}O were proven to be useful for the structure characterization of fragment ions in GC/MS spectra of benzophenones and hydroxy steroids. In all cases, the compound structure and conformation of functional groups was known before analyses, which naturally makes fragment ion assignment less speculative than for entirely unknown compounds or metabolites.

Isotopic labelling helps to generate hypotheses concerning fragment ion generation and composition, which can subsequently be substantiated with high-resolution mass spectrometry (HRMS) and MS/MS experiments. In those cases where HRMS only is not sufficient to differentiate between several possible elemental compositions for a specific fragment ion because of similar mass errors, or in cases where a HRMS instrument is not available, especially ^{18}O-labelled derivatives were crucial: the preferred position for trimethylsilanol (TMSOH) elimination of ten different androgens, for example, was established employing unit mass resolution GC/MS only (Manuscript 2).

4.1 Method development and applicability

The ^{18}O-labelling procedure developed for the experiments with benzophenones and hydroxy steroids is based on the formation of an intermediate imine product, as described in detail in Manuscripts 1 and 2. In short, oxo groups react with isopropyl amine to form an imine derivative with titanium tetrachloride ($TiCl_4$) as catalyst. The imine function is cleaved off with $H_2{}^{18}O$ in a second step and the original ^{16}O replaced with ^{18}O. For the cleavage of the imine function in the benzophenone derivatives, diluted hydrochloric acid was required but not in case of the hydroxy steroids, which demonstrates that the labelling method needs to be adapted to the respective substance class on which it is applied. Another vital factor that significantly influences the applicability of the method is the fact that the analyte needs to be soluble in toluene, which serves as a solvent of the first reaction with $TiCl_4$ and isopropyl amine. $TiCl_4$ polarizes the carbonyl bond and speeds up the reaction. Because it reacts with alcohols to produce salts, for example titanium isopropoxide ($Ti\{OCH(CH_3)_2\}_4$) with isopropanol, polar solvents cannot be used.

^{18}O-labelling with the polar steroid ecdysterone was not successful using this method because the compound was not soluble in toluene. In this case the acid-catalyzed exchange method described by Lawson et al[52] might

be more suitable for labelling although complete conversion to the respective ^{18}O-labelled derivative cannot be granted.

The described ^{18}O-labelling method can easily be combined with other workflows such as derivatization procedures or as the last step of metabolite synthesis. Reduction of the obtained products to their hydroxy counterparts with sodium borohydride was demonstrated and described in Manuscript 2. Theoretically, the reaction can be reversed using unlabelled water and replacing the ^{18}O with ^{16}O again, but there is little need for this.

^{18}O-labelling might be applied as an alternative to [$^{2}H_9$]TMS-labelling, especially in case of LC-MS approaches or for compounds not amenable for silylation, such as (2RS)-2-(3-benzoylphenyl)-propanenitrile (BP07) and 3-ethylbenzophenone (BP08, Manuscript 1). Additional mass shifts of +2 or +4 *m/z* units can be observed in fragment ions of GC/MS mass spectra that do not contain a TMS function, which is an important additional information not obtained otherwise.

Because the method is only applicable for oxo groups that can react to form imines and not hydroxyl oxygens or oxygen atoms that are incorporated into the steroid backbone (for example in oxandrolone), the lack of typical mass shifts in the mass spectra does not necessarily mean that the observed fragment ions consist of C, H and Si (if derivatized with TMS) atoms only. Thus, more general information about the analyte is mandatory before interpreting the structure of specific fragment ions.

As mentioned in Chapter 2.3.1 (p. 6), the silylating reagent typically used in anti-doping research for steroid analysis is MSTFA in the mixture MSTFA/NH_4I/ethanethiol (1000:2:3, v:w:v)[76]. Its deuterated counterpart [$^{2}H_9$]-MSTFA would consequently have been the best choice for deuteration experiments performed in this study. But because the reagent is very costly and the yield of its synthesis was very low (Annex 7.1, p. 93), [$^{2}H_9$]$_2$-BSA, as one of the educts for synthesis, was chosen instead. Method development for different silylation procedures using [$^{2}H_9$]$_2$-BSA, NH_4I and mercaptoethanol (as a well-established alternative to the antioxidant ethanethiol) is described in Manuscript 2 and a detailed derivatization protocol can be found in Annex 7.2 (p. 95) and Manuscript 3.

Because BSA possesses weaker silylation potential than MSTFA, reaction time needs to be longer (90-120 vs. 15-20 minutes for MSTFA) and reaction temperature higher (90°C vs. 60°C for MSTFA). The derivatization mixture with BSA promotes the generation of both 2,4- and 3,5-diene and only partly silylated derivatives in 3-keto-4-ene-steroids. This results in more peaks in the chromatograms as compared to steroids treated with MSTFA/NH_4I/ethanethiol, where predominantly the 3,5-diene derivative is observed. These “crowded” chromatograms might be problematic for analyses of unknown compounds and are the reason why no more

than three to four different hydroxy steroids should be analyzed together in one GC/MS run. These hurdles do not necessarily play a role in case of MS/MS experiments, where co-eluting peaks in chromatography can be overcome.

Disadvantages of enolization and TMS derivatization in general may include artifact formation[77,78], TMS migration[54,79] and the loss of stereochemical information (for example in position C-6 in case of 6β- and 6α-hydroxyandrostenedione[80]). To distinguish between isomers, the chromatographic retention times and, if applicable, the abundance of certain fragment ions must be evaluated additionally. Nevertheless, the fact that silylation is a key principle to provide reliable and reproducible GC/MS spectra which can easily be compared with the ample data collected in spectral libraries over many years, justifies its use for the introduction of deuterium via [$^2H_9$]TMS in the presented work.

4.2 Workflow for stable isotope labelling of hydroxy steroids

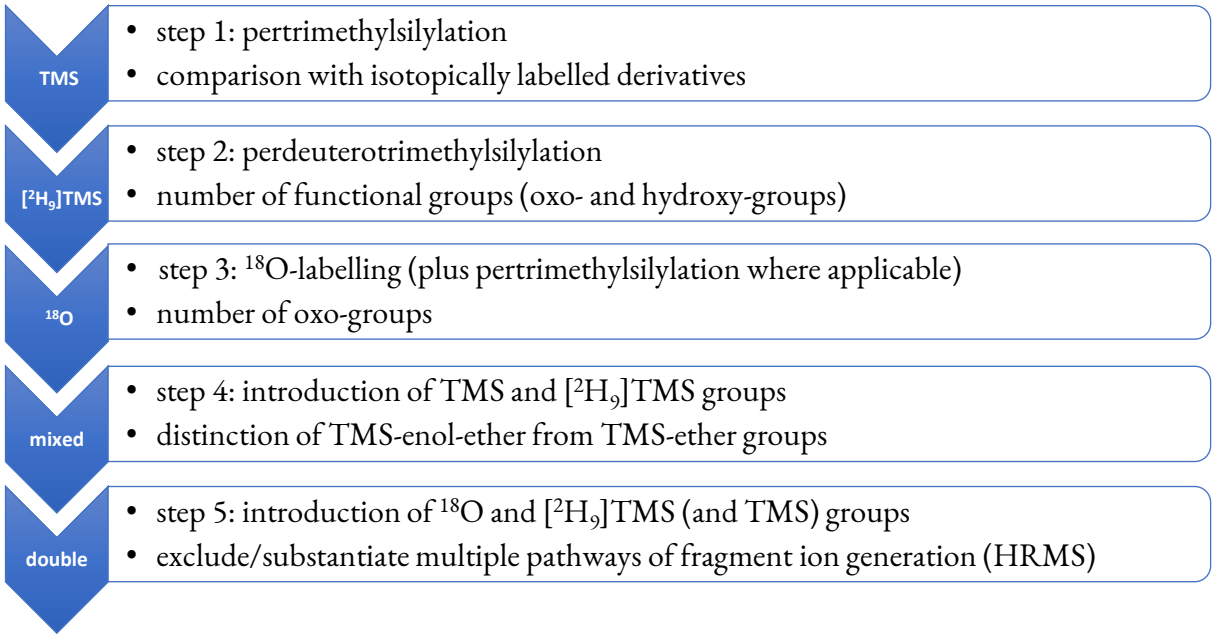

Figure 3: Workflow for the subsequent isotopic labelling of hydroxy steroids for fragment ion characterization

Figure 3 summarizes the workflow of the different isotopic labelling and derivatization protocols used for GC/MS fragment ion characterization in Manuscripts 2, 3 and in adapted form also for ketoprofen and its impurities in Manuscript 1. The five-step sequence starts with pertrimethylsilylation (step 1) to produce reference spectra for comparison with the spectra generated in the following steps. Perdeuterotrimethylsilylation (step 2) is useful to acquire information on the total number of functional groups in the compound or fragment ion and ^{18}O-labelled compounds (step 3) directly reflect the number of

oxo groups. If no mass shift in the GC/MS spectrum is observed after ^{18}O-labelling, the following two steps are not applicable, because they rely on the presence of oxo groups.

The preparation of "mixed" derivatives which involves the introduction of both TMS and [$^{2}H_9$]-TMS groups into the compound, can also be useful to detect the number of oxo groups as enol-ethers and could replace ^{18}O-labelling. But because it is more laborious and time-consuming than ^{18}O-labelling, it is proposed as step 4 and not step 3. Mixed deuterated TMS derivatives help to assign specific fragment ions to their correct place of origin in the steroid backbone, for example.

The last labeling procedure (step 5), which consists of the combination of ^{18}O and [$^{2}H_9$]-TMS labelling, is only advisable for measurements with high-resolution and if at least some information about the analyzed compound is already known (for example a hydroxy group in a specific position) and new insights are expected, that cannot be derived from the previous labelling steps 2-4. The combination of different mass shifts can be quite confusing, and the possible elemental composition of each single fragment ion must be individually evaluated. Fragment ion *m/z* 169 in the mass spectrum of formestane for example, is shifted to *m/z* 178 after perdeuterotrimethylsilylation (+9 *m/z* units), to *m/z* 171 after ^{18}O-labelling (+2 *m/z* units) and to *m/z* 180 (+9+2 *m/z* units) in the doubly labelled derivative. For the characterization of this specific fragment ion double labelling was unnecessary because the two previous experiments already confirmed the presence of a TMS- and an oxo-group.

With the combination of ^{18}O- and [$^{2}H_9$]TMS-labelling, fragment ions generated through several routes of formation can be detected with the help of the respective mass shifts, for example ions *m/z* 356, and *m/z* 341 in the mass spectrum of formestane (Manuscript 3): it was revealed that both fragment ions contain a TMS functions in position C-17 and a hydroxy group in either position C-3 or C-4, which indicates that at least two different structures for both ions can be proposed.

The advantage of acquiring new information by preparing doubly labelled steroid derivatives was demonstrated with the mass spectra obtained for the two mixed deuterated derivatives of [$^{18}O_2$]-formestane, 4-[$^{2}H_9$]TMS, 3,17[$^{18}O_2$]-bis-TMS-formestane (**1**) and 4-TMS, 3,17[$^{18}O_2$]-bis-[$^{2}H_9$]TMS-formestane (**2**) (Figure 4, Table 2, p. 85 and mass spectra in Annex 7.3, p. 96). Next to the expected fragment ions *m/z* 440 ([M – TMS^{18}O]$^+$), 433 ([M – [$^{2}H_9$]TMSO]$^+$), 424 ([M – CH_3 – TMS^{18}OH]$^+$) and 417 ([M – CH_3 – [$^{2}H_9$]TMSOH]$^+$) in the mass spectrum of **1**, a number of unexpected fragment ions are observed, namely *m/z* 442 ([M – TMSO]$^+$), 431 ([M – [$^{2}H_9$]TMS^{18}O]$^+$), 426 ([M – CH_3 – TMSOH]$^+$) and 415 ([M – CH_3 – [$^{2}H_9$]TMS^{18}OH]$^+$). The route of formation (blue dashed line, Figure 4) of the ions expected for **1** (blue numbers in Table 2) corresponds to the route of formation (orange dashed line, Figure 4) of the ions

unexpected for **2** (orange numbers in Table 2) and vice versa. For example, fragment ion [M – [$^{2}H_{9}$]TMSO]$^{+}$ was expected to be observed in the mass spectrum of **1** (*m/z* 433.2752, 1c, Table 2), because the deuterated TMS group is attached to the unlabelled oxygen in position C-4 (Figure 4) and this fragment represents the cleavage of these two functional groups. In case of **2** however, the formation of fragment [M – [$^{2}H_{9}$]TMSO]$^{+}$ was unexpected (m/z 442.3260, 2c, Table 2), because the TMS group attached to the C-4 oxygen is not isotopically labelled (Figure 4).

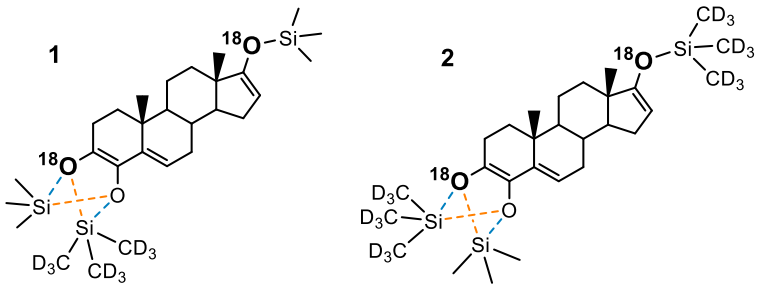

*Figure 4: 4-[$^{2}H_{9}$]TMS, 3,17[$^{18}O_{2}$]-bis-TMS-formestane (**1**) and 4-TMS, 3,17[$^{18}O_{2}$]-bis-[$^{2}H_{9}$]TMS- formestane (**2**) with proposed route of formation of expected ions (blue dashed line) and unexpected ions (orange dashed line) observed in the respective mass spectra (Annex 7.3, Figure 7, p. 96)*

No.	fragment ion	1	Δm	2	Δm
-	[M]$^{\bullet+}$	531.3706	1.13	540.4280	0.56
a	[M – TMSO]$^{+}$	442.3241	10.85	451.3826	6.20
b	[M – TMS^{18}O]$^{+}$	440.3214	7.49	449.3733	17.58
c	[M – [$^{2}H_{9}$]TMSO]$^{+}$	433.2752	6.23	442.3260	6.56
d	[M – [$^{2}H_{9}$]TMS^{18}O]$^{+}$	431.2593	20.64	440.3200	10.67
e	[M – CH_{3} – TMSOH]$^{+}$	426.3070	22.05	435.3436	24.12
f	[M – CH_{3} – TMS^{18}OH]$^{+}$	424.2960	6.13	433.3740	234.26
g	[M – CH_{3} – [$^{2}H_{9}$]TMSOH]$^{+}$	417.2388	5.75	426.2925	11.96
h	[M – CH_{3} – [$^{2}H_{9}$]TMS^{18}OH]$^{+}$	415.2335	8.19	424.2916	4.24

*Table 2: Expected (blue) and unexpected (orange) fragment ions with calculated mass errors observed in the mass spectra of 4-[$^{2}H_{9}$]TMS, 3,17[$^{18}O_{2}$]-bis-TMS-formestane (**1**) and 4-TMS, 3,17[$^{18}O_{2}$]-bis-[$^{2}H_{9}$]TMS- formestane (**2**), proposed structures of fragment ions in Annex 7.3, Figures 8-11, pp. 97-98*

This finding can be explained with a reciprocal exchange of the two TMS groups in positions C-3 and C-4 and was described for pregnanes with vicinal TMS groups in positions C-17 and C-20 before[53,55]. Apart from the required proximity of the involved TMS groups, the non-binding electrons of the oxygen atoms together with the empty 3d orbitals of the silicon atoms seem to play an important role in this "intramolecular scramble"[53].

Other 3-keto-4-ene hydroxy steroids with vicinal or functional groups close enough for bonding such as 2-, 6- or 16-hydroxy-androstenedione should be doubly labelled with ^{18}O and [$^{2}H_9$]-TMS in future studies to evaluate if this mutual exchange of TMS groups is a typical feature of the entire steroid class in general or occurs only in formestane. As a result, the obtained MS/MS data indicates that the reciprocal exchange of TMS groups is not limited to the side chain of pregnanes but also occurs in the A-ring of formestane and should be kept in mind when evaluating other fragment ions derived from this part of the compound, for example *m/z* 147 or vicinal TMS functions in general.

The above-described workflow is limited to those steroids that bear hydroxy- and oxo groups only, are not sterically hindered and can be converted into TMS ether and enol ether via derivatization in a complete manner. This excludes the applicability of this approach to the analysis of corticosteroids or ecdysterone, for example. If the position of a functional group not amenable for silylation is well described, however, even for these compounds new information about GC/MS fragment ions can be derived. In case of 3-ethylbenzophenone (BP-08, Manuscript 1), for example, ^{18}O-labelling helped to clarify the origin of fragment ion *m/z* 105: this ion was shown to correspond to both an ethyl-phenyl ion ($[C_8H_9]^+$) and a benzoyl fragment ion ($[C_7H_5O]^+$), the latter of which was shifted to *m/z* 107 after ^{18}O-labelling. The presence of double bonds in specific fragment ions (except for those generated during enolization) cannot be detected directly with this isotopic labelling approach but may be presumed with the help of accurate mass calculations.

The proposed workflow was only tested on steroids with known structure and is suitable for academic research or in cases where time-consuming procedures are acceptable. The multi-step labelling workflow, however, may be impractical for screening purposes. Overall, the described approach should be regarded as an additional method next to, for example NMR analysis, to gather structural information of partly characterized or new compounds or drug metabolites.

4.3 Interpretation of GC/MS spectra of isotopically labelled derivatives

Electron ionization as a hard-ionization technique provides mass spectra fragmentation patterns and fragment ion abundances that directly correlate with the compound's structure as well as its steric properties.

Fragmentation rules are only applicable to a limited extent and the "one fits all" approach for steroid analysis is neglectable in most cases. Derivatization with TMS significantly affects the observed fragmentation patterns[70] and it is therefore necessary to differentiate between common (TMS-) fragment ions and diagnostic subclass- or substance-specific ions.

For the interpretation of mass spectral data, the observed fragment ions of TMS derivatized compounds can roughly be classified into four different selectivity categories: fragment ions generated through the loss of a TMS group, for example ion $[M - TMSOH]^{\bullet+}$, or methyl group or ion *m/z* 73 (category 1) are considered to be least selective, because they can be found in basically every mass spectrum of TMS-derivatized compounds containing hydroxy and methyl groups. Only when comparing the abundance of the $[M - TMSOH]^{\bullet+}$ ions of different isomers like 5α- and 5β-androstane-3,17-diols (Manuscript 2), valuable stereochemical information may be derived.

Ion *m/z* 181 in case of the benzophenones (Manuscript 1) and ion $[M - 2xTMSOH]^{\bullet+}$ (*m/z* 256.2) in case of 5α-androstane-3α,17β-diol (Manuscript 2) are generated through the cleavage of all formerly attached TMS groups and are referred to as the "backbone ions" (category 2). For these ions, no mass shift is observed after perdeuterotrimethylsilylation and a shift of +2 (or +4) *m/z* units after ^{18}O-labelling is only detectable if oxo-functions are present. Although the abundance of these ions is usually low, they contribute some diagnostic value, especially if the molecular ion is undetectable in the mass spectra.

Category 3 consists of fragment ions that are entirely made up of TMS groups but can serve as diagnostic markers because their formation depends on specific structural features in the analyzed compound: *m/z* 147 for example indicates vicinal or TMS groups in close proximity[9], whereas $[M - TMSO]^{+}$ stands for the (stepwise) cleavage of TMS groups from a part of the steroid molecule where no hydrogen is available for binding and subsequent TMSOH elimination, which in turn indicates the presence of double bonds (Manuscript 3).

Fragment ions that convey the most helpful and diagnostic information for structural assignments belong to category 4. They are generated through partial cleavage of the analyzed compound upon electron ionization and additional rearrangement of TMS groups in some cases. This makes the characterization of their probable structure most challenging, albeit the elemental composition can be described with the help of GC coupled to high-resolution MS.

M/z 169 as a marker for 17-oxo-[11,81], *m/z* 143 for 17α-methyl-[11], *m/z* 206 for 1,4-diene-3-keto-steroids[82], *m/z* 129 and ion $[M - 129]^{\bullet+}$ for dehydroepiandrosterone (DHEA)[83,84] and *m/z* 319 for 6-hydroxy-4-ene-3-ketosteroids[80] are diagnostic subclass or substance-specific ions from category 4, to name a few.

Stable isotope labelling helps to better characterize fragment ions from all four selectivity categories. In fact, fragment ions that were generally considered to be well-described, for example ion $[M - 31]^+$ in the mass spectrum of ketoprofen or the origin of methyl cleavage in 3-keto-4-ene hydroxy steroids, turned out to be generated differently at a closer look. The focus of the presented isotopic labelling approach was the detection and characterization of fragment ions from category 4 and to test their usefulness for the analyte's identification among other closely related compounds.

In case of formestane and its 2-hydroxy isomer 2α-hydroxyandrostenedione, fragment ions *m/z* 267 and 269 were characterized in detail using [$^2H_9$] and [^{18}O]-labelling and the multiple reaction monitoring (MRM) transitions *m/z* 503 → 269 for formestane and *m/z* 503 → 267 for 2α-hydroxyandrostenedione were proposed (Manuscript 3). In this way these two very similar compounds can be distinguished.

5 Summary and Outlook

The proper identification of anabolic androgenic steroids with GC/MS in anti-doping research remains an important topic and stable isotope labelling is a convenient and straightforward way to increase confidence in detection through mass spectral structure characterization. The different labelling methods for the introduction of [$^{2}H_{9}$]TMS and ^{18}O presented in this work were shown to be suitable for the interpretation of benzophenone and hydroxy steroid GC/MS data. Together with HRMS and MS/MS experiments, fragmentation pathways were elucidated and unexpected differences to previously described assumptions uncovered for both substance classes.

The practical applicability was confirmed with the self-explanatory mass shifts observed in the respective mass spectra and the comparatively fast preparational steps of labelled derivatives even with low amounts of analyte. Especially the newly developed ^{18}O-labelling method proved to be valuable for confirmatory analysis. It can be employed independently from silylation procedures and thus also for LC-MS approaches, and no migratory tendencies as opposed to TMS groups were observed in the mass spectra.

All labelling methods described in this work can be performed with the usual laboratory equipment within a few hours. When used for the characterization of unknown metabolites, the number of reference standards required for unequivocal identification, is expected to be narrowed down and unnecessary laborious and time-consuming synthesis avoided.

With the help of isotopically labelled derivatives, differences in the mass spectra of structurally closely related analytes can be determined, which plays a role in the characterization of similar metabolic patterns of endogenous and exogenously administered steroids. In these cases, costly gas chromatography-combustion-isotope ratio mass spectrometry (GC/C-IRMS) for compound identification can be reduced to a minimum or even replaced.

Future work may focus on metabolite identification with the developed stable isotope labelling methods or fragment ion elucidation of other relevant steroid subclasses, such as 17-alkyl or 1,4-diene steroids. The use of protecting groups may be helpful to establish derivatization procedures to isotopically label every single functional group in steroids separately. Furthermore, the ^{18}O-labelling method can be further optimized to be applicable for other compound classes as well and should also be tested in LC-MS(/MS) approaches.

6 Zusammenfassung

Die korrekte Identifizierung anaboler androgener Steroide mittels GC/MS in der Anti-Doping-Forschung bleibt ein wichtiges Thema. Die Markierung mit stabilen Isotopen ist dabei eine praktische und vielseitige Herangehensweise für die massenspektrometrische Strukturaufklärung. Die in dieser Arbeit vorgestellten Markierungsmethoden für die Einführung von [$^{2}H_{9}$]TMS und ^{18}O erwiesen sich als gut geeignet für die Interpretation von Massenspektren von Benzophenonen und Hydroxysteroiden. Zusammen mit hochauflösender Massenspektrometrie und MS/MS-Experimenten konnten Fragmentierungswege aufgeklärt und unerwartete Unterschiede zu bereits beschriebenen Annahmen für beide Substanzklassen aufgedeckt werden.

Die praktische Anwendbarkeit der Markierungsmethoden wurde u. a. durch die selbsterklärenden Massenverschiebungen, die in den jeweiligen Massenspektren beobachtet wurden, bestätigt. Zusätzlich stellt die einfache und schnelle Erzeugung markierter Derivate, die selbst bei sehr geringer Substanzmenge gelang, einen großen Vorteil dar. Insbesondere die neu entwickelte ^{18}O-Markierungsmethode erwies sich als wertvoll für die Strukturbestätigung bestimmter Fragment-Ionen. Diese kann unabhängig von Silylierungsreaktionen, und damit auch für LC-MS Methoden, eingesetzt werden. Außerdem kommt es mit ^{18}O im Gegensatz zu TMS-Gruppen zu keinen Positionsänderungen der Markierung innerhalb des Moleküls

Alle in dieser Arbeit beschriebenen Markierungsmethoden können mit der üblichen Laborausrüstung innerhalb weniger Stunden durchgeführt werden. Bei der Charakterisierung unbekannter Metaboliten wird erwartet, dass die Anzahl der für eine eindeutige Identifizierung erforderlichen Referenzstandards reduziert und somit unnötige, aufwändige und langwierige Synthesen vermieden werden können.

Mit Hilfe isotopenmarkierter Derivate können Unterschiede in den Massenspektren strukturell eng verwandter Analyten bestimmt werden, was vor allem bei der Charakterisierung ähnlicher Stoffwechselmuster von endogen vorkommenden und exogen verabreichten Steroiden eine Rolle spielt. In diesen Fällen kann die kostspielige gaschromatographische Isotopenverhältnis-Massenspektrometrie (GC/C-IRMS) zur eindeutigen Herkunftsunterscheidung auf ein Minimum reduziert oder sogar ersetzt werden.

Mögliche Schwerpunkte zukünftiger Projekte sind die Metaboliten-Identifizierung mit den entwickelten Markierungsmethoden oder die Fragment-Ionen Aufklärung anderer relevanter Steroid-Unterklassen, wie z. B. 17-Alkyl- oder 1,4-Dien-Steroide. Die Verwendung von Schutzgruppen könnte hilfreich sein für die

Entwicklung von Derivatisierungsmethoden, mit denen jede einzelne funktionelle Gruppe in Steroiden separat isotopenmarkiert werden kann. Darüber hinaus kann die ^{18}O-Markierungsmethode weiter optimiert werden, um auch für andere Verbindungsklassen anwendbar zu sein, und sollte mit LC-MS(/MS)-Methoden getestet werden.

7 Annex

7.1 MSTFA Synthesis

Several attempts were made to synthesize N-mehtyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) according to a procedure first described by Donike in 1969[85] (Figure 5). In a round bottom flask on a Schlenk line under argon atmosphere 2.9 ml triethylamine was added to 25 ml dried benzene. While stirring, 2.67 g (=0.021 mol) N-methyltrifluoroacetamide (MTFA) was dissolved in the mixture and 2.6 ml trimethylsilyl chloride (TMCS) was added. After stirring for 30 minutes at room temperature, the precipitate triethylamine hydrochloride was separated. The filtrate was fractionated under vacuum using a Vigreux column. To improve the yield, TMCS was added within 20 minutes in the next run and the reaction mixture was heated for two hours in a water bath under reflux.

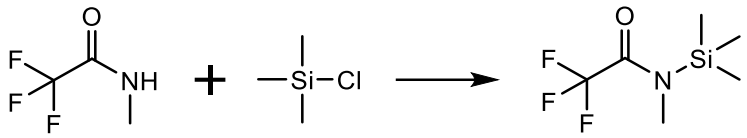

Figure 5: Reaction scheme of MSTFA synthesis after Donike[85] with MTFA and TMCS as educts

The described method proved to be unsuitable due to several reasons: filtering off the byproduct triethylamine hydrochloride resulted in hydrolysis of the product compound and the yield of the synthesis was reduced. The residue was slightly pink in color and turned dark purple when left to stand, indicating impurities. During rectification only the solvent was removed from the reaction mixture, the educt MTFA crystallized out in white needles in the Liebig cooler and no overflow of MSTFA could be observed when reaching the expected boiling temperature. NMR measurements revealed that some product was present in the starting flask but in very small amounts (yield 0.9%) and highly contaminated.

The synthesis is described in literature for a much larger scale (Donike 1 mol MTFA = 127 g, Herebian et al[86] 0.1 mol = 13 g). To keep investment for the deuterated educt [$^2H_9$]TMCS reasonable, however, only the minimum amount of starting material required for the laboratory's glassware was used in the described approach. An alternative synthetic route to MSTFA can be found in an American patent from 1987[87]. In this procedure the silylating agent N, O-bis(trimethylsilyl)acetamide (BSA) serves as the second educt instead of TMCS (Figure 6, p. 94).

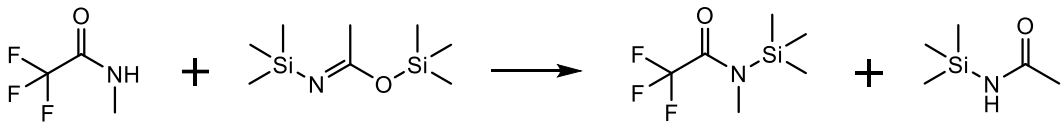

Figure 6: Reaction scheme of MSTFA synthesis after US Patent 4663471[87] with MTFA and BSA as educts and N-trimethylsilyl acetamide as byproduct

A mixture of 5,08 g (= 0,04 mol) MTFA and 11,74 ml BSA was heated in an inert gas atmosphere for four hours at 100 °C under reflux. Distillation was performed at 53 mbar and the desired product was transferred to the product flask at a temperature of 52-54 °C. The yield of about 80% was much higher than in the first procedure. Another advantage of this method is that no other solvent than BSA itself is required and the second product N-trimethylsilyl acetamide does not have to be filtered. As a result, for the synthesis of MSTFA and $[^2H_9]$-MSTFA the second protocol with BSA or $[^2H_9]_2$-BSA as educt is proposed.

7.2 Derivatization protocol for hydroxy steroids with BSA or $[^2H_9]_2$BSA

1. prepare solution of 1 mg/ml steroid in acetonitrile or methanol
2. check number of groups to derivatize per µL of this solution (e.g., formestane: 2 oxo groups, 1 hydroxy group = 3 groups in total)
    a. if 6 µL of formestane 1 mg/ml solution are to be derivatized, the amount of BSA needed must be multiplied by 18 (a different multiplier (6 in the example) must be used and no catalyst is required, if only the hydroxy groups are to be derivatized)
    b. per group to derivatize 0.004 µmol of BSA are needed, which is 18 x 0.004 µmol = 0.072 µmol BSA in total for the example
    c. calculate the volume of BSA needed with its molecular mass (M_{BSA} = 203.43 g/mol) and density (ρ = 0.869 g/L):

        0.072 µmol x 203.43 µg/µmol = 14.657 µg BSA

        14.657 µg / 0.869 µg/µL = 16.867µL ≈ 17 µL

3. prepare BSA/catalyst solution:
    a. solve approximately 50 mg NH_4I in 1 mL mercaptoethanol at 80° C. Add 5 µL of this catalyst solution to 100 µL BSA (colorless solution can turn slightly yellow)
    b. if you need less of the catalyst solution, calculate according to this ratio
    c. you do not need to vortex, as you might lose too much substance
    d. always prepare fresh mixtures of BSA + NH_4I/mercaptoethanol solution, the catalyst solution itself can be used at least six months if stored below 8° C
4. add BSA + catalyst solution to the dried steroid sample
5. put in heating block for 2 hours 75° C or 30 min 90° C
6. inject in GC/QTOFMS (or other instrument), chose standard method for steroid analysis
7. use the molecular mass of 221.54 µg/µmol in the equation above for derivatization with $[^2H_9]_2$-BSA

7.3 Mass spectra of doubly labelled formestane derivatives and structures of fragment ions

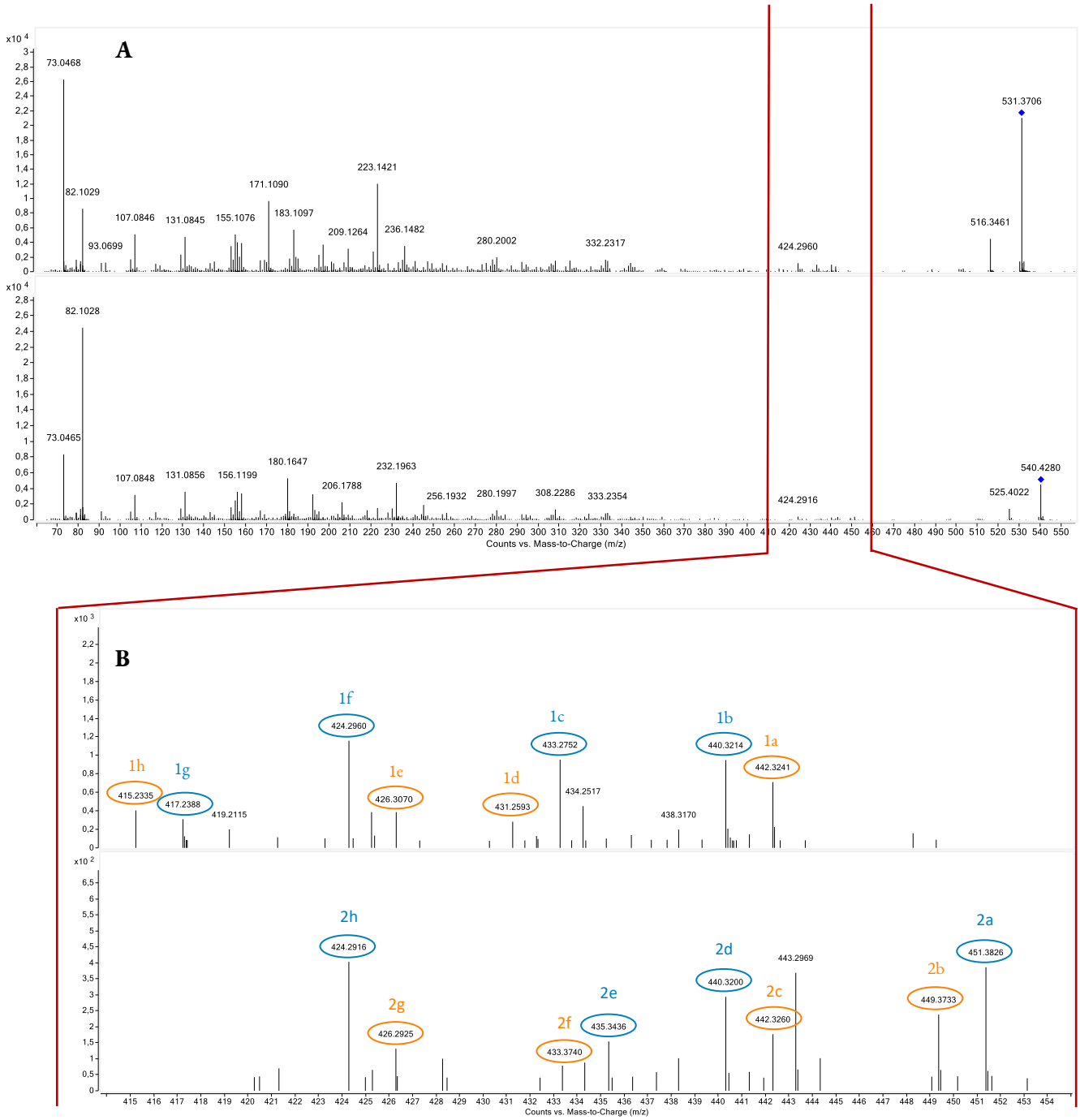

*Figure 7: **A**: MS/MS spectra of 4-[$^{2}H_{9}$]-TMS, 3,17[$^{18}O_{2}$]-bis-TMS-formestane (**1**, above, 25 eV) and 4-TMS, 3,17[$^{18}O_{2}$]-bis-[$^{2}H_{9}$]TMS-formestane (**2**, below, 30 eV), precursor $[M]^{\bullet+}$ **B**: Zoom of **A**, blue circles represent masses of expected, orange circles masses of unexpected fragment ions (see Figures 4,p. 85, 8-11, pp. 97-98 and Table 2, p. 85 for structures and calculated mass errors)*

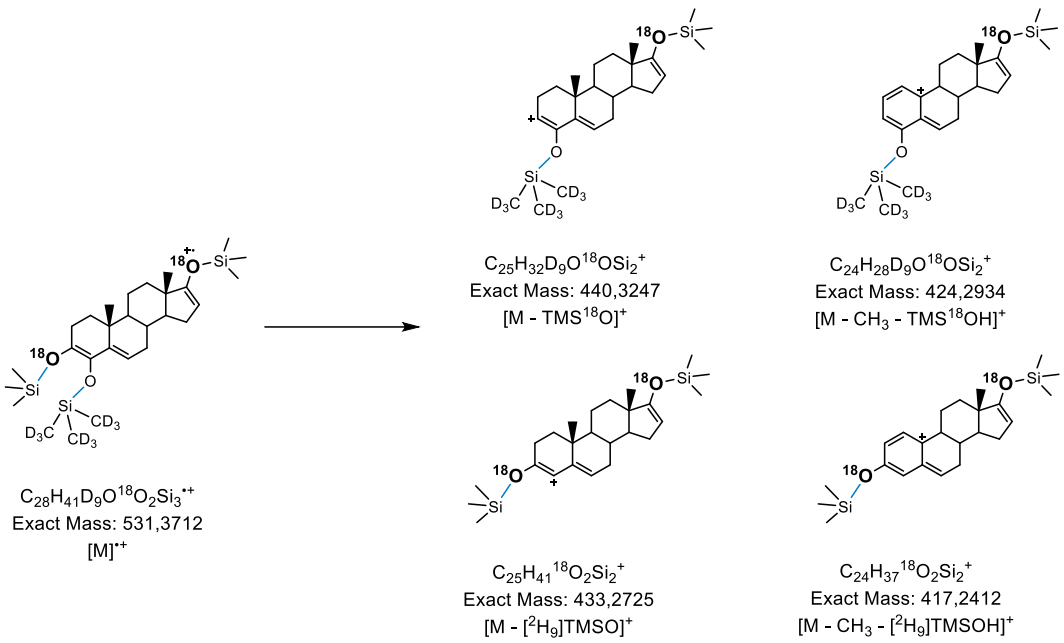

*Figure 8: Proposed structures of expected fragment ions of 4-[$^2H_9$]-TMS, 3,17[$^{18}O_2$]-bis-TMS-formestane (**1**, see Table 2, p. 85 for calculated mass errors)*

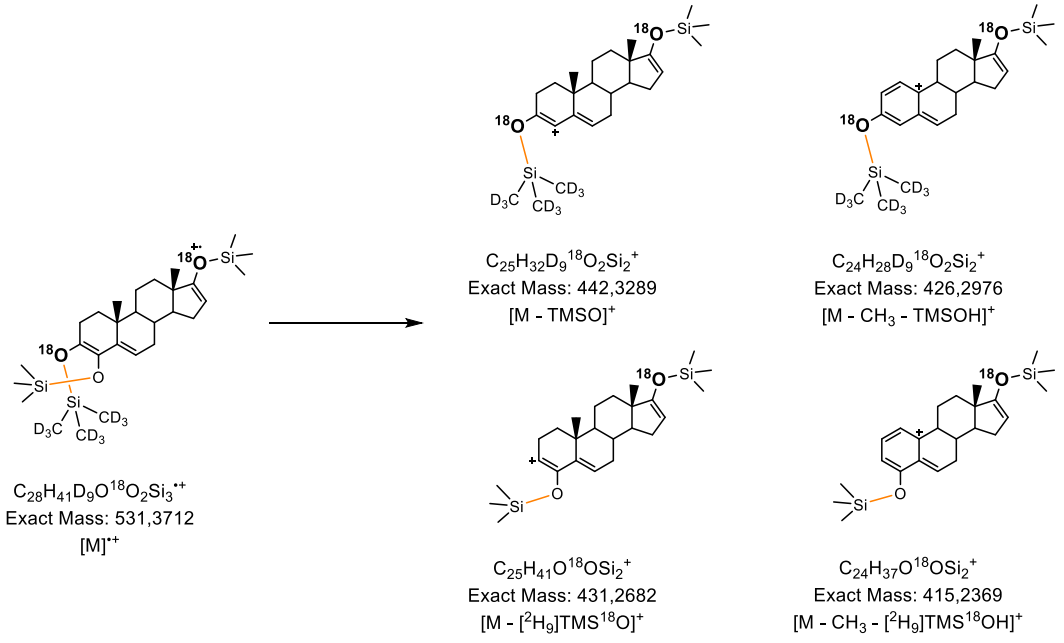

*Figure 9: Proposed structures of unexpected fragment ions of 4-[$^2H_9$]-TMS, 3,17[$^{18}O_2$]-bis-TMS-formestane (**1**, see Table 2, p. 85 for calculated mass errors)*

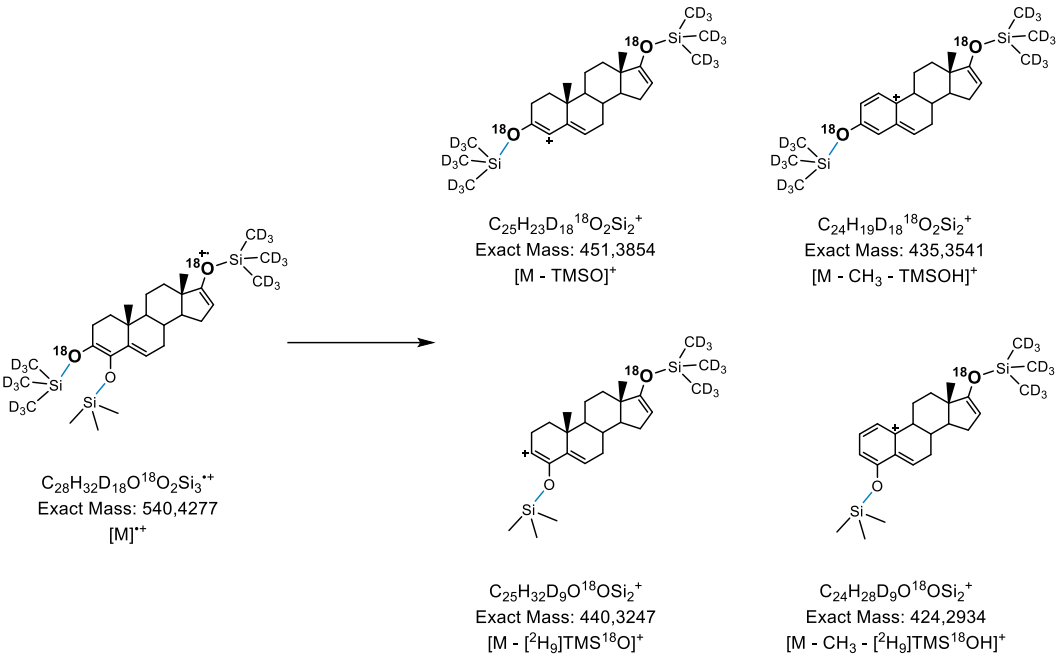

*Figure 10: Proposed structures of expected fragment ions of 4-TMS, 3,17[$^{18}O_2$]-bis-[$^2H_9$]TMS-formestane (**2**, see Table 2, p. 85 for calculated mass errors)*

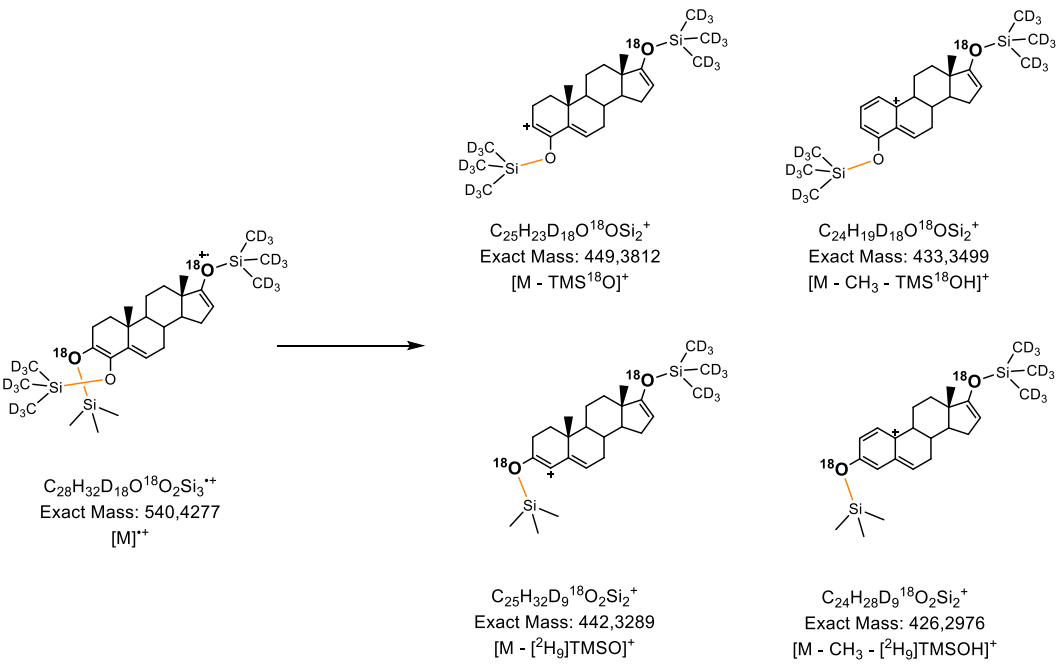

*Figure 11: Proposed structures of unexpected fragment ions of 4-TMS, 3,17[$^{18}O_2$]-bis-[$^2H_9$]TMS-formestane (**2**, see Table 2, p. 85 for calculated mass errors)*

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9 Declaration of own contribution

In the following, the author's contribution to the three publications used for this cumulative work are disclosed:

Manuscript No. 1

- conception and design of the ^{18}O-labelling experiments for ketoprofen and related benzophenones
- execution and adjustment of experiments to respective compound class
- evaluation of the obtained data in cooperation with the co-authors
- manuscript preparation in cooperation with the co-authors

Manuscript No. 2

- conception and design of isotopic labelling experiments
- sample preparation and execution of experiments
- evaluation of the obtained data in cooperation with the co-author
- manuscript preparation in cooperation with the co-author

Manuscript No. 3

- conception and design of isotopic labelling experiments
- sample preparation and execution of experiments in cooperation with the co-authors
- evaluation of the obtained data in cooperation with the co-authors
- manuscript preparation in cooperation with the co-authors

10 Publications and Conference Proceedings

Assaf J, Joseph JF, Kollmeier AS, Gomes DZ, Wuest B, Gautschi P, Parr MK. Mass spectrometric characterization of ketoprofen impurities. *27th International Symposium on Pharmaceutical and Biomedical Analysis (2016), Guangzhou, China*

Kollmeier AS, Joseph JF, Müller C, Botrè F, Parr MK. Mass spectral characterization of trimethylsilyl derivatized androgens. *DPhG Landesgruppentagung Berlin-Brandenburg (2016), Berlin*

Parr MK, Schmidtsdorff S, Kollmeier AS. Nahrungsergänzungsmittel im Sport – Sinn, Unsinn oder Gefahr? *Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz (2017) 314-322*

Kollmeier AS, Cetinkaya E, Joseph JF, Jardines D, de la Torre X, Botrè F, Müller C, Parr MK. Derivatization and mass spectral characterization of isotopically labelled androgens. *GDCh-Wissenschaftsforum Chemie (2017), Berlin*

Assaf J, Kollmeier AS, Müller C, Parr MK. Reconsidering mass spectrometric fragmentation in electron ionization mass spectrometry – new insights from recent instrumentation and isotopic labelling exemplified by ketoprofen and related compounds. *Rapid Communications in Mass Spectrometry (2019) 215-228*

Kollmeier AS, Parr MK. Mass spectral fragmentation analyses of isotopically labelled hydroxy steroids using low-resolution gas chromatography / mass spectrometry: A practical approach. *Rapid Communications in Mass Spectrometry (2020) e8769*

Kollmeier AS, de la Torre X, Müller C, Botrè M, Parr MK. In-depth GC-MS/MS Fragmentation Analysis of Formestane and Evaluation of Mass Spectral Discrimination of Isomeric 3-Keto-4-ene Hydroxy Steroids. *Rapid Communications in Mass Spectrometry (2020) e8937*

11 Curriculum Vitae