TECHNISCHE UNIVERSITÄT WIEN

DIPLOMARBEIT

Synthesis, characterization and application of a marker substance for monitoring 17-keto-modifications in endogenous steroids caused by microbiological activity

Ausgeführt

am Institut für Angewandte Synthesechemie

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unter der Anleitung von

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Abstract

Endogenous anabolic androgenic steroids (EAAS) are a common substance class used for doping in sports. One of the most frequently reported anabolic androgenic steroids used in doping cases is testosterone. The urinary steroid profile for monitoring the individual testosterone level of athletes includes concentrations and ratios of various endogenously produced steroidal hormones and metabolites. Due to microbiological contamination the concentrations of these endogenous steroids may alter, which leads to misinterpretation of analytical results. Cases have been observed were alterations in position 17 of the endogenous steroids happened due to microbiological activity. The 17-keto group is reduced to a 17ß-hydroxy group, leading to changes in concentrations of the urinary steroid profile. The aim of this work is to expand the monitoring system for microbiological contamination of urine samples. A synthesized substance, which is also a suitable substrate for 17-keto reduction is used to be a marker for this kind of microbiological activity. It should be implemented in routine analysis of doping control laboratories as internal standard. The proof, that one of the synthesized substances, 3ß-ethoxy-5αH-androstane-17-one, is fit for purpose, is shown by treatment with bacterial contaminated urine which leads to conversion into 3ß-ethoxy-5αH-androstane-17ß-ol.

Deutsche Kurzfassung

Endogene anabole androgene Steroide (EAAS) sind eine Substanzklasse, die häufig für Doping in Sport angewendet wird. Bei einer der am öftesten verwendeten Substanzen dieser Klasse handelt es sich um Testosteron. Das aus dem Harn gemessene Steroid Profil zur Überwachung der individuellen Testosteronkonzentration der Sportler umfasst Konzentrationen und Verhältnisse verschiedener endogen produzierter Steroidhormone und derer Metaboliten. Aufgrund mikrobieller Kontamination des Harns kann es zu Änderungen dieser Konzentrationen kommen, was zu Fehlinterpretationen analytischer Ergebnisse führen kann. Es traten bereits Fälle auf, bei denen es zu Änderungen des Steroidprofils aufgrund von mikrobiologischer Aktivität kam. Die Ketogruppe in Position 17 der endogenen Steroide wird zu einer 17ß-Alkoholgruppe reduziert, was zu Konzentrationsänderungen der analysierten Steroidhormone führt. Das Ziel der Arbeit ist, das Überwachungssystem der mikrobiologischen Aktivität im Harn zu erweitern. Eine Substanz wird synthetisiert, welche ein geeignetes Substrat für das beschriebene Enzym darstellt. Die Substanz soll in die Routineanalytik von Dopingkontrolllaboratorien als interner Standard eingeführt werden. Der Beweis, dass eine der hergestellten Substanzen, 3ß-ethoxy-5αH-androstan-17-on, ihren Zweck erfüllt, wird mittels mikrobiologisch infizierten Harns erbracht, welcher die Substanz in 3ß-ethoxy-5αH-androstan-17ß-ol umwandelt.

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1. Introduction

1.1 Doping in Sports

Doping can be understood as an attempt of athletes to improve their performance in sports through administration of illegal pharmaceutical substances or the application of prohibited methods. Even thousands of years ago athletes were trying to achieve an advantage towards their competitors by manipulating equipment, corruption or the intake of different mushrooms or special plants to enhance their physical performance. These early attempts would possibly be referred to the understanding of “doping” nowadays.

In the 19th and 20th century the usage of doping has risen with the medical and pharmaceutical knowledge and production. More and more attempts of enhancing the performance in sports were conducted and reported – as well with humans and animals (e.g. horse racing). (1) (2)

The World Anti-Doping Agency (WADA), established in 1999, is an independent organization which fights against doping in sports in all forms. Their mission is to create a doping-free environment and greater sportsmanship in competitions. Every year an updated “List of prohibited substances” is released. The substances and methods on this list are prohibited, because they enhance sport performance, are a health risk to the athlete or against the spirit of sports. The focus for this work is on the substance class containing steroids which contribute almost a half of all positive doping cases. (3) (4)

1.2 Steroids

The basic ring structure of steroids derives from a phenanthrene ring structure to which a pentano ring is attached. It is also called sterane ring structure and is pictured in Figure 1.

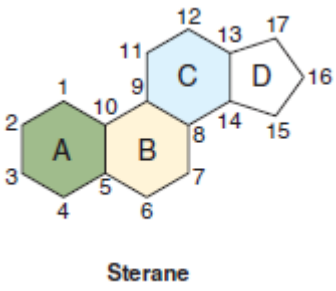


Figure 1: basic ring structure of steroids (5)

The nomenclature of the rings A-D as well as the numbering of the core is defined by IUPAC. Methyl groups at angular positions 10 and 13 are described with the numbers 18 (position 13) and 19 (position 10). The numbers are used to describe the position of double bonds or

substituents. An important structural feature of steroids is the presence of asymmetric carbon atoms. Any steroid compound contains at least six chiral centers. In all natural compounds the configuration at several centers (8ß, 9α, 10ß, 13ß) is constant which leads to less possible configurational isomers. α describes the position of substituents below the plane rings, ß is referred to substituents above the plane ring fusion. The junction between the rings A and B is trans, if the 19-methyl group and the hydrogen in position 5 are on different sides of the ring fusion (5α hydrogen). If A and B are merged cis (5ß hydrogen), the A ring is bent into a second plane that is at approximately a right angle to the B:C:D rings.

The sterane core structure of steroids is lipophilic and is mostly modified on the periphery with polar groups. Although the analysis of many steroids is possible without derivatization by means of GC-MS, a better separation and elucidation of the analytes is accomplished by trimethylsilylation of hydroxyl and carbonyl groups. (1) (5) (6)

1.3 Endogenous anabolic androgenic steroids (EAAS)

1.3.1 Biosynthesis of Testosterone

The most prevalent steroid in all animals is cholesterol, which is also the starting point in the biosynthesis of testosterone and other androgens. The group of androgens is characterized by the presence of a keto-function in position 3 and an absence of a side chain on carbon-17 containing 2 carbons. The steroids with androgenic function which appear naturally most of all in the human body are 5α-dihydrotestosterone (5α-DHT), testosterone (T), androst-4-ene-3,17-dione (A-dione) and dehydroepiandrosterone (DHEA), which are shown in Figure 2.

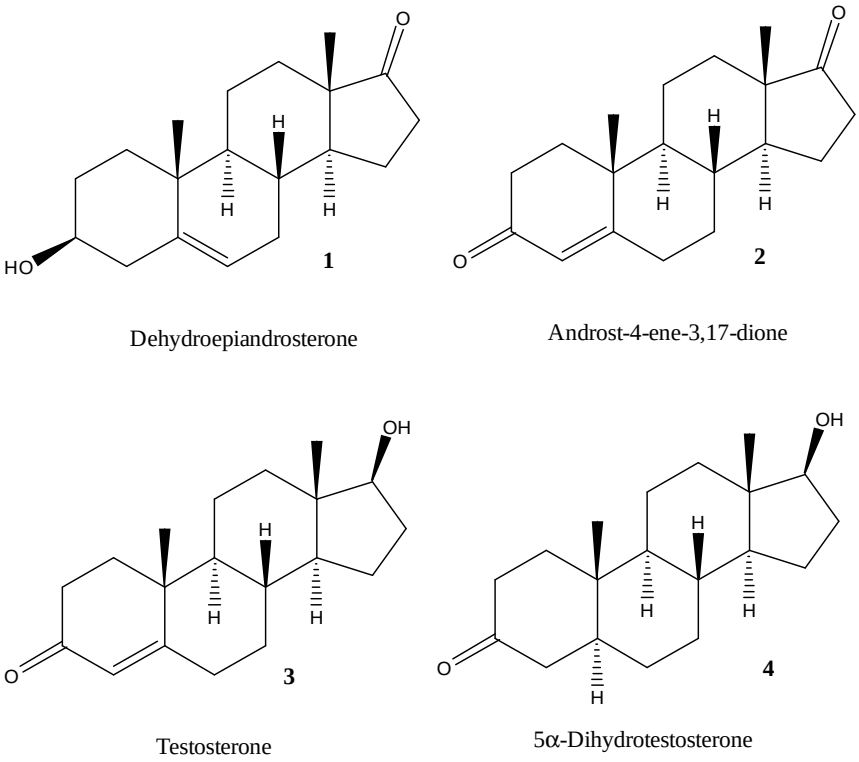


Figure 2: naturally most occurring androgens in the human body

The androgens are produced mainly in the testis in males and in much smaller amounts, mainly by the ovaries, the placenta and the adrenal gland in women. There are two metabolic pathways leading from cholesterol via pregnenolone to testosterone (Figure 3). From pregnenolone, the path splits in the Δ5- and Δ4-pathway. In the Δ5-pathway, the cleavage of carbon 20 and 21 takes place in a first step, followed by the oxidation of the 3ß-hydroxyl group and the migration of the double bond from the Δ5 to the Δ4 position. In the Δ4-pathway, the order of the two steps is reversed. In humans, the Δ5-pathway takes place predominantly and is illustrated in Scheme 1. (5) (7)

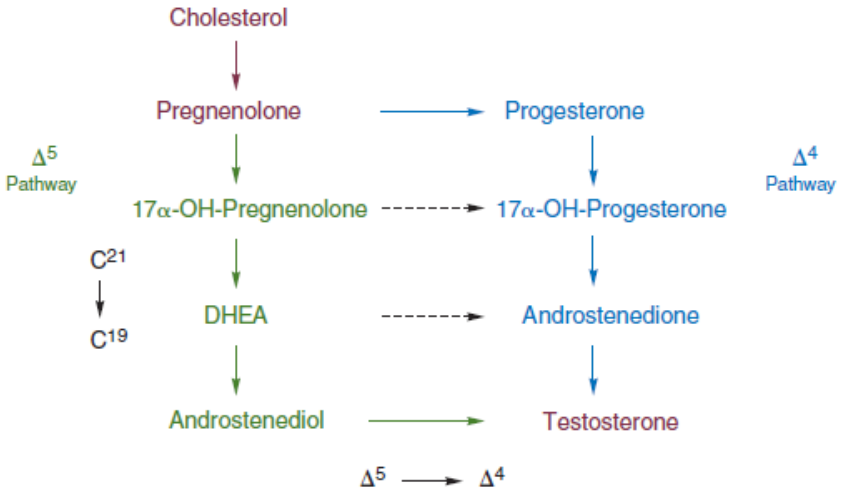
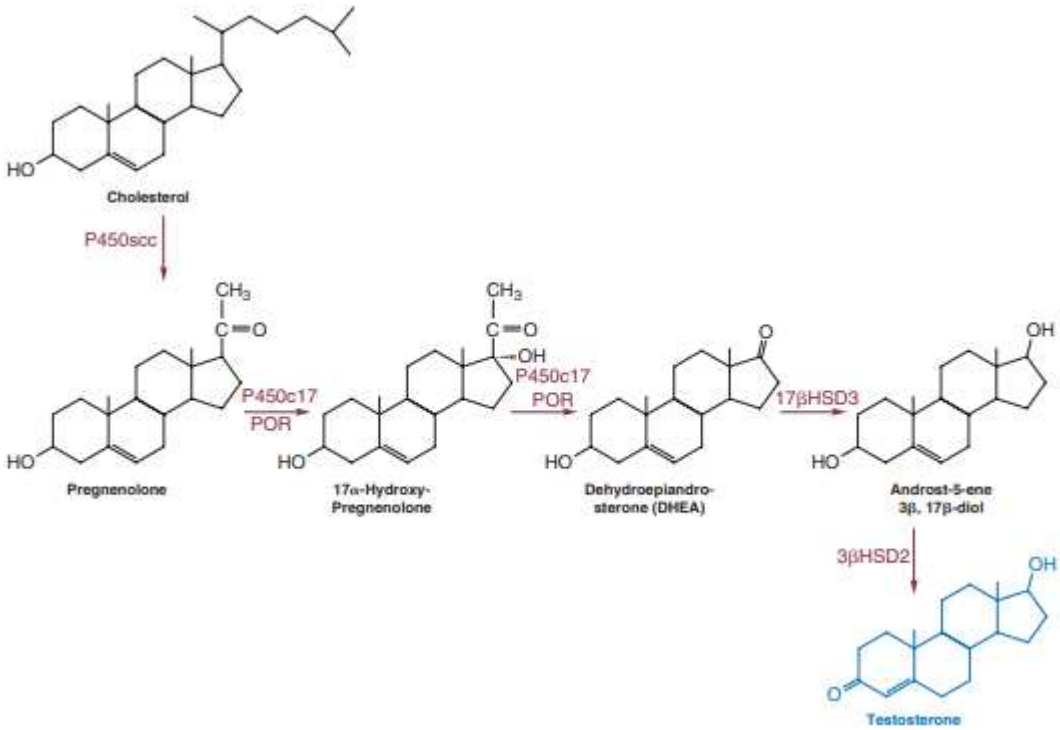


Figure 3: Δ5- and Δ4- metabolic pathway leading to testosterone (5)



Scheme 1: biosynthesis of testosterone via the predominantly Δ5-pathway (5)

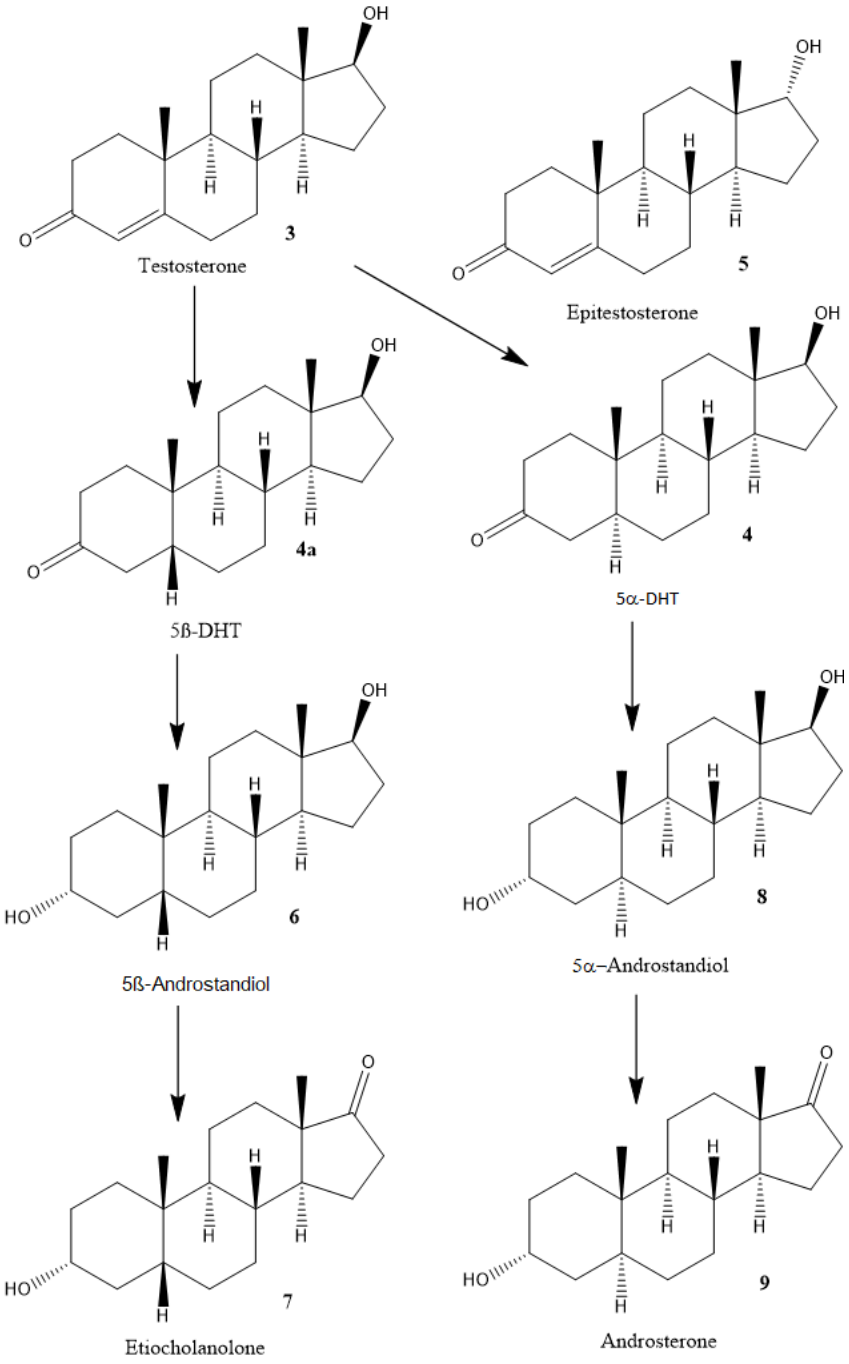
The main androgenic steroid hormone testosterone plays an important role in the maintenance and growth of the sexual reproductive tissues. Also, the growth of secondary sex characteristics and the growth of the skeletal and skeletal muscles are dependent on the androgen.

In the 1930s, the isolation and crystallization of testosterone as a pure substance was carried out successfully for the first time by E. Lacqueur and coworkers. The elucidation of the structure and synthesis of the compound was published by A. Butenandt, who won the nobel prize in 1939 for his achievement. (8)

1.3.2 Metabolism

The goal of metabolism is to increase polarity of endogenous and exogenous substances, to excrete them in urine or bile. In phase I metabolism, enzymatic catalysed reactions like hydrolysis, reduction or oxidation take place. Phase II metabolism includes enzymatic conjugation with glucuronic acid or sulphates, leading to more polar compounds which are excreted more readily.

In the liver testosterone undergoes reduction of the double bond, forming 5α and 5ß isomers, followed by hydrolysis of the keto group in position 3. The alcohol in position 17 can be oxidized. An overview of the metabolism leading to the most important metabolites in doping analysis is given in Scheme 2. In phase II, the metabolites are further metabolized via conjugation with glucuronic acid and, to a much smaller amount, with sulphate. The substances are released back into the circulation and are excreted from the body via the urine. The stereoisomer of testosterone, epitestosterone, which is also shown in Scheme 2, is a physiologically inactive metabolite which is built during the biosynthesis of testosterone as a side product. (5) (9)



Scheme 2: phase I metabolism of testosterone in the liver

1.3.3 Role in Doping

Using endogenous substances for doping is very common and popular among athletes. One of the most frequently reported anabolic androgenic steroids found in doping cases is testosterone. It is available in a wide range of application forms and dosages. It can be used

as gel or film for transdermal absorption, as injection of suspensions or dissolved in oil. Converted to a 17ß-undecanoate ester, it is available for oral uptake.

In sports, anabolic androgens are misused by athletes to build strength and muscle mass. The use of androgens for this purpose encloses a big variety of sport disciplines, especially for power sports and bodybuilding.

Excess androgens at any point in adult life can have masculinizing effects on the female, manifested as excess hair growth, voice changes and changes in body composition. Negative side effects on male and female with prohibited androgen use are overall increased mortality, cardiac and liver pathology, abnormal plasma lipoprotein levels and changes in the behaviour. As mentioned before, androgens have an important role in promoting the growth of the male reproductive system, including the prostate gland. It is proven, that the early stages of prostate cancer are androgen dependent. (2) (5)

1.3.3.1 Synthetic anabolic steroids

Anabolic steroids are synthetic analogs of testosterone, which show selectivity for the anabolic effects on skeletal muscle rather than androgenic effects, like stimulating growth of the sexual reproductive tissues. The reason for this selectivity is better interaction with the androgen receptor in target skeletal tissues and the resistance of the molecule to metabolize into dihydrotestosterone or estradiol. For medical purposes anabolic steroids are administered to treat diseases with muscle wasting, like cancer or AIDS, and in special forms of osteoporosis. (2) (5)

1.3.4 Athlete's Biological Passport (ABP)

After the prohibition of testosterone in sports, the first proposal as marker for its misuse was the ratio of testosterone to epitestosterone (T/E ratio), excreted as glucuronoconjugates in urine. As mentioned before, epitestosterone is a side product in the biosynthesis of testosterone and is almost not influenced by the intake of exogenous testosterone. Because of that it is chosen as a reference value, which remains the same, also if exogenous testosterone is applied. A threshold of 10, later reduced to 6 for this value was introduced by Manfred Donike in the beginning. A value exceeding this threshold was considered to reflect the administration of testosterone. Shortly after the introduction it became obvious, that some athletes have naturally elevated urinary T/E values. (2)

It was recognized, that the detection and calculation of the T/E ratio in the initial testing procedures (ITP) allows no final conclusion about an abuse, because the concentrations and

ratios of endogenous steroids show individual differences. To expand the monitoring of the endogenous steroids, in 2014, the Athlete's Biological Passport (ABP) was implemented in all WADA accredited laboratories. The steroidal module uses six steroidal parameters as well as ratios of these parameters. Whenever an athlete's urine sample is tested, the values are added to his passport and the changes of the steroid profile can be observed over the time.

Ratios of the six relevant "markers of the steroid profile" are shown in Figure 4. The passport includes testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α- (5a-diol) and 5ß-androstanediol (5b-diol).

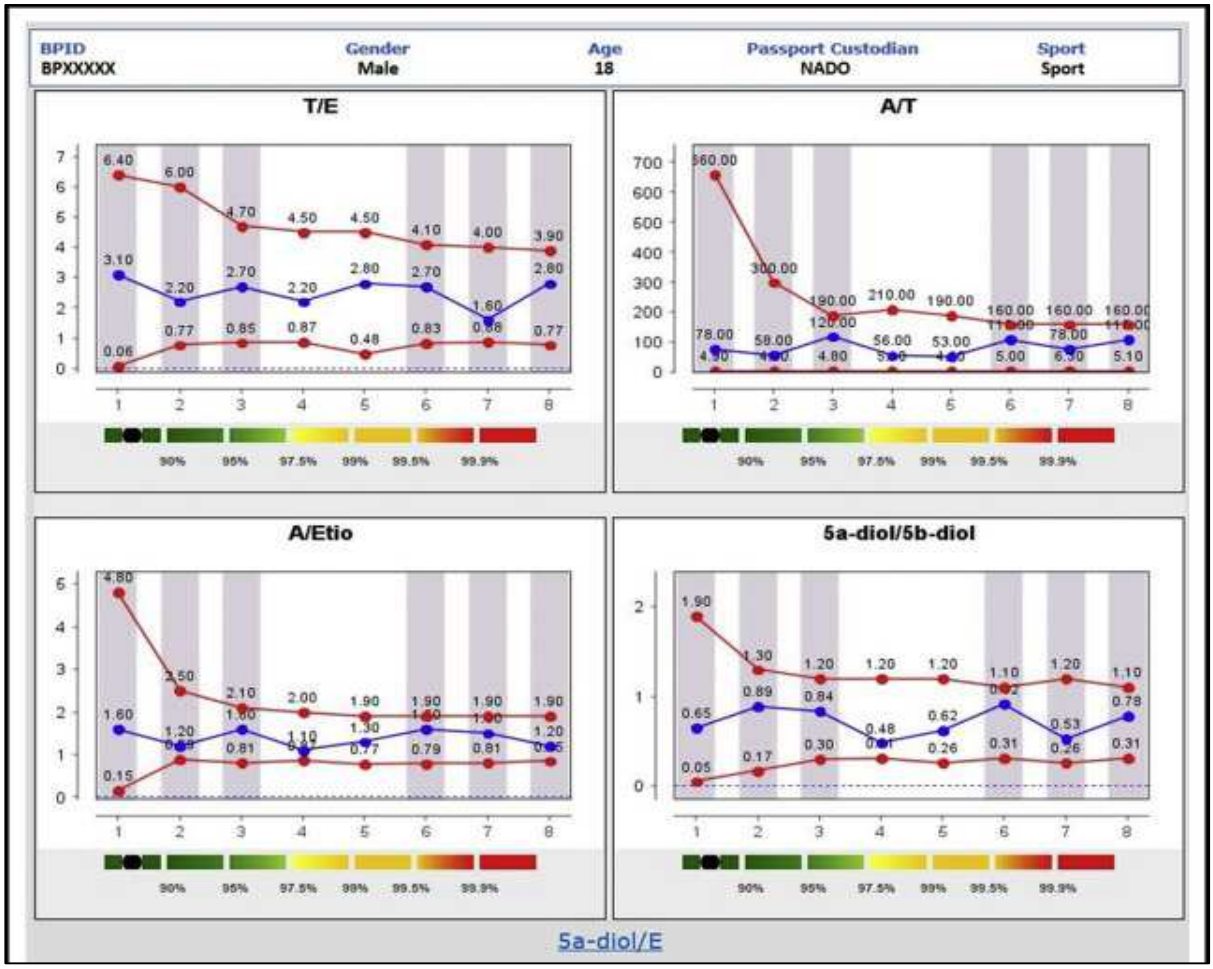


Figure 4: ratios determined for the ABP of an athlete. 8 samples of one athlete were tested and are shown over the time. The figures for 4 calculated ratios are shown. (10)

There are also some other challenges to overcome when it comes to the quantification of testosterone and its metabolites. There are some "confounding factors" which can influence the concentration of the markers of the steroid profile. For example, a rise of the T/E ratio can be caused by the intake of alcohol. Substances, like 5α-reductase inhibitors, can interfere with the natural metabolism of testosterone and change the concentration of its metabolites.

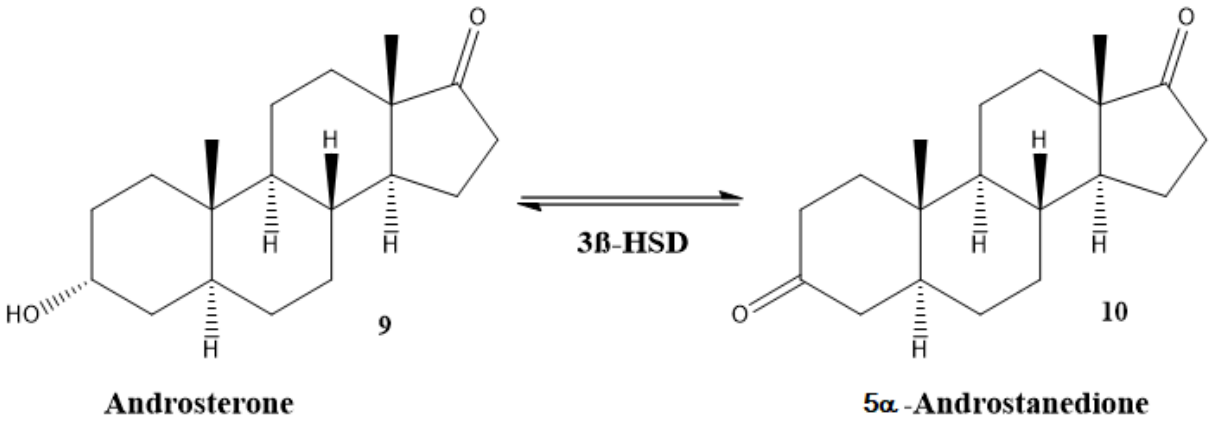
Another confounding factor is microbiological growth, which will be discussed in detail in the following chapter. (11)

1.3.5 Microbiological growth

Bacteria can be present in urine due to urinary tract infections or the urine can be contaminated by non-sterile conditions during the sample collection. Also, elevated temperatures during the transport or the storage of the urine samples can lead to bacterial contamination and growth. There have been many studies about the transformation of steroids caused by microorganisms found in human urine. The hydrolysis of glucuronide and sulphate conjugates has been observed. In addition, the steroid structure can be modified by oxidoreductive reactions (12) (13)

WADA is compiling this knowledge in the form of technical document (TD EAAS). Markers for microbiological contamination are 5α-androstanedione and 5ß-androstanedione. These substances occur normally at very low levels but can increase by reactions caused by microorganism. (11)

In Scheme 3, such a reaction is shown for the formation of 5α-androstanedione. If the ratio 5α-androstanedione/androsterone is ≥ 0.1 (or 5ß-androstanedione/etiocholanolone ≥ 0.1), the sample is regarded as invalid. The determined values for the steroid profile are not added to the ABP.



Scheme 3: reaction occurring by microbiological infected urine monitored according to TD EAAS

As mentioned before, also the deconjugation of the steroid from glucuronic acid can be an indicator for bacterial contamination. If the deconjugation rate is higher than 5 % of the total amount of testosterone, the sample is also regarded as invalid.

The observation of these parameters is applied in the routine analysis for monitoring microbiological contamination of urine samples.

As the consequence of initial testing of a certain number of urine samples significant alterations of some parameters of the steroid profile under certain conditions have been observed.

In Figure 5 the concentrations of the endogenous steroid marker substances of one sample are shown, which was prepared under two different conditions. The urine sample has been analysed on receipt, before it got frozen and stored under -20 °C. The hydrolysis took place at 37 °C overnight. Later, after the sample was frozen, it was prepared and analysed again with the hydrolysis step taking place at 50 °C for 2 hours. Both preparation conditions are standard operating procedures.

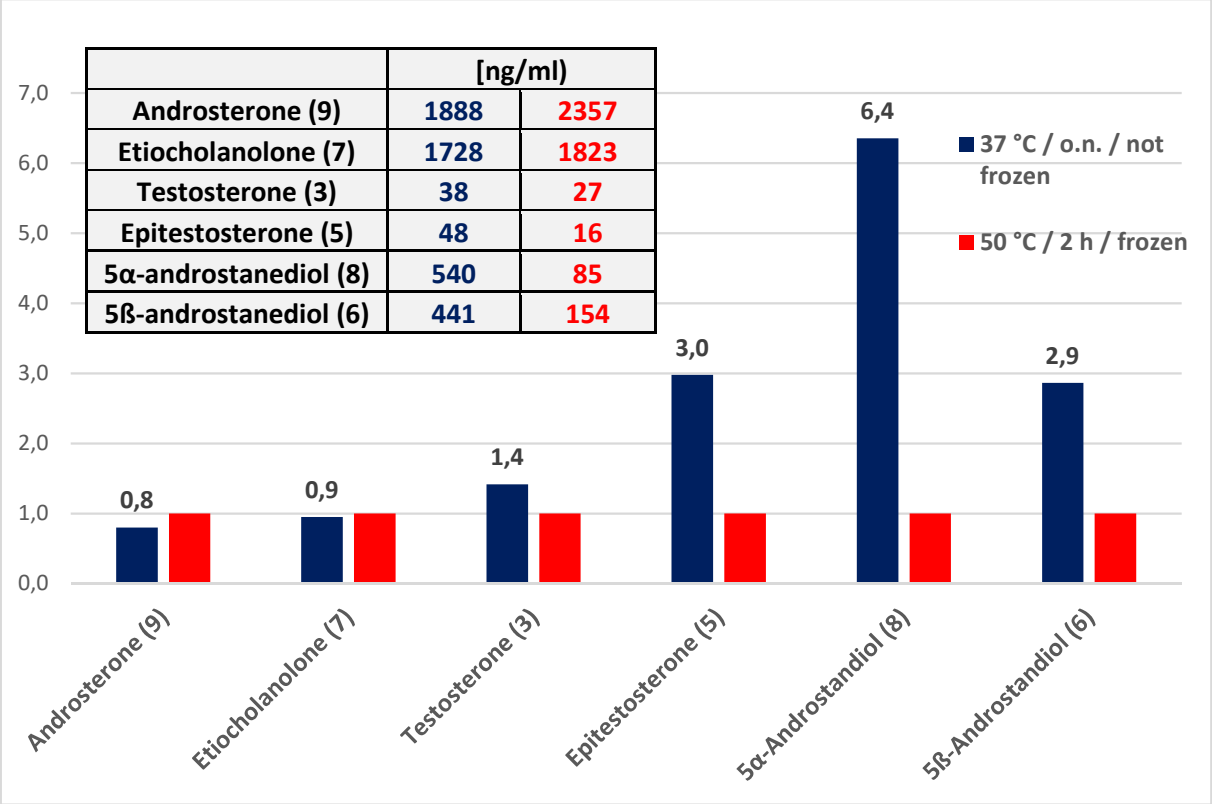
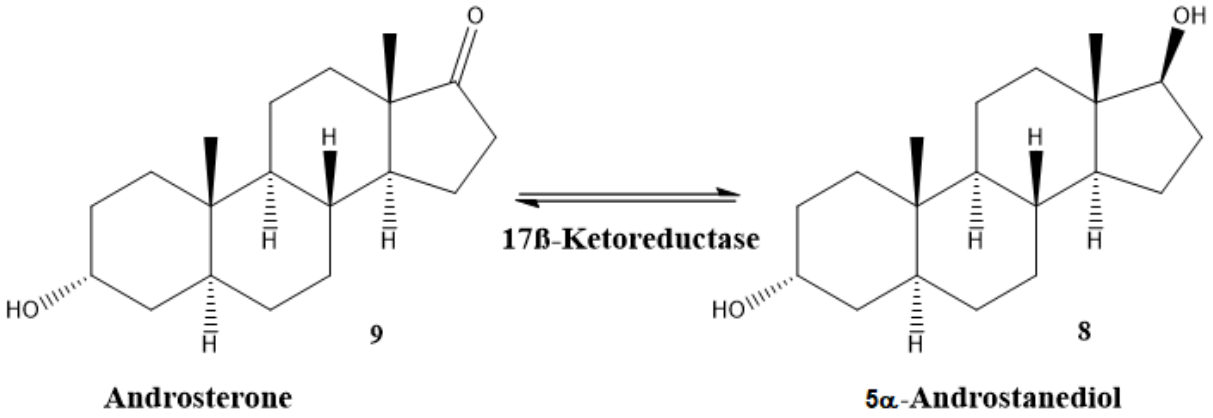


Figure 5: Concentrations of endogenous steroids marker substances:
37 °C/o.n./not frozen (blue): Preparation/analysis before freezing. Hydrolysis: Incubation 37 °C overnight.
50 °C/2h/frozen (red): Preparation/analysis after freezing. Hydrolysis: Incubation 50 °C 2 hours.

Comparing the concentrations of the markers of the steroid profile for the two cases, significant differences in the concentrations especially for **6** and **8** were observed.

This sample and the other affected samples were not invalid regarding the definition in the TD EAAS. The formation of the 5α- and 5ß-androstanediones was not taking place in a significant

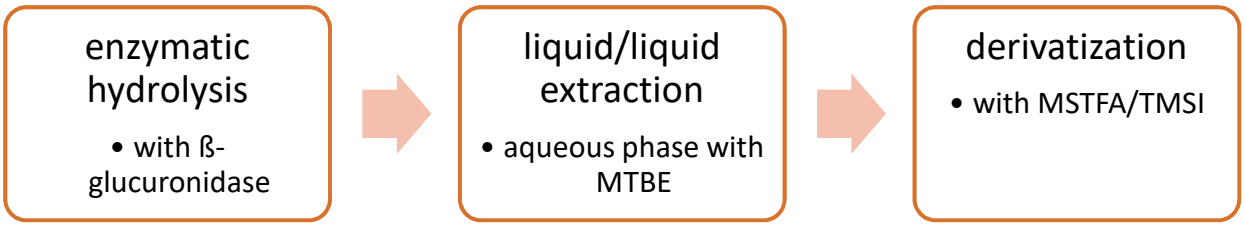
scale. A different reaction, which is not monitored by now, occurred and is shown in Scheme 4. Reduction of the keto group in position 17 took place.



Scheme 4: ketoreduction reaction occurring by microbiological infected urine

1.4 Analytical Methods

The general work up strategy for the analysis and quantification of EAAS includes three important steps, shown in Scheme 5.

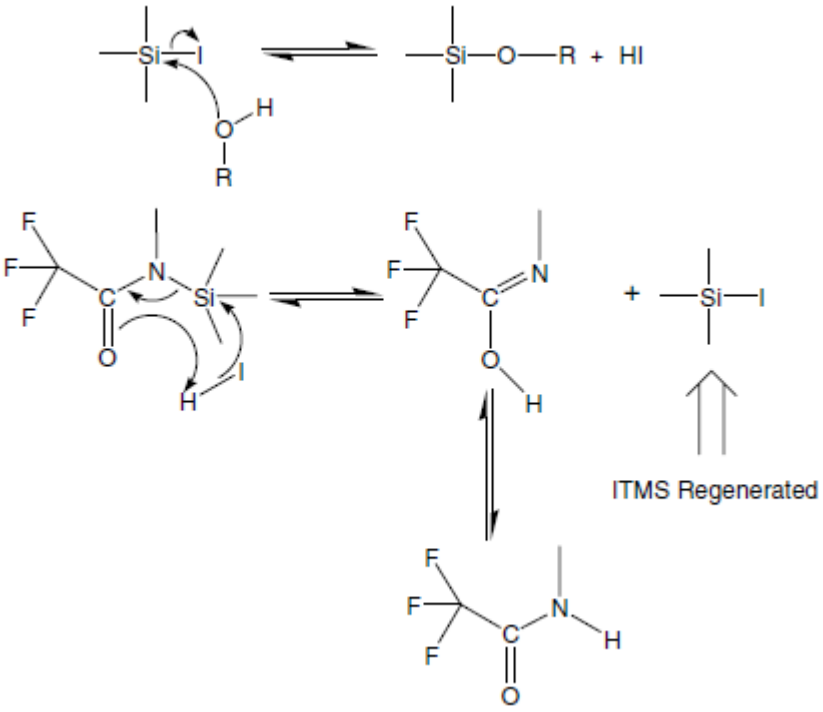


Scheme 5: general work up process for analyzing EAAS

Enzymatic hydrolysis with the enzyme ß-glucuronidase is carried out at pH = 6.8 at elevated temperatures to cleave the glucuronic acid off the steroids. After changing the pH to 9.8 the activity of the enzyme is stopped. To separate the free steroids from salts, sugars and other matrix components a liquid/ liquid extraction with the organic solvent methyl-t-butyl ether (MTBE) is carried out. The biphasic solution is shaken and centrifuged. The organic phase is separated from the aqueous phase and is evaporated. The samples are dried in a vacuum chamber to be water-free for the derivatization step. The dry residue is mixed with the

derivatization agent mixture N-methyl-N-(trimethylsilyl)trifluoroacetamide/trimethylsilyliodine (MSTFA/TMSI) and heated up to 60 °C. (14)

To obtain full derivatization of the analysed compounds (e.g. the IS d3-testosterone), MSTFA including TMSI is used for the derivatization of steroids. TMSI is activating the MSTFA, for double and triple silylation. A possible reaction mechanism for silylation with MSTFA/TMSI is shown in Scheme 6.



Scheme 6: catalytic activity of TMSI for silylation (6)

1.4.1 GC-MS(MS)

The analysis of the derivatized samples is carried out via gas chromatography – tandem mass spectrometry. The substances separated by chromatography enter the mass spectrometer where electron ionization (EI) is employed. The measurements are carried out in selected reaction monitoring mode (SRM). For each substance, a precursor ion with defined mass/charge ratio (m/z) is selected in the first quadrupole, is taking to collision in the second quadrupole with argon and the fragments (product ions) with defined m/z are selected in the third quadrupole for detection. The selected masses of precursor and product ions form a characteristic “transition” of each substance. SRM mode is highly specific and eliminates

matrix background. It is the method of choice for quantification of low levels of target compounds in the presence of high sample matrix background.

1.4.2 Confirmation of exogenous testosterone by GC-C-IRMS

The gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) is carried out to confirm the endogenous origin of the steroid hormones. This important test can be triggered by the ABP, if there is a value or ratio outside defined borders or requested by the Testing Authority (TA) based on other information. The GC-C-IRMS testing shows a higher percentage of Adverse Analytical Findings (AAFs) compared to other methods. (4)

1.4.3 Identification criteria

WADA declares in the TD IDCR the *"Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes"*. The chromatographic criteria for identification of a substance is that the retention time (RT) shall not differ more than ±0.1 minutes. The MS criteria for identification are based on the presence and relative abundance of at least two ions which are characteristic for the substance and are determined by the laboratory. Relative abundances shall be calculated by dividing the area of the less intense transition by the area obtained from the transition of the most abundant diagnostic ion taken as the base peak. There are tolerance windows for the relative abundances to ensure the identification of a substance, shown in Table 1. (15)

Table 1: Maximum tolerance windows for relative abundances to ensure appropriate confidence in identification (15)

Relative Abundance in the reference specimen [5] (% of base peak)	Maximum Tolerance Windows for the Relative Abundance in the *Sample*	Examples	
		Relative Abundance (% of base peak)	Tolerance Window (% of base peak)
50 - 100	±10 (absolute)	60 95	50-70 85-105
25 - 50	± 20% (relative)	40	32-48
1 - 25	±5 (absolute)[6]	10 3	5-15 >0 [6] – 8

[5] Spiked sample, Reference Collection sample, or Reference Material analyzed in the same analytical batch.

[6] The diagnostic ions must always be detected in the *Sample* (S/N > 3:1).

1.5 Objectives

The above-mentioned degradation processes of endogenous steroids caused by microorganism can have a tremendous influence on the urinary endogenous steroid profiles and can lead to misinterpretation of analytical results. Therefore, the monitoring of bacterial activity in urine samples should be extended. Beside the reactions caused by the enzyme alcohol-dehydrogenase, which is already monitored and defined in the WADA technical document TD EAAS, also 17-keto reduction activity should be monitored. It is important to have an independent instrument for monitoring this activity and to improve the understanding of alterations in the steroid profile. With the proof that a sample is microbiological contaminated retesting of the athlete can be required to get valid parameters for the ABP.

The main goal of the project is to synthesize a substance, similar to an endogenous anabolic steroid, for monitoring the bacterial contamination of urine samples. The steric and electronic properties of the synthesized substance should not differ too much from the analyzed endogenous steroids, because it should be taken as substrate by the same enzyme. After observation that the effect of 17-keto reduction in microbiological contaminated urine happens preferentially on androsterone, a substance sterically like this steroid with similar electronical properties would be required. It is also important, that the synthesized substance cannot occur in the urine from human metabolism so that a clear statement can be made, if there is microbiological activity causing ketoreduction in the urine.

Beside the synthesis of this substance also its full characterization and analysis under ITP conditions is important to find a substance, which can be used for monitoring 17-keto activity in the initial testing of urine samples in anti-doping control laboratories. Also, the proof, that the synthesized substance is capable of its purpose, should be shown by treatment with the enzyme ketoreductase or urine showing bacterial contamination.

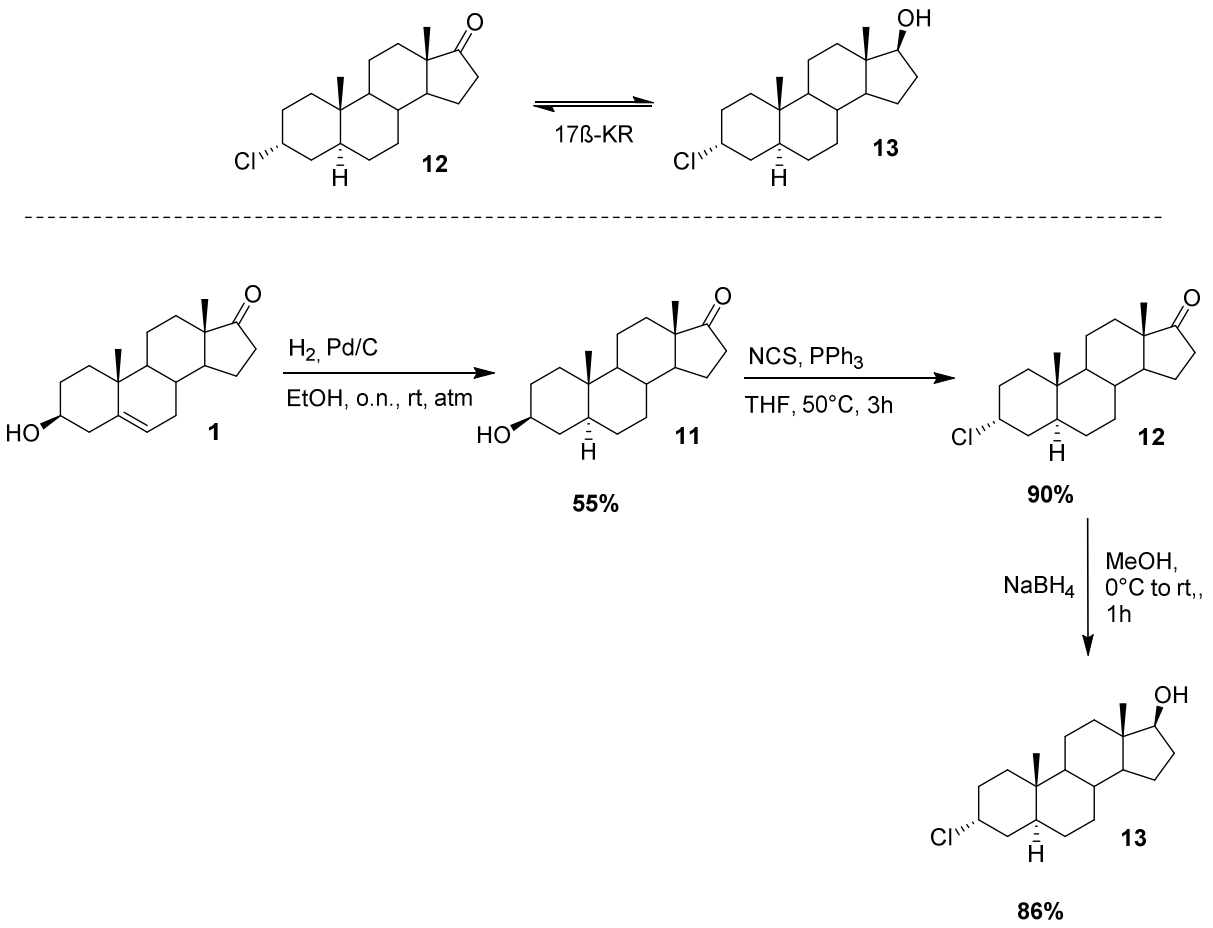
2. Results and discussion

The characterization of the substances was carried out by NMR and GC-MS(MS) methods. The corresponding spectra and chromatograms are listed in the ANNEX.

2.1 3α-Chloro-5αH-androstane-17-one/3α-chloro-5αH-androstane-17ß-ol

2.1.1 Synthesis

The synthetic steps leading to 3α-chloro-5αH-androstane-17-one (**12**) and the corresponding reduced compound 3α-chloro-5αH-androstane-17ß-ol (**13**) were carried out according to literature and are pictured in Scheme 7. (16) (17) (18)



Scheme 7: synthetic route for 3α-chloro-5αH-androstane-17-one and 3α-chloro-5αH-androstane-17ß-ol

The first step in the synthesis was the reduction of the starting material **1** via stereoselective hydrogenation in presence of the catalyst palladium to get the 5αH stereoisomer **11**. For

installing the chlorine in position 3 the first approach was via an Appel reaction. (19) Poor yields were obtained; a different approach with thionyl chloride was carried out, leading again to poor yields. (20) The chlorination via N-chlorosuccinimide and triphenylphosphine shown in Scheme 7 was the method of choice for the synthesis of **12**, with a yield of 90 %. For the reduction of the 17-keto group, the mild reduction reagent $NaBH_4$ was used to obtain **13** (Yield 86 %).

2.1.2 Analytical evaluation

GC-MS without derivatization

The chromatograms and mass spectra of **12** and **13** without derivatization are shown in the ANNEX.

GC-MS(MS) with derivatization

The analysis of the two substances was carried out by derivatization with the derivatization reagent MSTFA/TMSI. The full scan chromatogram of the samples shows, next to the peak of the internal standard (IS) d3-testosterone and the expected substance, a third peak. The chromatogram including mass spectra is shown in Figure 6.

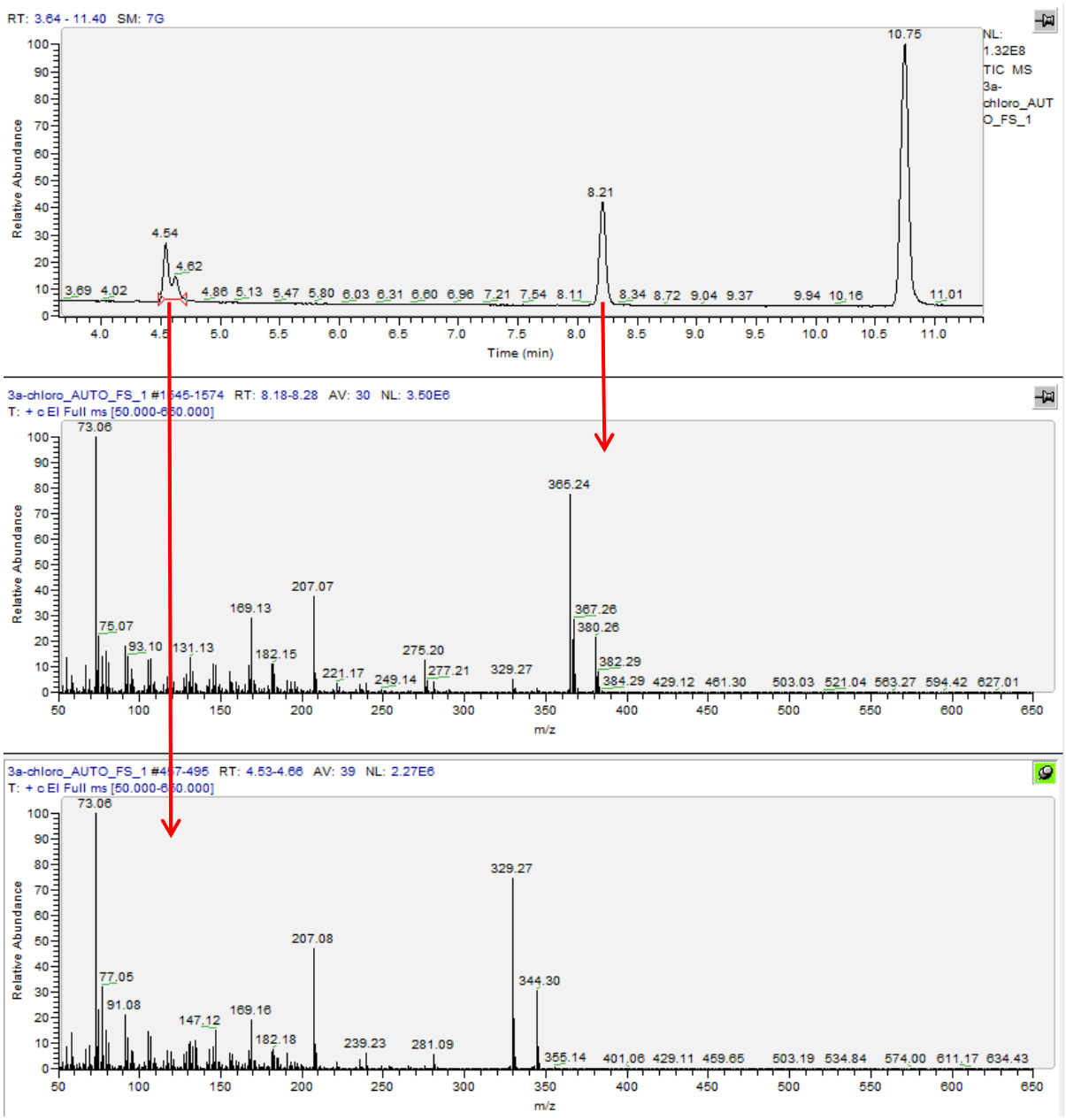
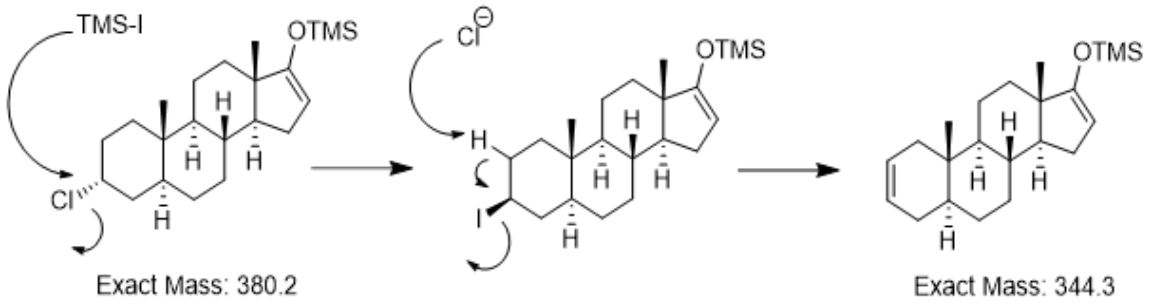


*Figure 6: full scan chromatogram of internal standard d3-testosterone (RT 10.75), **12** (RT 8.21) and its elimination product (RT 4.54). The corresponding mass spectra for **12** and its elimination product are shown below.*

An elimination within the synthesized substance occurred. Almost 40 % of product after elimination of HX was obtained, both for **12** and **13**. A substitution/elimination reaction, described in Scheme 8, happened in presence of the catalytic species of the derivatization agent.



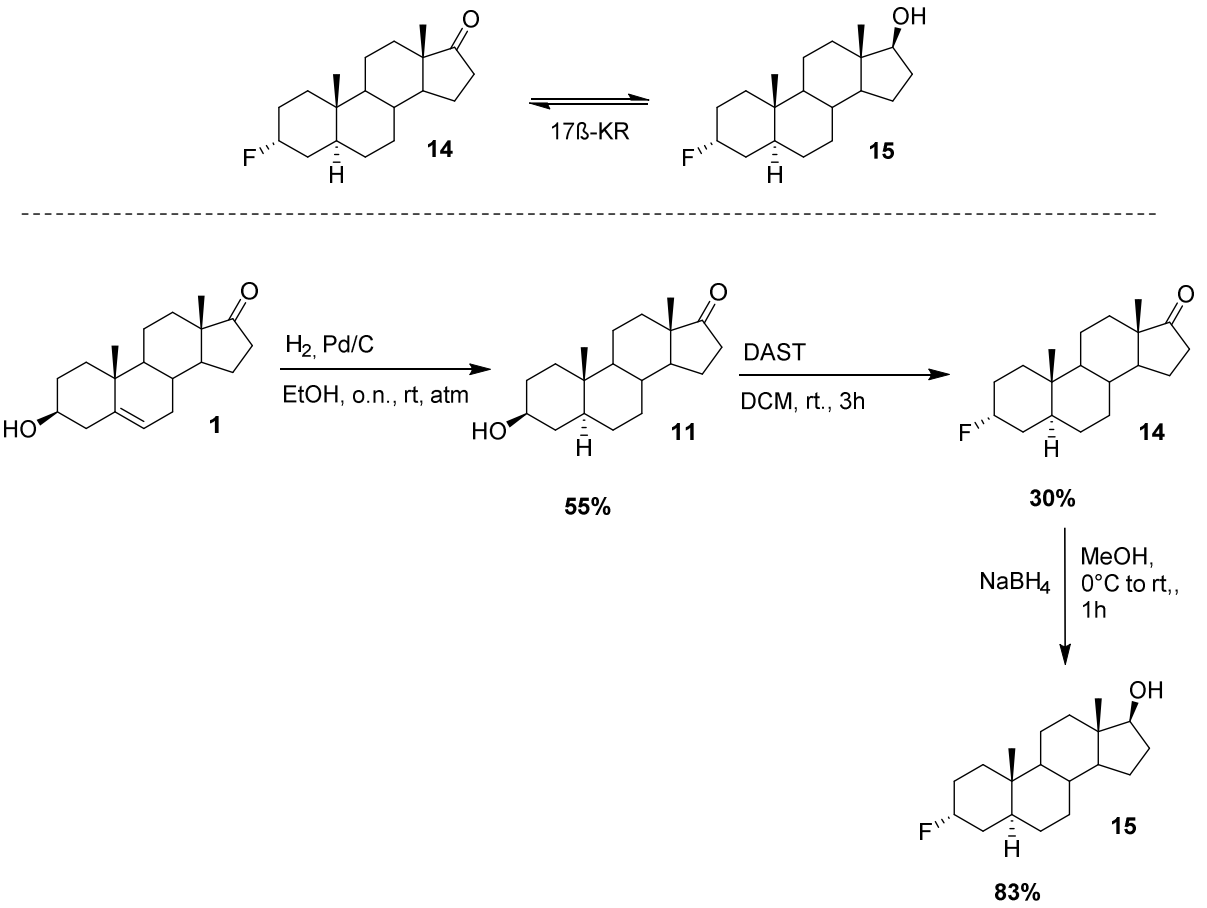
Scheme 8: Elimination process occurring during derivatization

In a first step, nucleophilic substitution of the chlorine with iodine takes place. In the following elimination reaction, the iodine is eliminated and HCl is formed. The 2,3 – elimination product is favoured. (21)

2.2 3α-Fluoro-5αH-androstane-17-one/3α-fluoro-5αH-androstane-17ß-ol

2.2.1 Synthesis

The synthetic steps leading to 3α-fluoro-5αH-androstane-17-one **14** and the corresponding reduced compound 3α-fluoro-5αH-androstane-17ß-ol **15** were carried out according to literature and are pictured in Scheme 9. (16) (22) (18)



Scheme 9: synthetic route for 3α-fluoro-5αH-androstane-17-one and 3α-fluoro-5αH-androstane-17ß-ol

The first step in the synthesis was the reduction of the starting material **1** via stereoselective hydrogenation in presence of the catalyst palladium to get the 5αH stereoisomer **11**. The first approach for installing the fluorine in position 3 was a reaction with the reagent bis(methoxyethyl)aminosulfur trifluoride (BAST). (23) As alternative, the similar reagent diethylaminosulfur trifluoride (DAST) was used; giving slightly better yields for **14** (30 %). DAST was one of the first practical deoxyfluorination reagents, followed by many derivatives like BAST. The yields obtained were similar to literature; a big amount (33 %) of elimination by-product with a double bond in position 2,3 was obtained. Starting material could be recovered (8 %). For the reduction of the 17-keto group, the mild reduction reagent $NaBH_4$ was used to obtain **15** (Yield 83 %).

2.2.2 Analytical evaluation

GC-MS without derivatization

The chromatograms and mass spectra of **14** and **15** without derivatization are shown in the ANNEX.

GC-MS(MS) with derivatization

The analysis of the two substances was carried out by derivatization with the derivatization reagent MSTFA/TMSI. The full scan chromatogram of the samples shows, next to the peak of the IS d3-testosterone and the expected substance, a third peak. The chromatogram including mass spectra is shown in Figure 7.

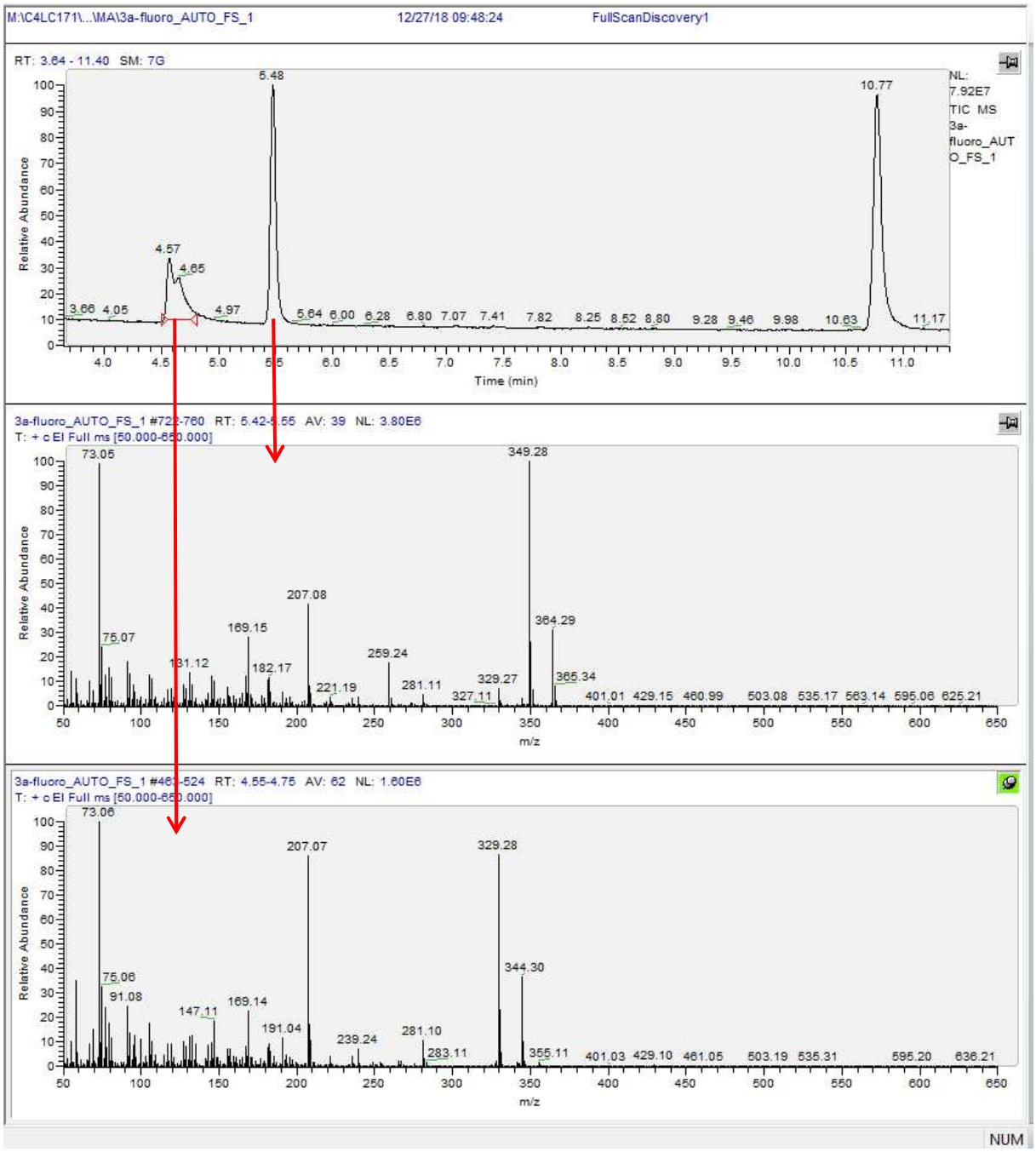


*Figure 7: full scan chromatogram of internal standard d3-testosterone (RT 10.77), **14** (RT 5.48) and its elimination product (RT 4.57). The corresponding mass spectra for **14** and its elimination product are shown below.*

The same elimination process like for **12** and **13** was observed for the substances **14** and **15**. Again, around 45 % of elimination product was obtained.

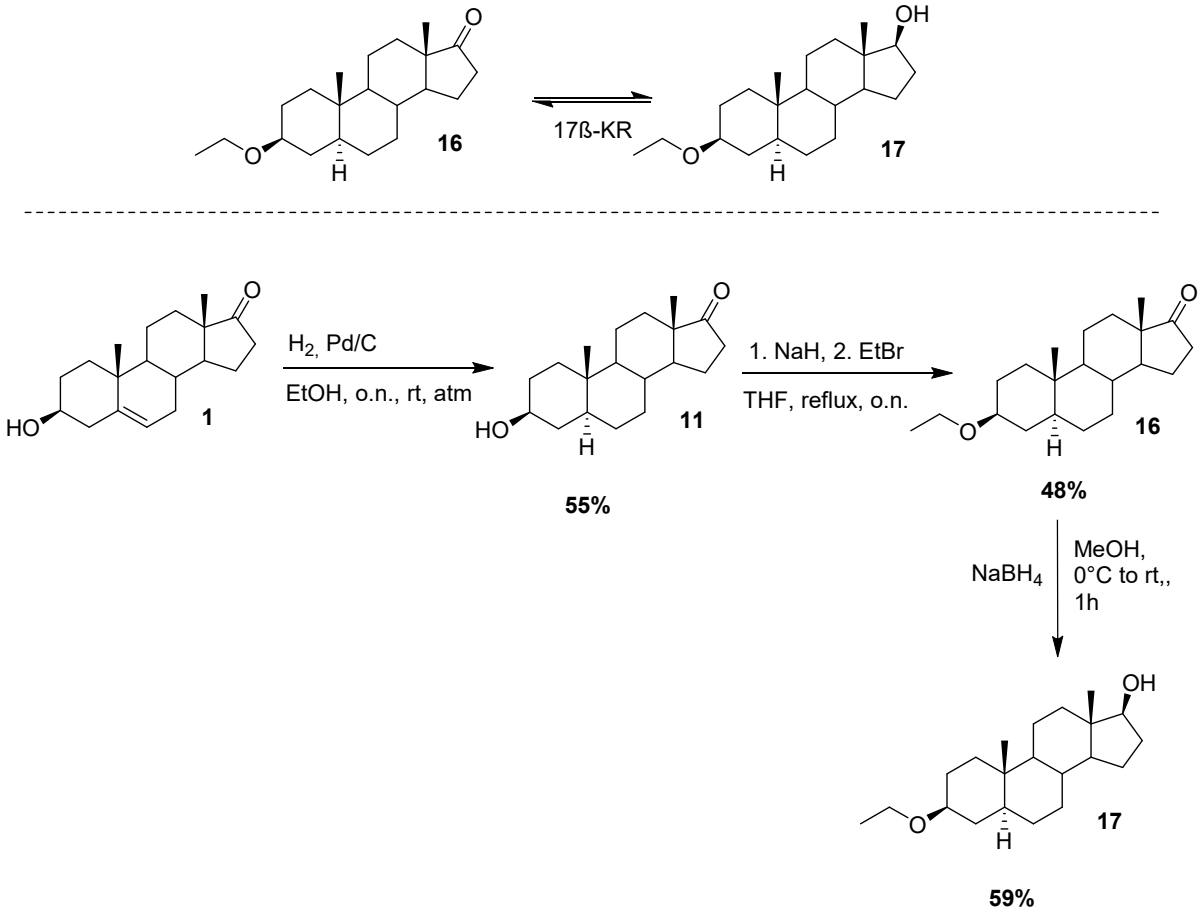
As it was defined before it is important to synthesize a substance which can be implemented in the routine analysis in doping control laboratories. It is not the goal to change the general work up conditions in routine analysis, but to find a substance which is stable under the present conditions. Due to the elimination reaction of the synthesized halogenated substances, they

are not suitable for the required purpose. The substances were not further tested. As alternative, a third substance was synthesized and is described in 2.3.

2.3 3ß-Ethoxy-5αH-androstane-17-one/3ß-ethoxy-5αH-androstane-17ß-ol

2.3.1 Synthesis

The synthetic steps leading to 3ß-ethoxy-5αH-androstane-17-one **16** and the corresponding reduced compound 3ß-ethoxy-5αH-androstane-17ß-ol **17** were carried out according to literature and are pictured in Scheme 10. (16) (24) (18)



Scheme 10: synthetic route for 3ß-ethoxy-5αH-androstane-17-one and 3ß-ethoxy-5αH-androstane-17ß-ol

The first step in the synthesis was the reduction of the starting material **1** via stereoselective hydrogenation in presence of the catalyst palladium to get the 5αH stereoisomer **11**. The first approach for the synthesis of **16** was tosylation and substitution with ethanol giving an overall yield of 19 %. (25) The easier and preferable synthesis was via Williamson ether synthesis. First, deprotonation of the alcohol was carried out. In a second step, the alkoxide was reacted

with ethyl bromide. **16** was obtained with 48 % yield. For the reduction of the 17-keto group, the mild reduction reagent $NaBH_4$ was used to obtain **17** (Yield 59 %).

2.3.2 Analytical evaluation

GC-MS without derivatization

The chromatograms and mass spectra of **16** and **17** without derivatization are shown in the ANNEX.

GC-MS(MS) with derivatization

The analysis of the two substances was carried out by derivatization with the derivatization reagent MSTFA/TMSI. The chromatograms including mass spectra for **16** and **17** are shown in Figure 8 and Figure 9.

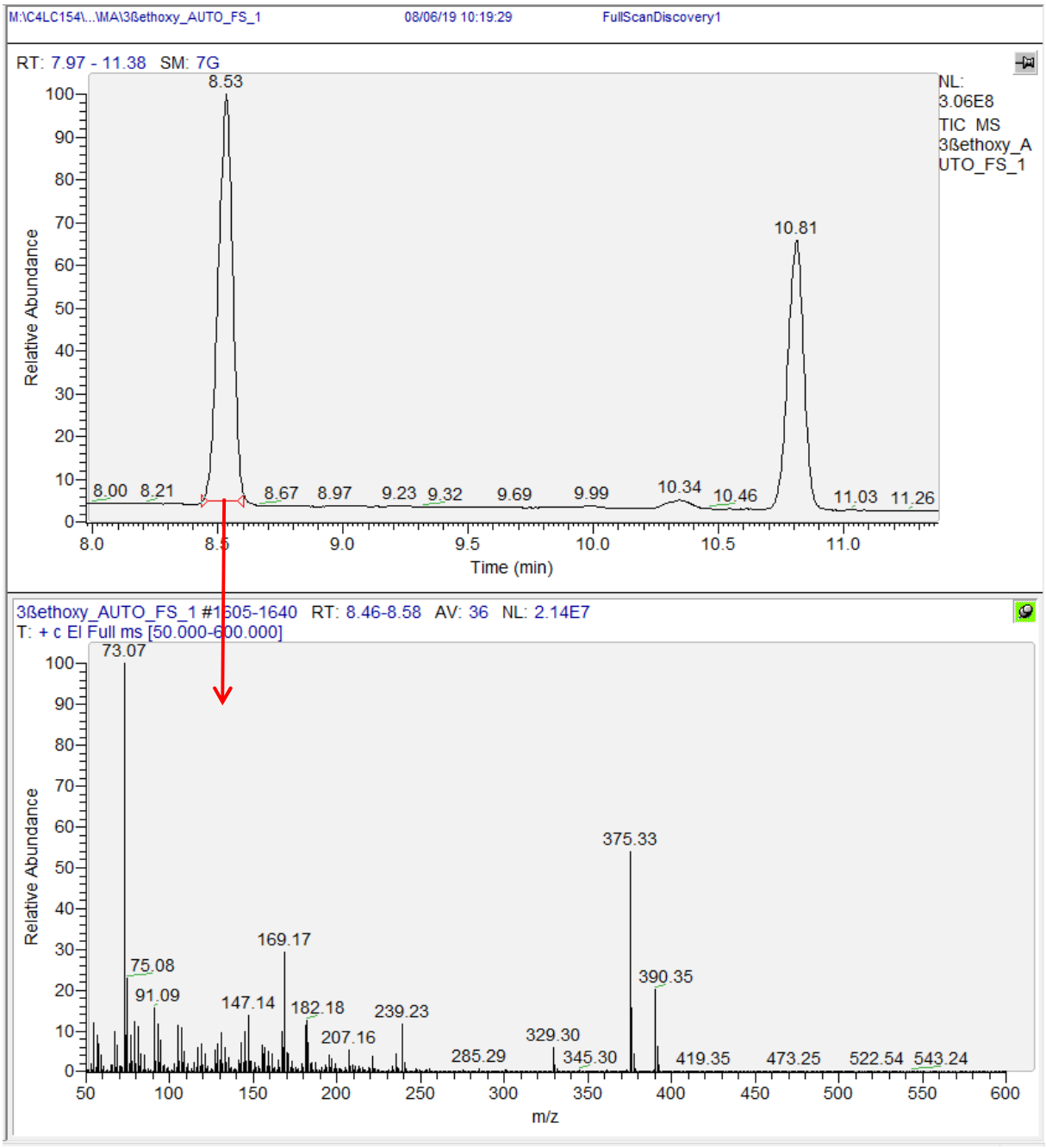


*Figure 8: full scan chromatogram of IS d3-testosterone (RT 10.81) and **16** (RT 8.53). The corresponding mass spectrum for **16** is shown below.*

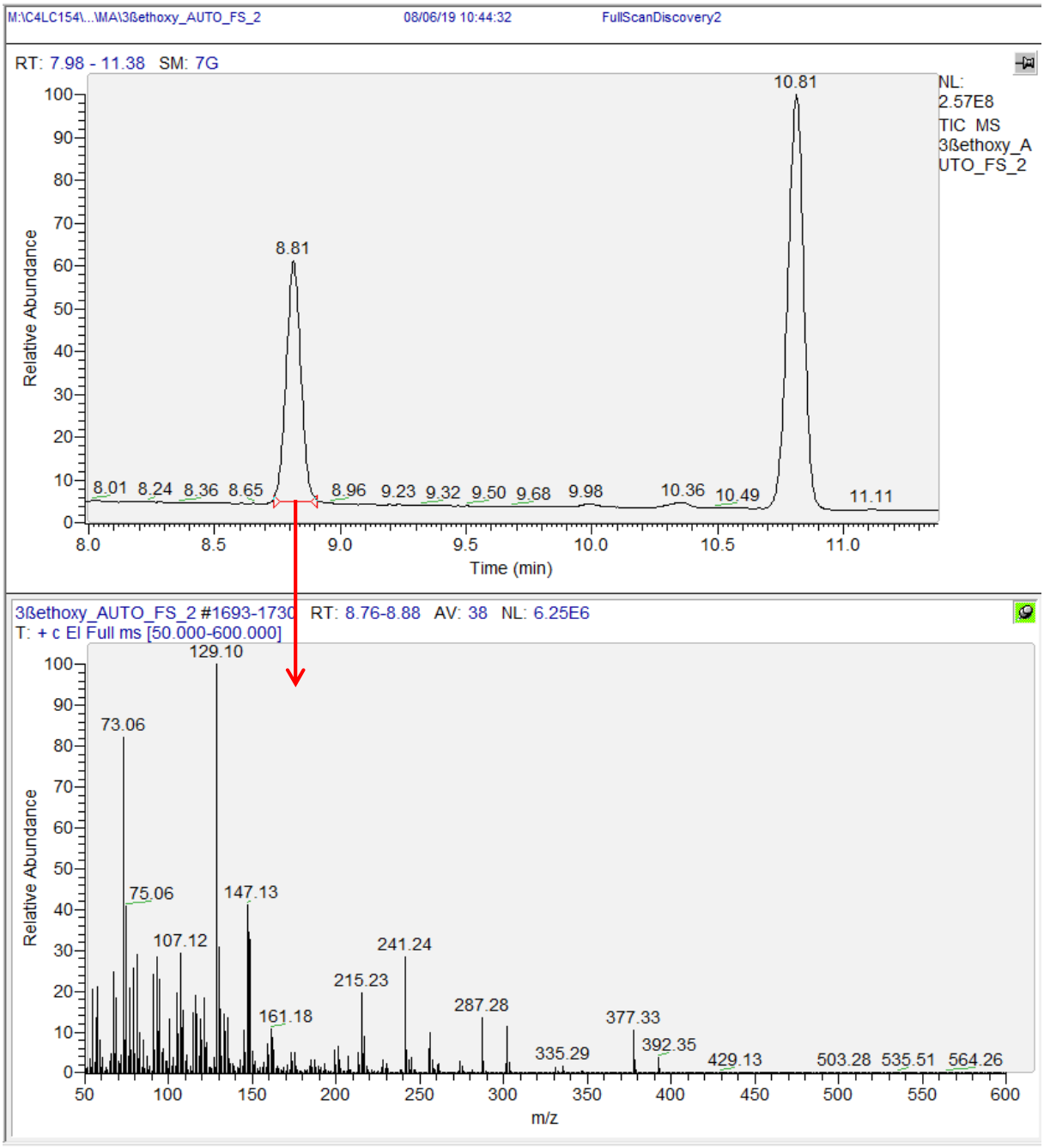


*Figure 9: full scan chromatogram of IS d3-testosterone (RT 10.81) and **17** (RT 8.81). The corresponding mass spectrum for **17** is shown below.*

In routine analysis the endogenous steroids are analysed in SRM mode. Therefore, the most intense and characteristic parent ions were selected. In a second quadrupole, further fragmentation trough collision with argon was carried out. The product ions were scanned and again the most abundant ions were selected. The transitions were measured with different collision energy. In ANNEX the four best transitions for **16** and **17** in water are shown. In Figure 10 the two most characteristic transitions for identification of both substances are shown.

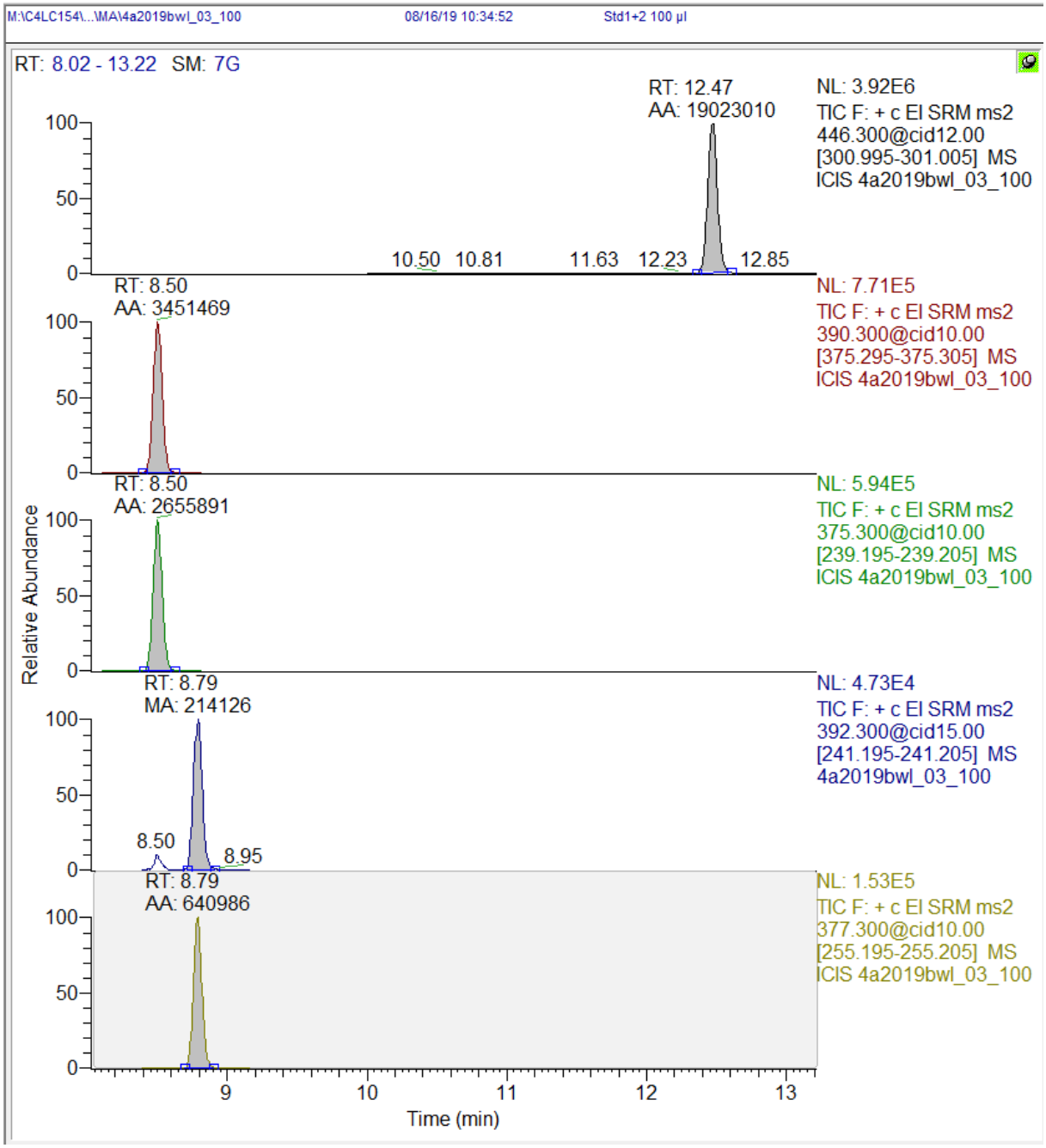


*Figure 10: transition for IS methyltestosterone (RT 12.47) and selected transitions for identification for **16** (RT 8.50) and **17** (RT 8.79) of a urine sample spiked with **16** and **17***

For **16** the selected transitions are most abundant and very characteristic for the substance, including the parent ion of the non-fragmented substance with an m/z = 390 $[M]^+$. The first transition describes the loss of a methyl group of the TMS group by collision with argon m/z = 375 $[M-15]^+$. For the second selected transition a methyl group is already lost through the EI

impact m/z = 375 $[M-15]^+$, either from the TMS group or of the steroid core structure (position 18 or 19). Through collision of the parent ion with argon the (rest of the) TMS-OH in position 17, the ethoxy group in position 3 (and a methyl group) are cleaved off the detected fragment $[M-90-46]^+$ or $[M-75-46-15]^+$.

For **17** the selected transitions are the most characteristic ones. The first transition describes again a loss from the non-fragmented parent ion m/z = 390 $[M]^+$. Through collision with argon the product ion m/z = 241 $[M-90-46-15]^+$ is formed. The second transition describes a parent ion with loss of a methyl group by EI m/z = 377 $[M-15]^+$ and a loss of TMS-OH, the ethoxy group and probably the formation of a double bond trough collision with argon m/z = 255 $[M-90-46-1]^+$. (6)

For comparison a blank water sample and a blank urine sample without the substances **16** and **17** are shown in Figure 11 and Figure 12. No interfering signals are visible for the selected transitions, showing the wanted selectivity.

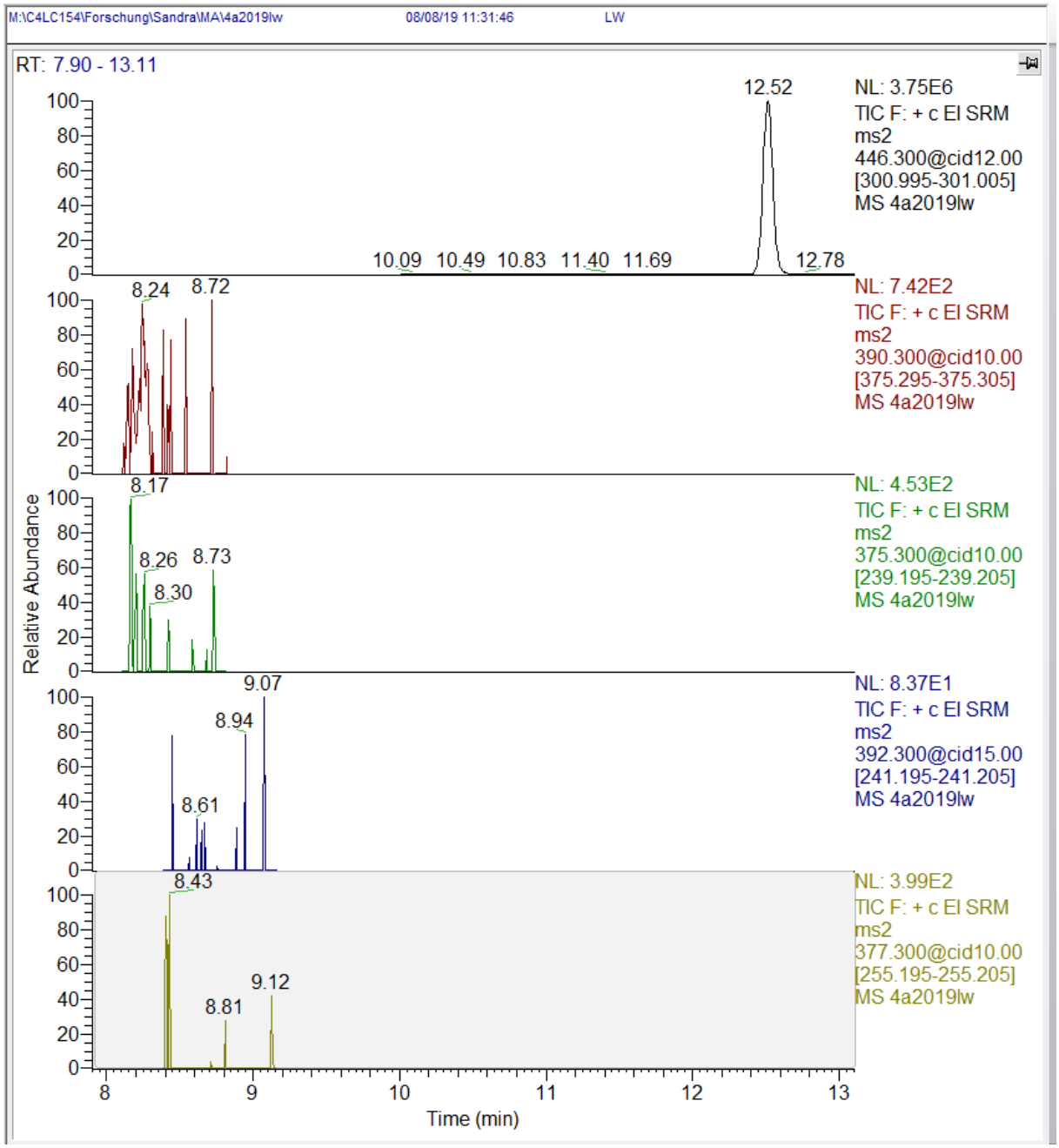


*Figure 11: transition for IS methyltestosterone (RT 12.52) and selected transitions for identification for **16** (RT 8.55) and **17** (RT 8.84) of a blank water sample without **16** and **17***

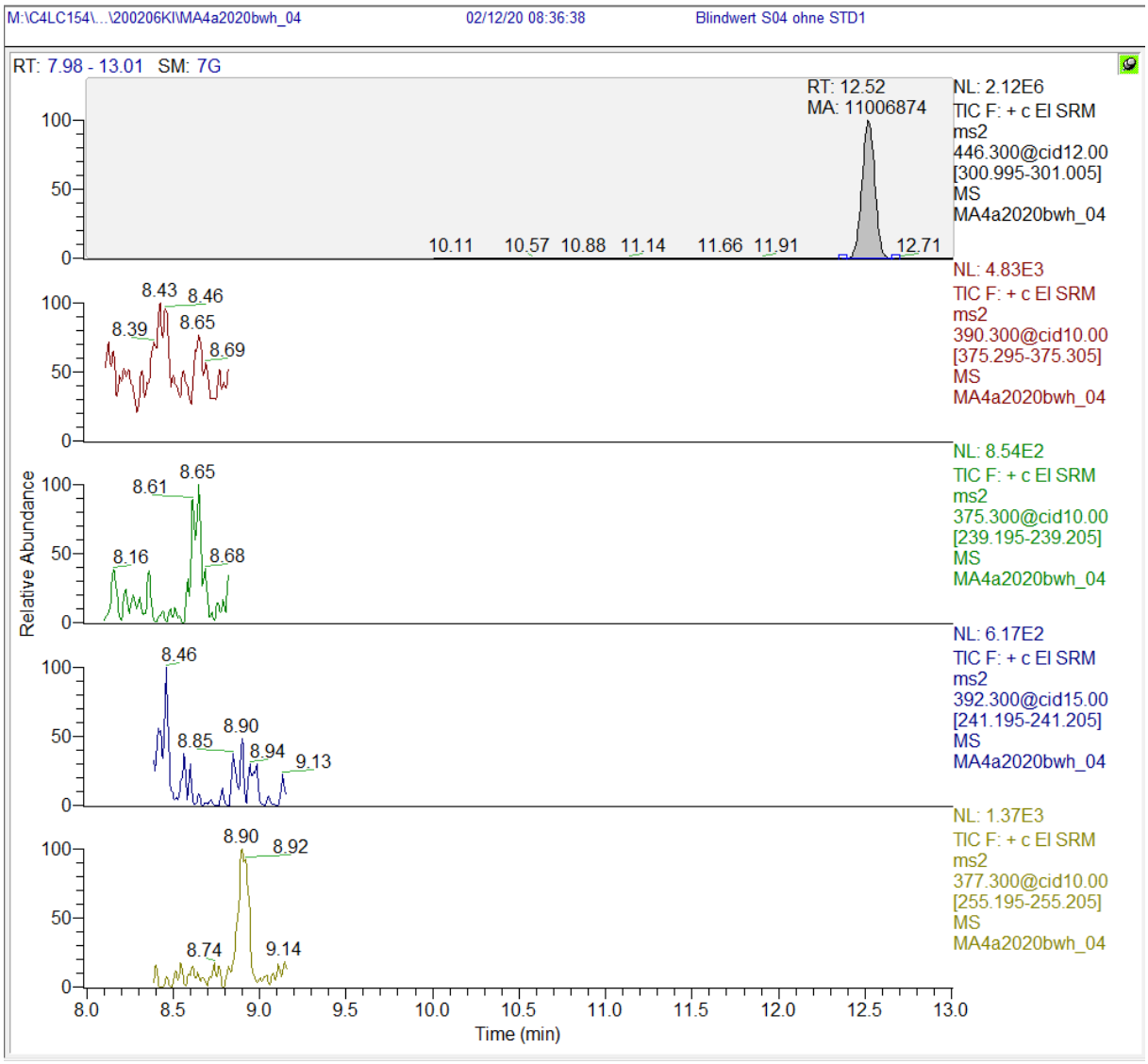


*Figure 12: transition for IS methyltestosterone (RT 12.52) and selected transitions for identification for **16** (RT 8.55) and **17** (RT 8.84) of a blank urine sample without **16** and **17***

Table 2 gives an overview over the identification criteria for the substances **16** and **17**.

*Table 2: identification criteria for **16** and **17** for IS methyltestosterone RT 12.47*

3ß-ethoxy-5αH-androstane-17-one					
RT	**Transition**	**Area**	**Relative Abundance**	**Lower Limit**	**Upper Limit**
8.50	390.3 → 375.3	3451469	100,0%	90,0%	110,0%
	375.3 → 239.2	2655891	76,9%	66,9%	86,9%
3ß-ethoxy-5αH-androstane-17ß-ol					
8.79	392.3 → 241.2	214126	33,4%	26,7%	40,1%
	377.3 → 255.2	640986	100,0%	90,0%	110,0%

2.4 Enzymatic Conversion

2.4.1 Enzyme ketoreductase

For testing the suitability of **16** to be an appropriate substrate for the enzyme, experiments with the enzyme ketoreductase were carried out. Urine samples were worked up under normal conditions, adding **16** as internal standard. The enzyme ketoreductase and co-substrate NADPH were added. No conversion of **16** could be observed.

The used enzyme had an activity of ≥ 0.2 U/mg. An enzyme with higher activity was not available. To verify the reaction conditions (e.g. amount of co-substrate) the enzyme alcohol dehydrogenase with an activity of ≥ 500 U/mL was also used. Under the same reaction conditions, the conversion of **17** to **16** was successful.

It was not possible to purchase the enzyme ketoreductase with higher activity within the time frame of this project/thesis, so the focus was on achieving the conversion by microbiological infected urine.

2.4.2 Microbiological infected urine samples

The collected urine samples were prepared under the conditions described in 3.4.

To reconstruct the cases observed, the samples were prepared under different incubation conditions. First, the samples were analysed frozen with the hydrolysis step at 50 °C for 2 hours. The analysis of one of these samples is shown in Figure 13.

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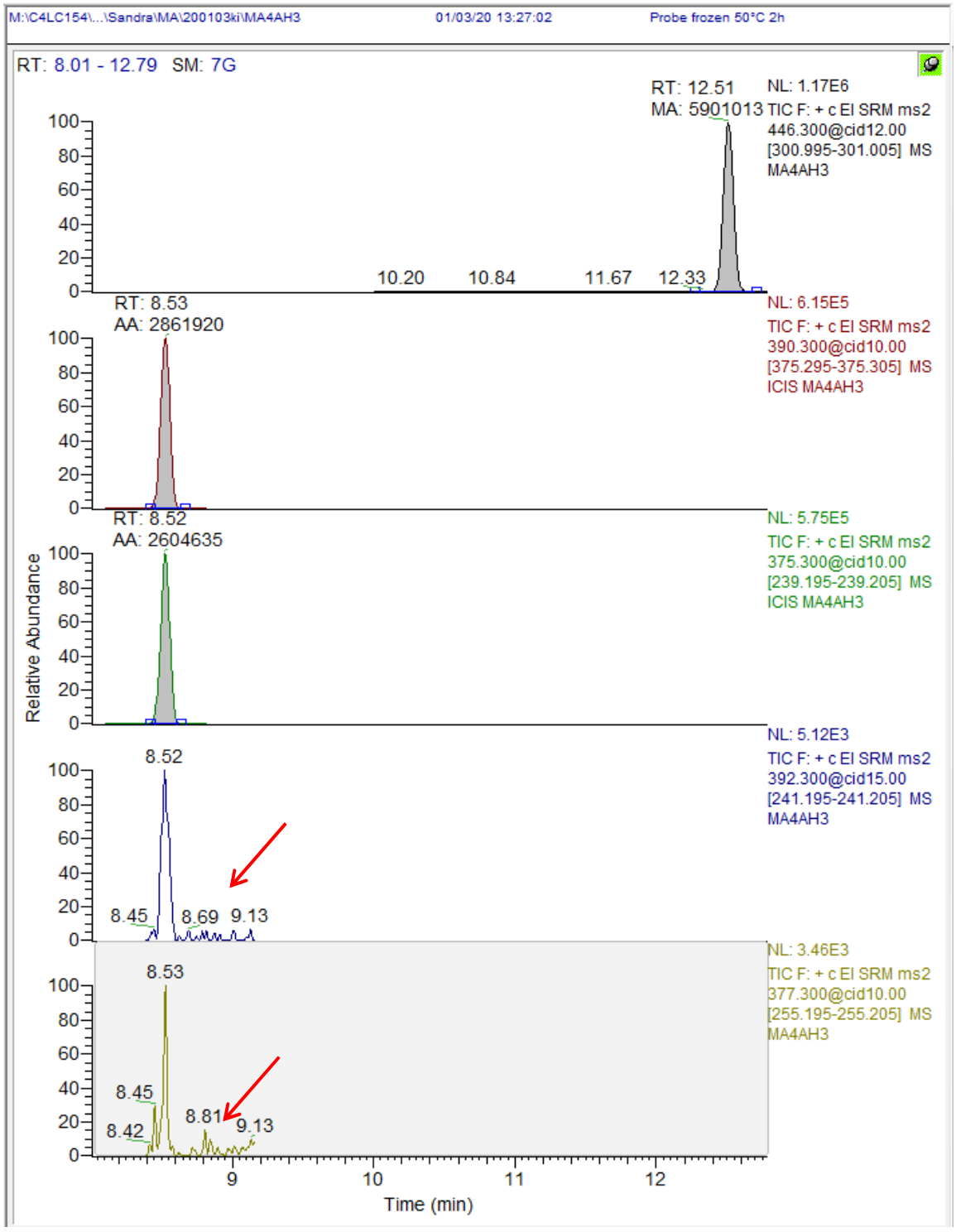


*Figure 13: Preparation/analysis after freezing. Hydrolysis: Incubation 50 °C 2 hours. transitions for IS methyltestosterone (RT 12.51) and selected transitions for identification for **16** (RT 8.53) and **17** (RT 8.82) of a urine sample with **16** added as IS.*

The analysis shows the expected result. No conversion of **16** occured. The same set of samples were also collected and stored at 8 °C. They were prepared non-frozen with the hydrolysis step at 37 °C overnight. The result of the analysis is shown in Figure 14.

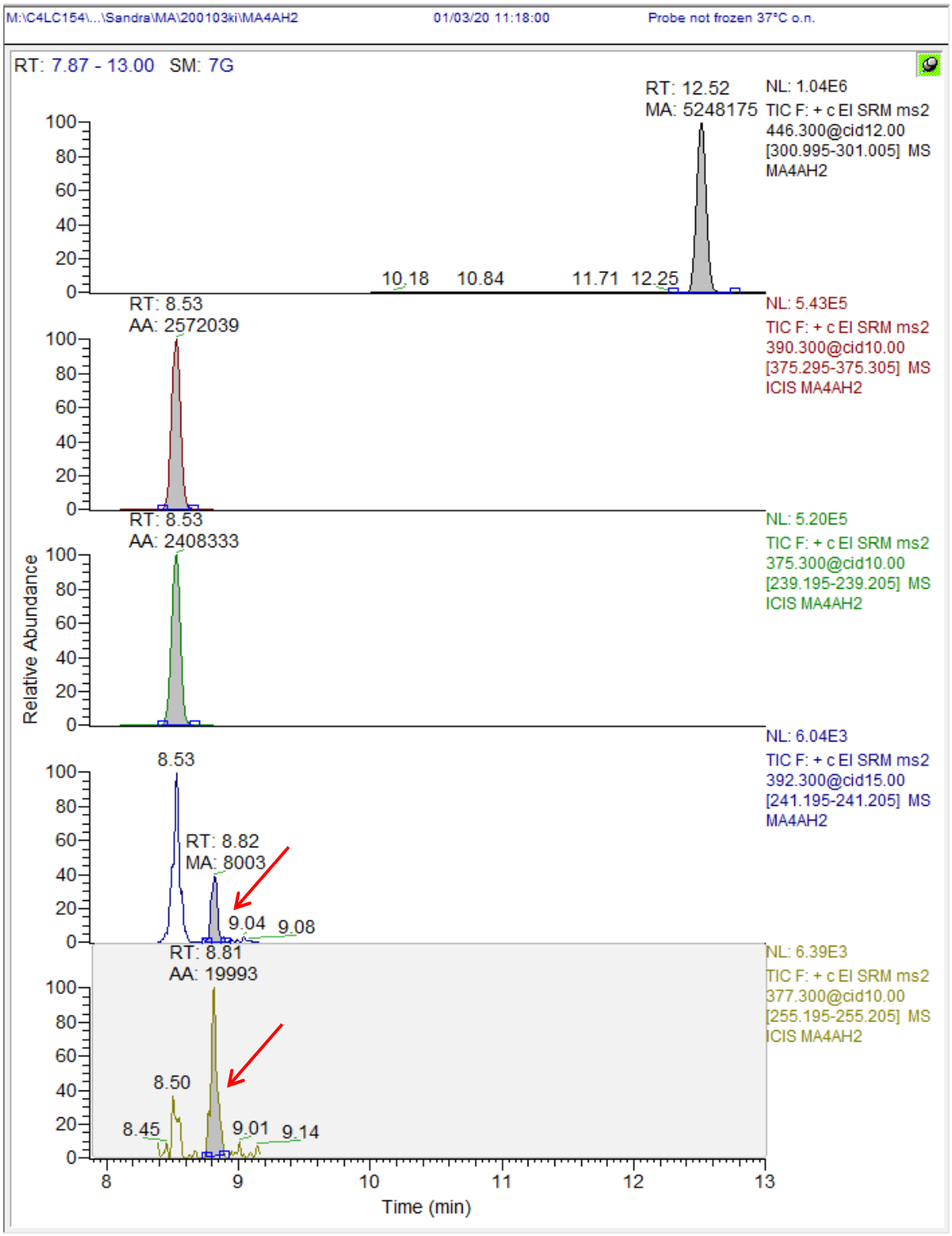


*Figure 14: Preparation/analysis before freezing. Hydrolysis: Incubation 37 °C overnight. Transitions for IS methyltestosterone (RT 12.52) and selected transitions for identification for **16** (RT 8.53) and **17** (RT 8.82) of a urine sample with **16** added as IS.*

For the workup of the non-frozen sample with the hydrolysis step carried out at 37 °C overnight (18 hours), a small peak for **17** occured. To get a more satisfying result, the incubation was

carried out also for a longer period, two times overnight (44 hours). The result is shown in Figure 15.

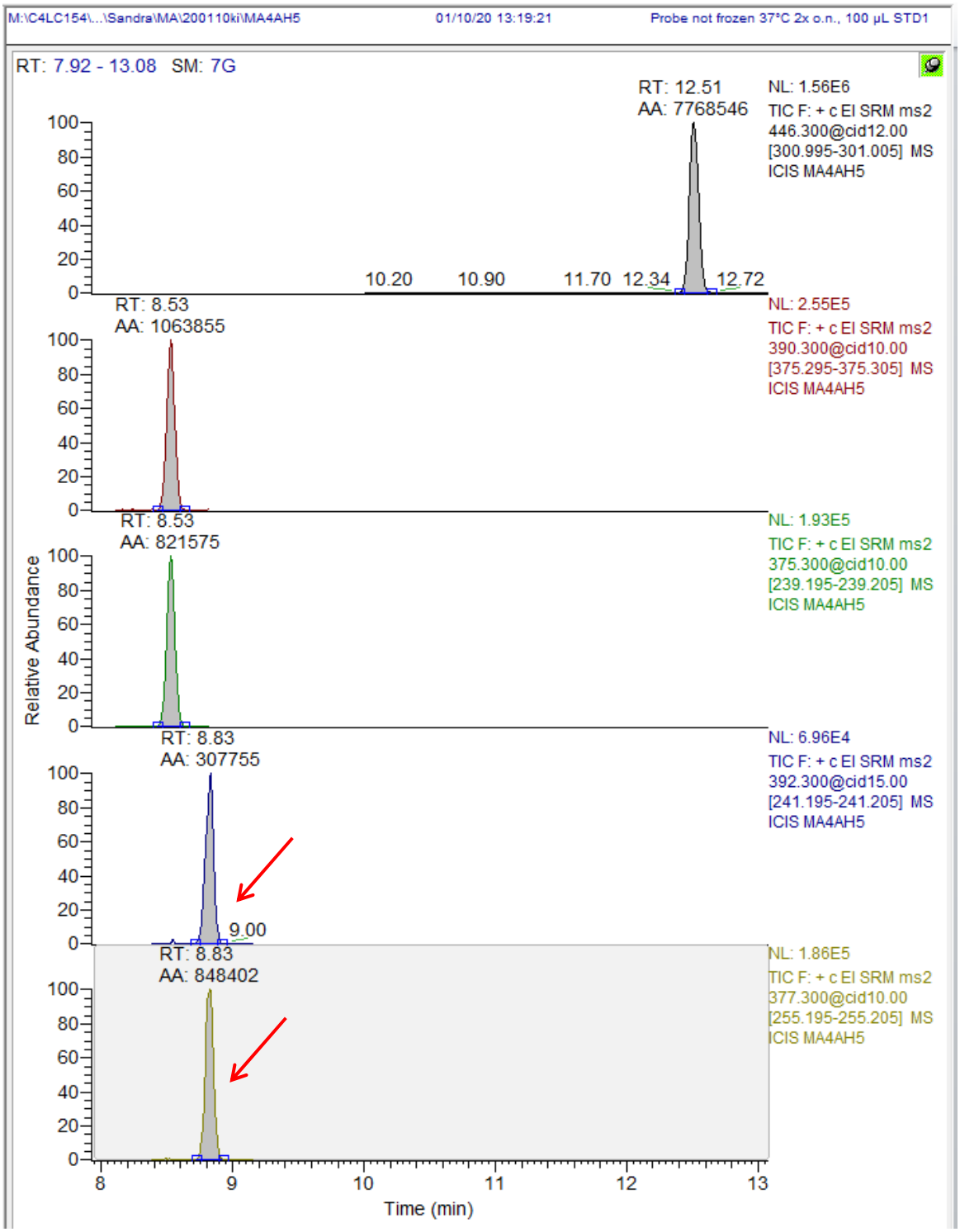


Figure 15: Preparation/analysis before freezing. Hydrolysis: Incubation 37 °C, ***two times*** *overnight. Transitions for IS methyltestosterone (RT 12.51) and selected transitions for identification for* ***16*** *(RT 8.53) and* ***17*** *(RT 8.83) of a urine sample with* ***16*** *added as IS.*

The conversion of **16** into **17** is clearly evident. In Table 3 the criteria for identification of the substance are summarized for the two cases and are compared with the synthesized compound.

*Table 3: identification criteria for **17** and identification for the conversion of **16** adjusted to IS methyltestosterone RT 12.47*

3ß-ethoxy-5αH-androstane-17ß-ol (Standard)					
RT	**Transition**	**Area**	**Relative Abundance**	**Difference**	**Evaluation**
8.79	392.3 → 241.2	214126	33,4%	26,7%	40,1%
	377.3 → 255.2	640986	100,0%	90,0%	110,0%
3ß-ethoxy-5αH-androstane-17ß-ol (incubation 18 hours)					
8.78	390.3 → 375.3	8003	40,0%	6,6%	Acceptable
	375.3 → 239.2	19993	100,0%	0,0%	Acceptable
3ß-ethoxy-5αH-androstane-17ß-ol (incubation 44 hours)					
8.80	392.3 → 241.2	307755	36,3%	2,9%	Acceptable
	377.3 → 255.2	848402	100,0%	0,0%	Acceptable

To check all possible sample preparation conditions, the samples were also prepared frozen at 37 °C overnight and not frozen at 50 °C for 2 hours. The results are shown in Figure 16 and Figure 17.

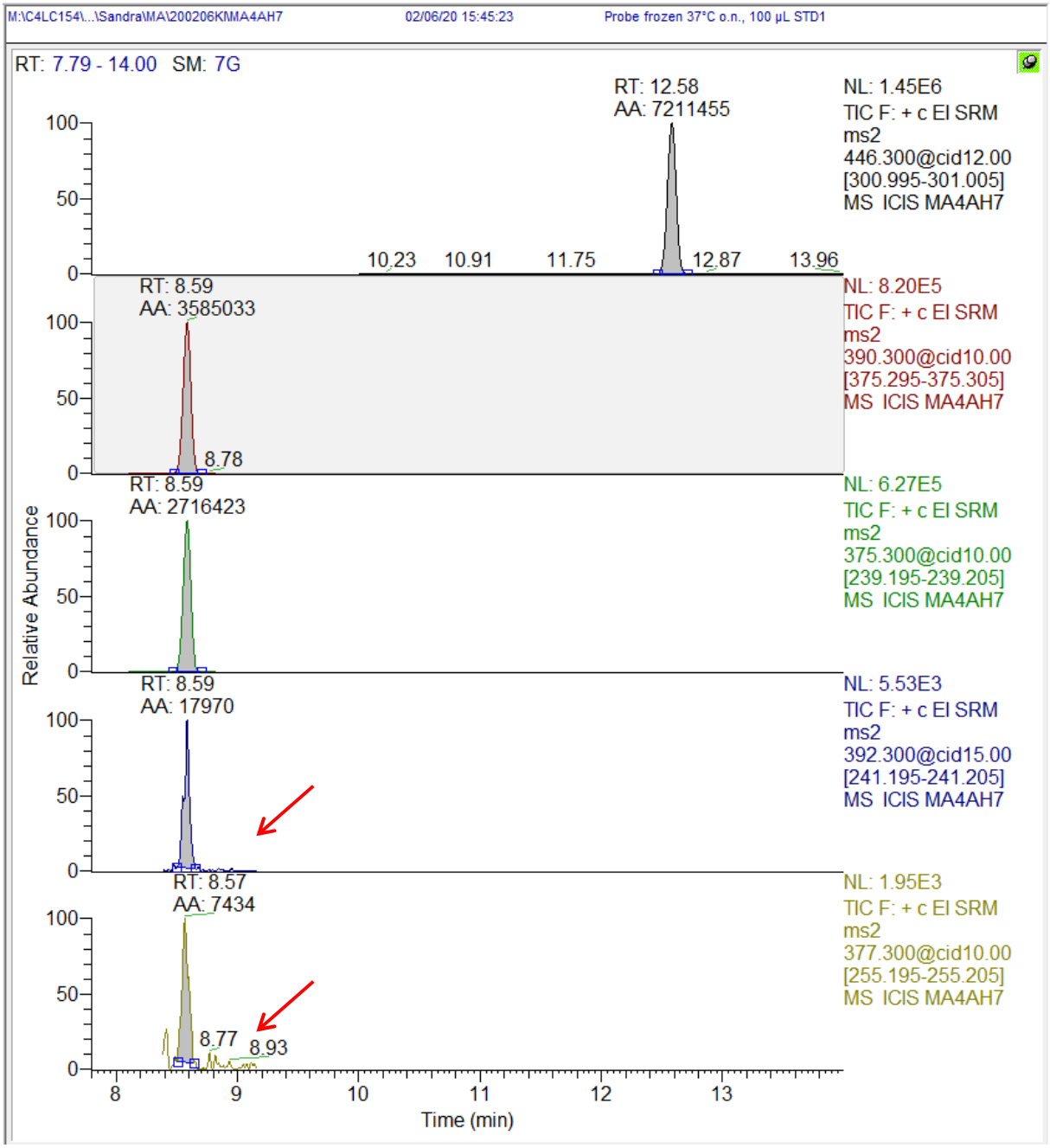


*Figure 16: Preparation/analysis after freezing. Hydrolysis: Incubation 37 °C, overnight. Transitions for IS methyltestosterone (RT 12.58) and selected transitions for identification for **16** (RT 8.59) and **17** (RT 8.89) of a urine sample with **16** added as IS. No conversion of 16 occurred.*

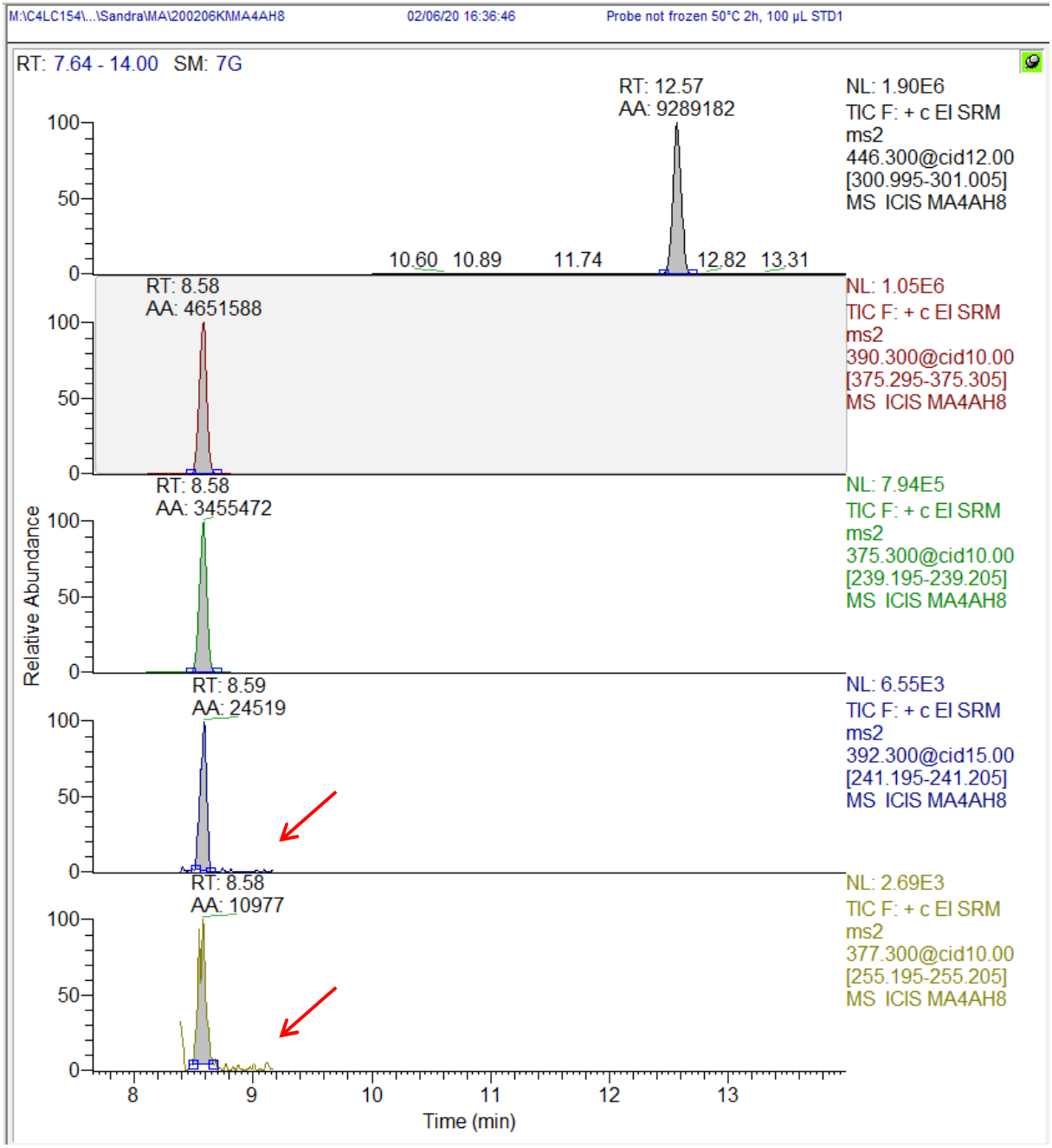


*Figure 17: Preparation/analysis before freezing. Hydrolysis: Incubation 50 °C, 2 hours. Transitions for IS methyltestosterone (RT 12.57) and selected transitions for identification for **16** (RT 8.57) and **17** (RT 8.88) of a urine sample with **16** added as IS. No conversion of 16 occurred.*

In both cases no conversion of **16** occurred. The activity is not observed for frozen samples and for not frozen samples for which the hydrolysis is carried out at elevated temperatures of 50 °C. In Table 4 the results of the different preparation conditions are summarized.

Table 4: comparison of different preparation conditions

Preparation conditions	17-keto activity observed
50 °C, 2 h, frozen	No conversion of **16** into **17** observed
50 °C, 2 h, not frozen	No conversion of **16** into **17** observed
37 °C, overnight (28 h), frozen	No conversion of **16** into **17** observed
37 °C, overnight (28 h), not frozen	Yes, approx. 5 % conversion of **16** into **17**
37 °C, 2 times overnight (44 h), not frozen	Yes, approx. 70 % conversion of **16** into **17**

If the concentrations of the markers of the steroid profile for the different incubation times are compared, an increase in the concentration of 6 and 8 and a decrease in the concentration of 7 and 9 are observed for longer incubation. An overview is given in Figure 18 for the markers of the steroid profile.

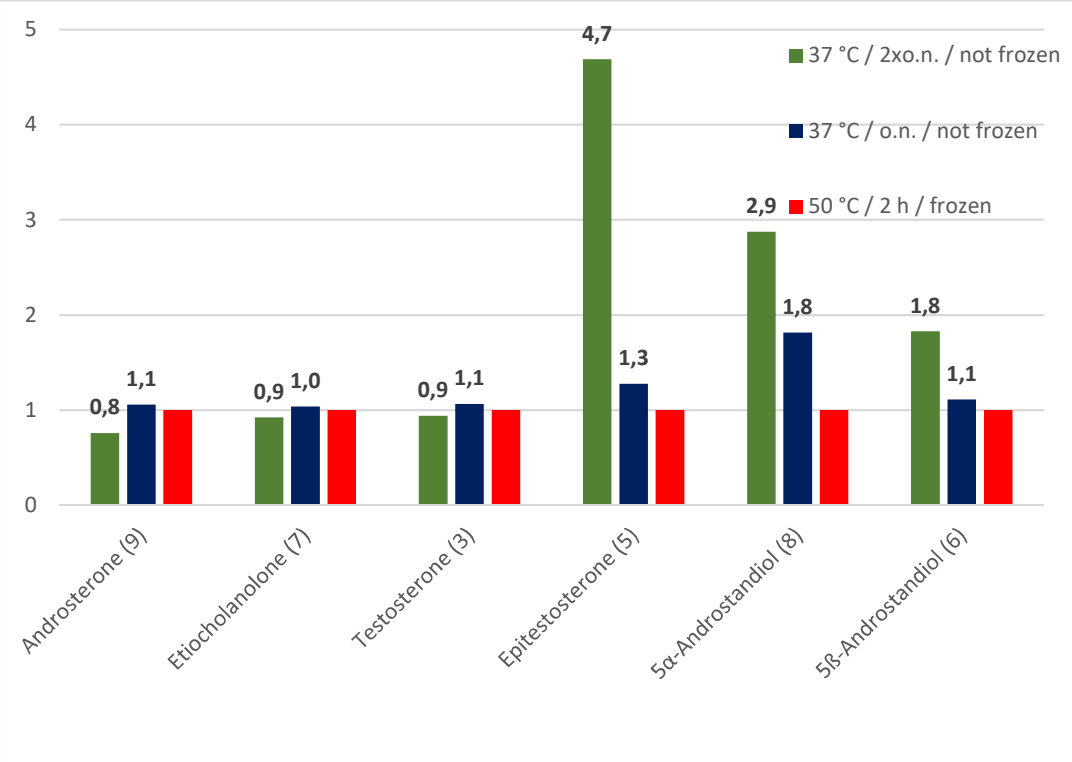


Figure 18: Concentrations of endogenous steroids marker substances: 50°C/2h/frozen (red): Preparation/analysis after freezing. Hydrolysis: Incubation 50 °C 2 hours. 37°C/o.n./not frozen (blue): Preparation/analysis before freezing. Hydrolysis: Incubation 37 °C overnight. 37°C/2x o.n./not frozen (green): Preparation/analysis before freezing. Hydrolysis: Incubation 37 °C two times overnight.

It is shown that the steroid markers changed after longer incubation. An increase of the diols **6** and **8** is observed, which results of the transformation of **7** and **9** by reduction of the 17-keto group. Also, the increase of **5** matches the already observed cases.

2.5 Conclusion

Although not naturally occurring as endogenous steroids due to the presence of chlorine or fluorine in the steroid structure the synthesized substances 3α-chloro-5αH-androstane-17-one **12** and 3α-fluoro-5αH-androstane-17-one **14** are not considered as suitable markers of 17-keto activity. The main reason for this is the stability of these substances at routine testing conditions, which is an essential and crucial point for suitability. The elimination of the chlorine or fluorine atom during derivatization does not lead to reproducible conditions for the detection of the product of 17-keto activity, the 17-hydroxy marker.

The idea of using halogenated substances originated because the low chance of naturally occurring chlorines or fluorines in the human body. An ethoxy ether derivate was chosen in the second approach, because human phase I metabolism does not form ethers for an increase of polarity. In phase II metabolism methylation is common. This is the reason, why an ethyl ether was preferred to a methyl ether, which might have fit better as a substrate for the enzyme because of sterically reasons.

It was shown, that despite the bulky rest in position 3, the synthesized substance 3ß-ethoxy-5αH-androstane-17-one (**16**) is converted to 3ß-ethoxy-5αH-androstane-17ß-ol (**17**) by 17-keto activity. Comparing the results with the cases happened several years ago, a similar picture is observed. For the experiments with the non-frozen samples and hydrolysis at 37 °C overnight, a conversion of approx. 5 % of 3ß-ethoxy-5αH-androstane-17-one (**16**) in 3ß-ethoxy-5αH-androstane-17ß-ol (**17**) is observed. It was shown, that an increase in the incubation time also leads to more conversion. In the experiment carrying out the hydrolysis step at 37 °C for 44 h, a conversion of approximately 70 % of 3ß-ethoxy-5αH-androstane-17-one (**16**) into 3ß-ethoxy-5αH-androstane-17ß-ol (**17**) was observed. For this experiment, also the decrease for etiocholanolone (**7**) and androsterone (**9**) and an increase for 5ß-androstanediol (**6**) and 5α-androstanediol (**8**) was apparent, matching the observed cases. Also, if only e.g. 5 % of etiocholanolone (**7**) and androsterone (**9**) are converted into 5ß-androstanediol (**6**) and 5α-androstanediol (**8**) it can lead to big differences in the steroid profile and false interpretations of the ABP.

The first step in synthesizing a suitable substance and proving its suitability was done successfully. It can be defined, that also very small amounts of

3ß-ethoxy-5αH-androstane-17ß-ol (**17**) are a signal for activity at the position 17. The monitoring system will be implemented into the standard analysis procedures to proof its applicability at routine conditions. More experiments must be carried out do determine a threshold for 3ß-ethoxy-5αH-androstane-17ß-ol (**17**).

The goal is to develop a stable monitoring system for the 17-keto activity over a longer time period with more positive cases. If this is achieved, 3ß-ethoxy-5αH-androstane-17-one (**16**) can be implemented as marker substance for monitoring activity in position 17 of endogenous steroids in all doping control laboratories as internal standard.

3. Experimental part

3.1 General remarks

Chemicals

Chemicals were purchased from chemical suppliers and used without further purification, unless otherwise noted.

Chromatography (TLC, column chromatography)

TLCs were performed on aluminum coated silica gel 60 F254. The spots were visualized with staining reagents (Cerium Molybdate).

Column chromatography was performed using silica gel 60 (230-400 mesh, Merck).

Eluents used for cc: LP (distilled), EtOAc

Reaction monitoring

All reactions were monitored by TLC on silica gel 60 F254-plates. Spots were visualized by staining by dipping into acidic phosphomolybdic acid/cerium sulfate solution and heating.

NMR spectroscopy

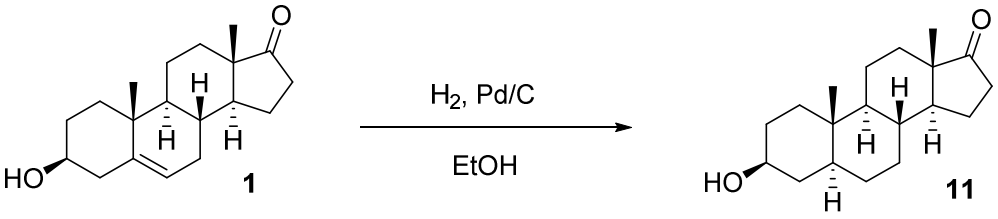
1H and 13C spectra were recorded in $CDCl_3$ on a Bruker Avance UltraShield 400 (400 MHz) or Avance III HD 600 (600 MHz) spectrometer and chemical shifts (δ) are reported in ppm using tetramethylsilane as internal standard[16], coupling constants (J) are given in Hertz (Hz). The multiplicities are given by the following abbreviations: s = singlet, d = duplet, t= triplet, q = quartet, m = multiplet, bs = broad singlet. All assignments are based on 2D-spectra (COSY, phase sensitive HSQC, HMBC and NOESY, depending on the molecule).

GC-MS(MS)

GC–MSMS runs of the derivatized samples were performed on a Thermo Scientific TSQ 8000 Triple Quadrupole instrument with TriPlus RSH Autosampler and Trace GC Ultra. GC-MS runs of the underivatized samples were performed on a Thermo Scientific Voyager Single Quadrupole instrument with A2000 S Autosampler and Trace GC 2000.

3.2 Synthesis of the Marker Substances

3.2.1 Reduction of DHEA



Lit.: *Steroids,* **1992**, vol. 57, March

			d	equiv.	MW
4,00 g	(13,87 mmol)	DHEA		1,00	288,43 g/mol
737,93 mg	(0,69 mmol)	Pd 10%		0,05	106,42 g/mol
69,34 g	(200,00 mmol)	H2			2,02 g/mol
300,00 mL		EtOH			46,07 g/mol
4,03 g	(13,87 mmol)	theoretical yield		1,00	290,45 g/mol
2,21 g	(7,61 mmol)	yield (purified)	55 %		

The white solid starting material **1** (4.00 g, 13.87 mmol, 1.00 equiv.) was dissolved in 300 ml dry Ethanol in a Schlenk tube. After approximately 30 minutes the solid was dissolved. The tube was flashed with argon. The catalyst Palladium (10%) on charcoal (740 mg, 0.69 mmol, 0.05 equiv.) was added to the stirring solution. The argon was removed by reduced pressure (vacuo until the ethanol begins to boil) and the flask was filled with hydrogen. The solution was stirred at room temperature/pressure overnight. After 18 hours the completion of the reaction was monitored by NMR.

The reaction mixture was filtered over celite to remove the Palladium and washed with ethyl acetate. The solvent was removed under reduced pressure. The white solid (4.60 g) was solved in ethyl acetate and flashed over silica, to separate the byproducts from the product (white crystals, 55 %).

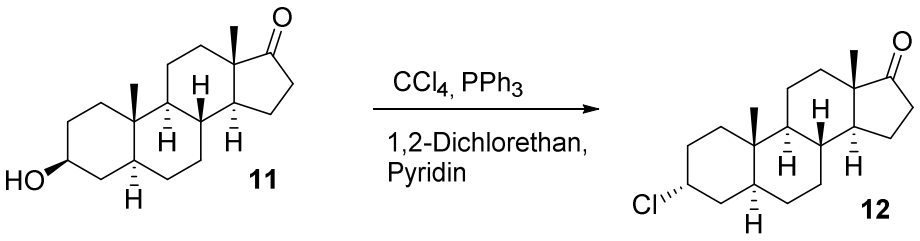
^{1}H NMR (400 MHz, CDCl3) δ 3.60 (tt, J = 11.0, 4.8 Hz, 1H), 2.44 (dd, J = 19.2, 8.8 Hz, 1H), 2.13 – 1.99 (m, 1H), 1.97 – 1.87 (m, 1H), 1.85 – 1.06 (m, 18H), 1.04 – 0.92 (m, 2H), 0.86 (s, 3H), 0.83 (s, 3H).

Comments:

The TLC gives not much information; the retention time for the product is almost the same as for the starting material.

3.2.2 3α-chloro-5αH-androstane-17-one

APPEL REACTION



Lit.: *Fujisawa Pharmaceutical Co.,* **2002,** Ltd. - US6384080, B1

			d		equiv.	MW
100,00 mg	(0,34 mmol)	Epiandrosterone			1,00	290,45 g/mol
180,61 mg	(0,69 mmol)	PPh_3			2,00	262,29 g/mol
2,50 ml	(25,84 mmol)	CCl_4	1,59	g/cm³	75,06	153,81 g/mol
2,50 ml		1,2-Dichlorethan				
8,00 drops		Pyridine				
106,35 mg	(0,34 mmol)	theoretical yield			1,00	308,89 g/mol
78,00 mg	(0,25 mmol)	yield	73	%		
77,000 mg	(0,25 mmol)	column purification SPF-005				308,89 g/mol
10,000 mg	(0,03 mmol)	yield	9	%		

Starting material **11** (100 mg, 0.34 mmol, 1 equiv.) was dissolved in 2.5 ml tetrachloromethane and 2.5 ml 1,2-Dichloroethane. To the stirring solution Triphenylphosphine (180.6 mg, 0.69 mmol, 2 equiv.) was added in one portion. A few drops of pyridine were added. The reaction mixture was heated up to 90 °C. After refluxing for 1.5 hours a TLC was performed, showing the conversion of starting material.

The solvent was removed under reduced pressure. From the residue a ^{1}H-NMR was performed.

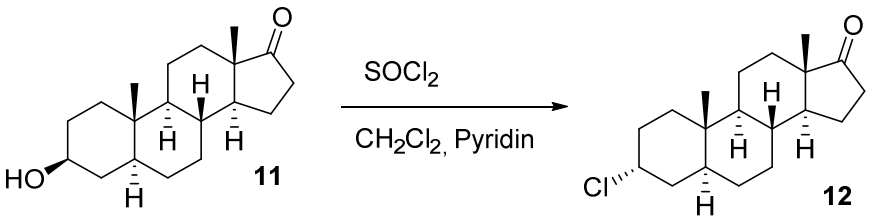
A column with 6 g silica was prepared. The products were eluted with a mobile phase PE:EE=50:1 → 20:1. Only 10 mg of the product were obtained. The column was eluted with pure EE and the solvent was removed under reduced pressure. From the residue also a ^{1}H-NMR was performed.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.61 – 4.37 (m, 1H), 2.44 (dd, *J* = 19.2, 8.9 Hz, 1H), 2.16 – 2.00 (m, 1H), 1.97 – 1.43 (m, 14H), 1.34 – 1.21 (m, 5H), 1.05 (qd, *J* = 12.1, 5.6 Hz, 1H), 0.86 (s, 3H), 0.81 (s, 3H).

Comments:

After the reaction mixture cooled down, a white precipitation could be observed, which was probably the triphenylphosphine oxide. Due the product is relatively nonpolar and stable; petrol ether was added to the reaction mixture and triphenylphosphine oxide precipitated. The suspension was filtered over a silica plug, the product was eluted with ether. The solvent was removed under reduced pressure, giving no residue. So, the silica plug was extracted again with 1,2-Dichloroethane to elute the product. Very low yields were achieved.

CHLORINATION VIA THIONYLCHLORIDE



Lit.: *Steroids 62*: **1997,** 543-545,

			c		equiv.	MW
50,00 mg	(0,17 mmol)	Epiandrosterone			1,00	290,45 g/mol
0,34 ml	(0,69 mmol)	Thionylchloride in DCM	2,00	mol/l	4,00	118,96 g/mol
0,30 ml		Pyridine				
53,17 mg	(0,17 mmol)	theoretical yield			1,00	308,89 g/mol
229,00 mg	(0,74 mmol)	yield	431	%		
		column purification SPF 006				
10,000 mg	(0,03 mmol)	yield	19	%		

The yellowish thionyl chloride was purified by distillation, giving a clear, slightly yellow liquid. A solution of thionyl chloride in dry DCM with a concentration of 2 mol/l was prepared. 0.35 ml of this solution (0.69 mmol, 4 equiv.) was added to the reaction flask. 0.3 ml of pyridine was added. To the smoking solution, the starting material **11** (50 mg, 0.17 mmol, 1 equiv.) was added. The solution was stirred at room temperature for 18 hours. The reaction was monitored via TLC.

The solvent was removed under reduced pressure. From the brown residue a ^{1}H-NMR was performed.

A column with 16 g silica was prepared. The products were eluted with a mobile phase PE:EE=20:1. Only 10 mg of the product was obtained. The column was eluted with pure EE

and the solvent was removed under reduced pressure. From the residue also a ^{1}H-NMR was performed.

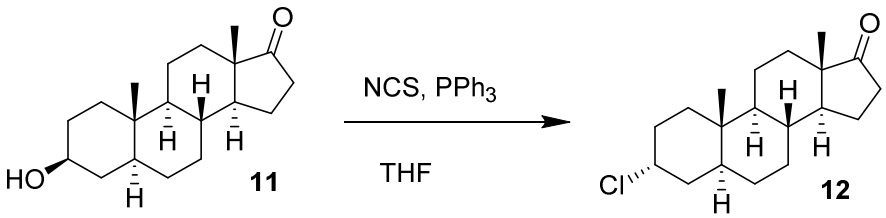
^{1}H NMR (400 MHz, CDCl3) δ 4.51 (s, 1H), 2.44 (dd, J = 19.2, 8.9 Hz, 1H), 1.99 (s, 1H), 1.96 – 1.43 (m, 14H), 1.28 (dd, J = 13.3, 8.2 Hz, 5H), 1.13 – 1.00 (m, 1H), 0.86 (s, 3H), 0.81 (s, 3H).

Comments:

In the literature no pyridine was added to the reaction, but for conversion of the stereocenter (S_N2) a mild base is required.

After 2 hours, the reaction TLC shows still starting material, so the reaction was stirred overnight. Low yields were achieved.

CHLORINATION WITH N-CHLORO-SUCCINIMIDE



Lit.: *Tetrahedron Letters*, **1973**, p. 3937

				equiv.	MW
650,00 mg	(2,24 mmol)	Epiandrosterone		1,00	290,45 g/mol
597,66 mg	(4,48 mmol)	NCS		2,00	133,53 g/mol
1173,92 mg	(4,48 mmol)	PPh_3		2,00	262,28 g/mol
25,00 ml		THF			
691,27 mg	(2,24 mmol)	theoretical yield		1,00	308,89 g/mol
1934,00 mg	(6,26 mmol)	yield crude	280 %		
621,000 mg	(2,01 mmol)	yield column purified	90 %		

NCS (600 mg, 4.48. mmol, 2 equiv.) was dissolved in 6 ml THF and heated up to 50 °C. PPh3 (1.175 g, 4.48 mmol, 2 equiv.) was also dissolved in 6 ml THF and added dropwise via syringe to the solution of NCS in THF. The formation of much white precipitation was observed immediately. The suspension turns violet colored. The starting material **11** (650 mg, 2.24 mmol, 1 equiv.) was solved in 6 ml THF and added to the suspension. The white precipitation was disappearing rather. The reaction mixture was stirred at 50 °C for three hours. The progress of the reaction was monitored via TLC.

The solvent was removed under reduced pressure. The violet residue oil was taken up in diethylether and water. The two phases were transferred into a seperation funnel. The organic

layer was washed with water three times, then dried over anhydrous MgSO4. The solvent was removed in vacuo. From the residue a ^{1}H-NMR was taken.

Column purification was carried out, using a 50 g silica column and a solvent gradient PE → PE:EE=50:1. The product was obtained with a yield of 90 % (621 mg, 2.01 mmol).

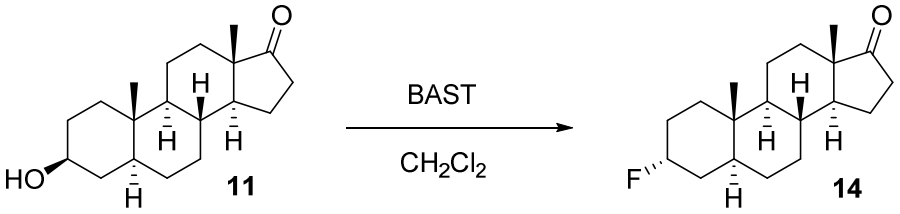
^{1}H NMR (400 MHz, CDCl3) δ 4.51 (s, 1H), 2.43 (dd, J = 19.2, 8.8 Hz, 1H), 2.07 (dt, J = 19.0, 9.0 Hz, 1H), 1.98 – 1.42 (m, 14H), 1.37 – 1.14 (m, 5H), 1.05 (ddt, J = 17.5, 12.0, 5.6 Hz, 1H), 0.86 (s, 3H), 0.81 (s, 3H).

Comments:

The NBS was recrystallized in acetic acid before use. The product shows a crystalline shape but has a pink color. Maybe because of this the reaction mixture turned pink.

3.2.3 3α-fluoro-5αH-androstane-17-one

FLUORINATION WITH BAST



Lit.: *J. Org. Chem.*, Vol. 64, No. 19, **1999**

			d		equiv.	MW
138.00 mg	(0.48 mmol)	Epiandrosterone			1.00	290.45 g/mol
0.30 ml	(0.81 mmol)	BAST	2.70	mol/l	1.70	221.24 g/mol
0.50 ml		CH_2Cl_2				
138.95 mg	(0.48 mmol)	theoretical yield			1.00	292.44 g/mol
130.40 mg	(0.45 mmol)	yield crude	94	%		
29.80 mg	(0.1 mmol)	yield column	21	%		

Starting material **11** (138 mg, 0.48 mmol, 1 equiv.) was dissolved in 0.5 ml DCM in a Schlenk tube. The tube was flushed with argon and the reagent BAST (0.3 ml, 0.71 mmol (2.7 M solution in Toluol), 1.7 equiv.) was added to the stirring solution at room temperature via syringe. The reaction was monitored via TLC. After three hours the reaction was stopped.

The reaction mixture was diluted with DCM and transferred into a seperation funnel. The organic layer was washed twice with aquous $KHCO_3$ solution and twice with water. At the first

contact with the aqueous phase, the organic layer turns white. The solvent was removed under reduced pressure, from the residue, a yellow oil, a ^{1}H-NMR was performed.

The crude residue was solved in DCM. A column with 10 g silica was prepared. The products were eluted with a mobile phase PE:EE=50:1.

^{1}H NMR (400 MHz, CDCl3) δ 4.81 (d, J = 48.7 Hz, 1H), 2.43 (dd, J = 19.2, 8.8 Hz, 1H), 2.07 (dt, J = 18.2, 9.0 Hz, 1H), 1.98 – 1.39 (m, 14H), 1.28 (ddd, J = 22.0, 8.5, 5.1 Hz, 5H), 1.02 (qd, J = 12.7, 4.8 Hz, 1H), 0.86 (s, 3H), 0.81 (s, 3H).

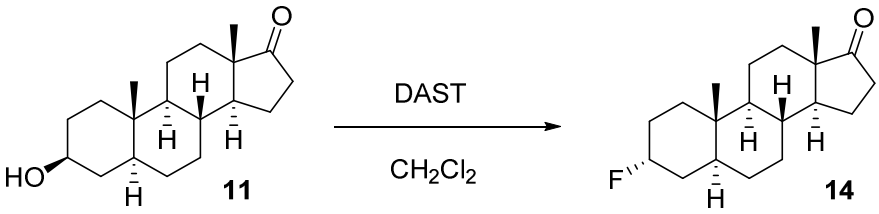
Comments:

The yield of the by-product (elimination product) was 25.5 mg (18%).

No starting material could be recovered.

Due to lower yields and availability of the reagent BAST further reactions were carried out with DAST.

FLUORINATION WITH DAST



Lit.: *Collect. Czech. Chem. Commun.*, **2002,** Vol. 67

			d		equiv.	MW
827,00 mg	(2,85 mmol)	Epiandrosterone			1,00	290,45 g/mol
917,91 mg	(5,69 mmol)	DAST	1,22	g/cm³	2,00	161,19 g/mol
30,00 ml		Dichlormethane				
832,67 mg	(2,85 mmol)	theoretical yield			1,00	292,44 g/mol
929,00 mg	(3,18 mmol)	yield crude	112	%		
250,000 mg	(0,855 mmol)	yield	30	%		

Starting material **11** (827mg, 2.85 mmol, 1 equiv.) was dissolved in DCM in a Schlenk tube. The reagent DAST (918 mg, 5.69 mmol, 2 equiv.) was added to the stirring solution at room temperature via syringe. After 3 hours the TLC shows almost full conversion of the starting material.

The reaction mixture was diluted with 20 ml DCM and transferred into a seperation funnel. The organic layer was washed with 5 ml aquous $NaHCO_3$ solution and with 5 ml brine. The seperation funnel was rinsed with DCM and the combined organig layers were dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure, from the crude residue (96 %) a ^{1}H-NMR was performed.

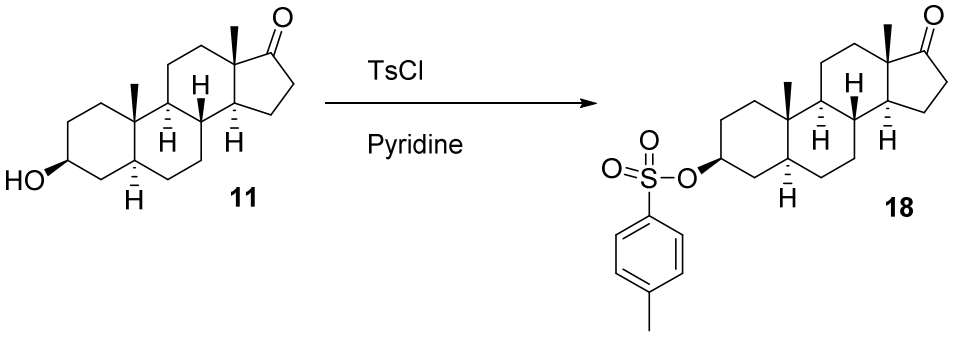
A column with 35 g silica was prepared. The products were eluted with a mobile phase PE (until the first product occurs) → PE:EE=50:1.

The pink color migrated to the aqueous phase during washing.

^{1}H NMR (400 MHz, CDCl3) δ 4.81 (d, J = 48.6 Hz, 1H), 2.43 (dd, J = 19.2, 8.8 Hz, 1H), 2.07 (dt, J = 18.3, 9.0 Hz, 1H), 1.96 – 1.44 (m, 14H), 1.32 – 1.23 (m, 5H), 1.03 (tt, J = 12.1, 6.1 Hz, 1H), 0.86 (s, 3H), 0.81 (s, 3H).

3.2.4 3ß-ethoxy-5αH-androstane-17-one

TOSYLATION AND SUBSTITUTION



Lit.: *Chemistry of Natural Compounds*, **2004**, Vol. 40, No. 2

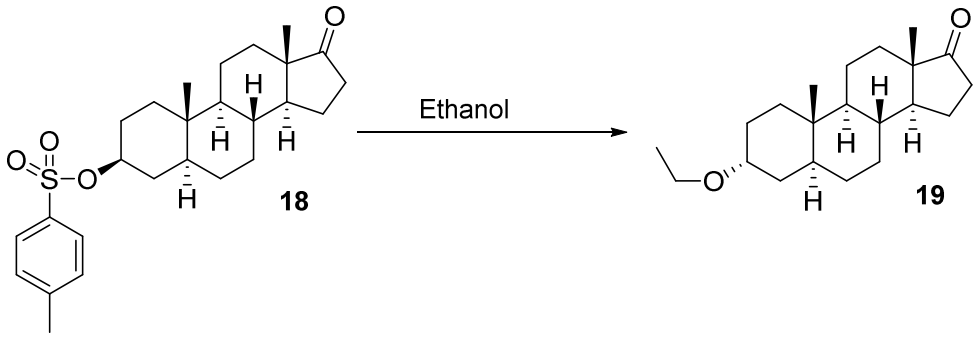
			d		equiv.	MW
265.00 mg	(0.91 mmol)	Epiandrosterone			1.00	290.45 g/mol
347.87 mg	(1.82 mmol)	4-Toluenesulfonyl chloride			2.00	190.64 g/mol
					0.10	172.20 g/mol
5.00 mL		Pyridine				79.10 g/mol
405.67 mg	(0.91 mmol)	theoretical yield			1.00	444.63 g/mol
569.00 mg	(1.28 mmol)	yield (crude)	140	%		
135.00 mg	(0.3 mmol)	yield (purified)	33	%		

Pyridine (6 ml) was freshly distilled. Starting material **11** (225 mg, 0.77 mmol, 1 equiv.) was dissolved in pyridine. The solution was cooled down to 0 °C with an ice bath. At this temperature, 4-Toluenesulfonyl chloride (295 mg, 1.55 mmol, 2 equiv.) was added in portions.

The reaction mixture was warmed up to room temperature and stirred overnight for 22 hours. The completion of the reaction was monitored via TLC.

The reaction mixture was poured in 20 ml ice water. The white precipitate was filtered off and washed with cold water. The filter was cleaned with DCM and the solvent was evaporated, giving 138 mg white crystals. The water phase appeared as a white solution, so the solvent was also removed, giving 431 mg of whitish crystals and a clear oil. For recrystallization, only the white crystals from the precipitation were used. The filtrated white crystals **18** (135 mg, 33 % Y) were used in the next reaction.

^{1}H NMR (400 MHz, CDCl3) δ 7.79 (d, J = 8.3 Hz, 2H), 7.38 – 7.31 (m, 2H), 4.41 (tt, J = 10.9, 5.1 Hz, 1H), 2.44 (s, 3H), 2.42 – 2.35 (m, 1H), 2.12 – 1.99 (m, 1H), 1.96 – 1.39 (m, 14H), 1.24 (ddd, J = 24.2, 13.3, 6.4 Hz, 5H), 0.94 (t, J = 5.1 Hz, 1H), 0.84 (s, 3H), 0.80 (s, 3H).



Lit.: *Gazzetta Chimica Italiana*, **1962**, vol. 92, p. 632 - 646

			d		equiv.	MW
135.00 mg	(0.30 mmol)	Epiandrosterone (protected)			1.00	444.63 g/mol
2.69 ml	(45.54 mmol)	Ethanol	0.78		150.00	46.07 g/mol
96.70 mg	0.30 mmol)	theoretical yield			1.00	318.5 g/mol
192.00 mg	0.60 mmol)	yield (crude)	199	%		
53.00 mg	(0.17 mmol)	yield (purified)	55	%		

The synthesized material **18** (135 mg, 0.3 mmol, 1 equiv.) was dissolved in 3.2 ml absolute Ethanol. The reaction mixture was heated up to 85 °C (oil bath). The material dissolved after 0.5 hours. The solution was stirred and refluxed for 96 hours. The reaction was monitored via TLC.

The solvent was evaporated under reduced pressure. After the crude NMR the crystals in yellow oil were solved in DCM. A column with 10 g silica was prepared. The products were eluted with a mobile phase PE:EE=50:1 → 20:1.

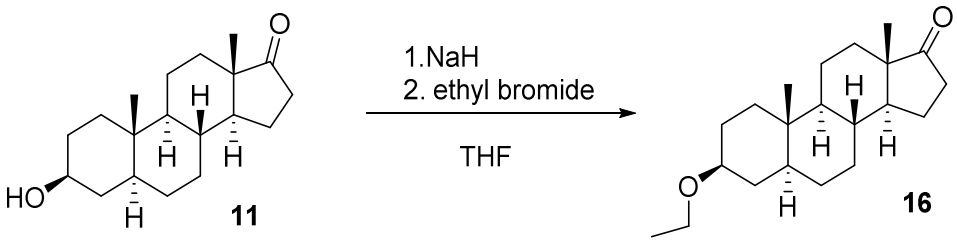
^{1}H NMR (400 MHz, CDCl3) δ 3.55 – 3.51 (m, 1H), 3.43 (ttd, J = 9.5, 7.0, 2.5 Hz, 2H), 2.42 (dd, J = 19.3, 8.2 Hz, 1H), 2.13 – 2.00 (m, 1H), 1.97 – 1.24 (m, 19H), 1.19 (t, J = 7.0 Hz, 3H), 1.02 (qd, J = 12.5, 4.8 Hz, 1H), 0.85 (s, 3H), 0.81 (s, 3H).

Comments:

The crude NMR shows still signals from the tosylate. But also, a quartett at 3.7 ppm appeared which might be a signal from the ether. It could also come from ethanol.

After purification the elimination by-product (F1) was obtained with 6 mg, the product **19** was obtained with a yield of 55 % (53 mg, F2).

WILLIAMSON ETHER SYNTHESIS



Lit.: *Journal of Medicinal Chemistry*, **2005**, vol. 48, # 16, p. 5257 - 5268

			d	equiv.	MW
299,00 mg	(1,03 mmol)	Epiandrosterone		1,00	290,45 g/mol
82,32 mg	(2,06 mmol)	NaH 60% suspension		2,00	23,99 g/mol
0,61 ml	(8,24 mmol)	Ethylbromide	1,47	8,00	108,97 g/mol
8,00 mL		THF			71,10 g/mol
327,88 mg	(1,03 mmol)	theoretical yield		1,00	318,5 g/mol
357,00 mg	(1,12 mmol)	yield (crude)	109 %		
158,00 mg	(0,5 mmol)	yield (purified)	48 %		

Starting material **11** (299 mg, 1.03 mmol, 1 equiv.) was dissolved in 8 ml dry THF. Under argon atmosphere the base NaH (82 mg, 2.06 mmol, 2 equiv.) in a 60 % suspension in mineral oil was added. The reaction mixture was heated up to 70 °C and refluxed for 1 hour. The reagent ethyl bromide (0.61 ml, 8.26 mmol, 8 equiv.) was added via syringe. The Schlenk tube was attached to a cooler under argon atmosphere and the reaction mixture stirred at refluxing temperature overnight (22.5 hours).

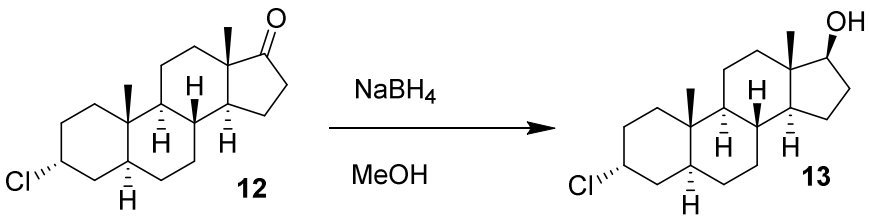
After a TLC the yellow/white reaction mixture was cooled to room temperature and diluted with 10 ml H_2O. In a separation funnel, the aqueous layer was extracted with EE. The organic phases were combined and were washed with brine and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure. A mixture of yellow oil and crystals were obtained with a crude yield of 109 %. A ^{1}H-NMR was performed.

Column purification was carried out, using 30 g of silica and a solvent mixture of PE:EE = 40:1 → 20:1.

^{1}H NMR (400 MHz, CDCl3) δ 3.45 (qd, J = 7.0, 1.0 Hz, 2H), 3.15 (td, J = 11.1, 5.5 Hz, 1H), 2.36 (dd, J = 19.2, 8.7 Hz, 1H), 2.05 – 1.93 (m, 1H), 1.91 – 1.14 (m, 19H), 1.12 (t, J = 7.0 Hz, 3H), 0.90 (dd, J = 12.9, 4.6 Hz, 1H), 0.79 (s, 3H), 0.76 (s, 3H).

3.3 Synthesis of the converted marker substances

3.3.1 3α-chloro-5αH-androstane-17ß-ol



Lit.: *Chemistry of Natural Compounds*, Vol. 43, No. 1, **2007**

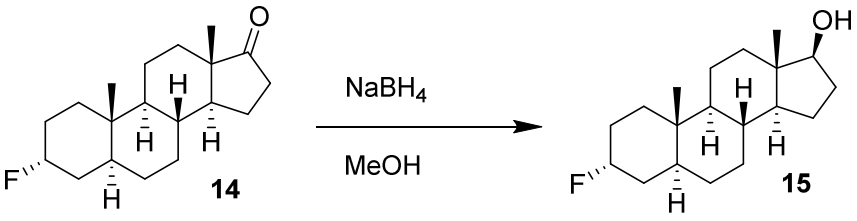
					equiv.	MW
164,00 mg	(0,53 mmol)	3a-chloro-5aH-androstan-17-one			1,00	308,89 g/mol
80,34 mg	(2,12 mmol)	$NaBH_4$			4,00	37,83 g/mol
10,00 ml		MeOH				
165,07 mg	(0,53 mmol)	theoretical yield			1,00	310,91 g/mol
142,00 mg	(0,46 mmol)	yield crude	86	%		

12 (164 mg, 0.53 mmol, 1 equiv.) was dissolved in 10 ml MeOH. The solution was cooled down to 0 °C with an ice bath. To the stirring solution, $NaBH_4$ (81 mg, 2.12 mmol, 4 equiv.) was added slowly. The reaction mixture stirred at room temperature. After one hour, the reaction was complete.

The reaction mixture was acidified with acetic acid (~3 ml) and poured into 10 ml H_2O. a white precipitation was obtained, which was filtered off and washed with water.

^{1}H NMR (400 MHz, CDCl3) δ 4.55 – 4.46 (m, 1H), 3.64 (t, J = 8.5 Hz, 1H), 2.06 (ddt, J = 18.7, 9.2, 4.9 Hz, 1H), 1.90 (ddd, J = 13.8, 6.7, 4.0 Hz, 1H), 1.86 – 1.38 (m, 14H), 1.29 – 1.16 (m, 5H), 0.98 (dd, J = 11.6, 4.1 Hz, 1H), 0.79 (s, 3H), 0.73 (s, 3H).

3.3.2 3α-fluoro-5αH-androstane-17ß-ol



Lit.: *Chemistry of Natural Compounds*, Vol. 43, No. 1, **2007**

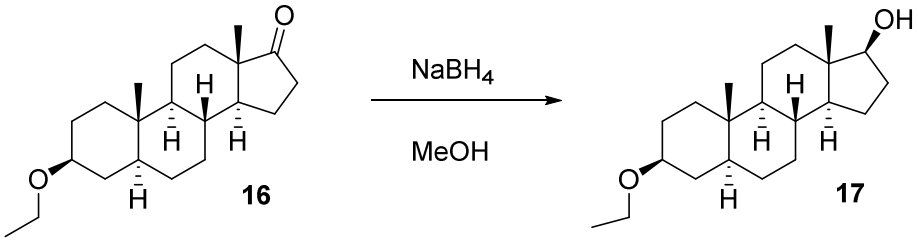
					equiv.	MW
105,00 mg	(0,36 mmol)	3a-fluoro-5aH-androstan-17-one			1,00	292,44 g/mol
54,33 mg	(1,44 mmol)	$NaBH_4$			4,00	37,83 g/mol
7,00 ml		MeOH				
105,72 mg	(0,36 mmol)	theoretical yield			1,00	294,45 g/mol
88,00 mg	(0,3 mmol)	yield crude	83	%		

14 (105 mg, 0.36 mmol, 1 equiv.) was dissolved in 7 ml MeOH. The solution was cooled down to 0 °C with an ice bath. To the stirring solution, $NaBH_4$ (55 mg, 1.44 mmol, 4 equiv.) was added slowly. The reaction mixture stirred at room temperature. After one hour the reaction was complete.

The reaction mixture was acidified with acetic acid (~3 ml) and poured into 10 ml H_2O. The white precipitation was filtered off and washed with water.

^{1}H NMR (400 MHz, CDCl3) δ 4.80 (d, J = 48.6 Hz, 1H), 3.63 (t, J = 8.5 Hz, 1H), 2.05 (ddt, J = 14.9, 9.2, 4.7 Hz, 1H), 1.89 (t, J = 12.9 Hz, 1H), 1.69 – 1.17 (m, 19H), 0.93 (dd, J = 12.3, 4.4 Hz, 1H), 0.79 (s, 3H), 0.73 (s, 3H).

3.3.3 3ß-ethoxy-5αH-androstane-17ß-ol



Lit.: *Chemistry of Natural Compounds*, Vol. 43, No. 1, **2007**

			d		equiv.	MW
90,00 mg	(0,28 mmol)	3ß-ethoxy-5aH-androstan-17-on			1,00	318,5 g/mol
54,52 mg	(1,44 mmol)	NaBH4			5,10	37,83 g/mol
5,00 mL		MeOH				32,04 g/mol
90,57 mg	(0,28 mmol)	theoretical yield			1,00	320,52 g/mol
100,00 mg	(0,31 mmol)	yield (crude)	110	%		
53,00 mg	(0,17 mmol)	yield (purified)	59	%		

16 (90 mg, 0.28 mmol, 1 equiv.) was dissolved in 5 ml MeOH. The solution was cooled down to 0 °C with an ice bath. To the stirring solution, $NaBH_4$ (55 mg, 1.44 mmol, 5.1 equiv.) was added slowly. The reaction mixture stirred at room temperature. After one hour the reaction was complete.

The reaction mixture was acidified with acetic acid (~3 ml) and poured into 5 ml H_2O. The white precipitation was filtered off (por.3) and washed with water.

^{1}H NMR (400 MHz, CDCl3) δ 3.62 (t, J = 8.6 Hz, 1H), 3.50 (dddd, J = 9.2, 7.0, 4.8, 2.4 Hz, 2H), 3.21 (ddd, J = 15.8, 11.1, 4.6 Hz, 1H), 2.03 (ddd, J = 13.5, 9.5, 5.8 Hz, 1H), 1.89 – 1.81 (m, 1H), 1.80 – 1.34 (m, 14H), 1.26 (ddt, J = 11.2, 6.4, 3.8 Hz, 5H), 1.19 (t, J = 7.0 Hz, 3H), 0.95 – 0.91 (m, 1H), 0.80 (s, 3H), 0.72 (s, 3H).

Comments:

The crude NMR showed the signals of the product (t, 3.6, 1H) and small amounts of acetic acid. Full characterization was carried out. No further purification was needed.

The low yield arrived maybe due to the use of a Büchner funnel with a great porosity. Although the washing solution was used further, maybe a part of the product was stuck in the sinter disk. Unfortunately, the funnel was not further treated with solvent. Also, maybe product was lost in the washing water, which was not further worked up.

3.4 Sample Preparation

The sample preparation followed the general workup procedure for endogenous steroids defined in AA/DOP/S04 according to TD EAAS. (11) [24]

A 2.5 ml aliquot is taken form the urine samples. 1 ml of 0.8 M phosphate puffer (pH = 6.8) is added. IS S04 is added (50 µL). For hydrolysis, 25 µL of the enzyme ß-glucuronidase are added. The solution is mixed and incubation in a water bath takes place (e.g. 50 °C for 2 hours). The hydrolysis is stopped by addition of 1 mL carbonate puffer (pH = 9.8). 5 mL MTBE are added. The aqueous phase is extracted by shaking of the samples for 10 minutes. After centrifugation the organic phase is separated and evaporated. The dry samples are mixed with 80 µL derivatization agent MSTFA/TMSI. After derivatization for 20 minutes at 60 °C the solution is transferred into autosampler vials and is measured with GC-MSMS.

For the derivatization agent 200 mg NH_4I and 600 µL ethanthiol-TMS are solved in 10 mL MSTFA. 3 units of this stock solution are mixed with 10 units MSTFA.

3.4.1 Preparation of standard solutions

The synthesized substances were solved in methanol. Stock solutions with a concentration of 1 mg/mL were prepared. For the analytical evaluation without derivatization 1 µL of the solution was mixed with 50 µL internal standard 5α-androstan-3ß-ol in MTBE (c = 17 µg/mL). For the measurements with derivatization 5 µL of the solution were mixed with 5 µL internal standard d3-testosterone (c = 1 mg/mL). The solvent was evaporated; the dry samples were mixed with 100 µL MSTFA/TMSI (60 °C, 20 minutes).

Dilutions with a concentration of 1 µg/mL were prepared. 100 µL of this dilution were added as internal standard to the experiments.

3.4.2 Enzymatic Conversion with Ketoreductase

For the experiments for enzymatic conversion by addition of enzyme ketoreductase (CAS 9028-12-0) was used. 1 mg of the white crystals with an enzyme activity of ≥ 0.2 U/mg was reconstituted in 1 ml phosphate puffer (pH = 6). 50 µL of this solution were added to the sample solutions. The needed co-substrate ß-nicotinamide adenine dinucleotide (NADPH) was dissolved in 10 mM NaOH and diluted (c = 1 µg/mL). 10 µL of this solution were added to the sample solutions.

3.4.3 Enzymatic Conversion with infected Urine

For these experiments urine samples were collected over a longer period during the summer months. The anonymized samples from men and women were stored at 2-8 °C.

3.5 GC-MS(MS)- conditions

3.5.1 GC-MS

The used temperature program for analysis of the underivatized samples is shown in Table 5. The samples were injected in splitless mode at a temperature of 275 °C, the carrier flow dropped from 2.2 mL/min to 1.3 mL/min after four minutes.

Table 5: temperature program for measurements with GC-MS

Rate [°C/min]	Temperature [°C]	Hold time [min]
-	80	0.5
30	230	0
4	280	0
30	300	5

The mass spectrometer conditions were positive EI mode with 70 eV collision energy, measured in full scan mode in a mass range from 42 – 410 amu.

3.5.2 GC-MSMS

The used temperature program for analysis of the underivatized samples is shown in Table 6. The sample was injected in split mode at a temperature of 270 °C, the carrier gas had a constant pressure over the time of 98 kPa.

Table 6: temperature program for measurements with GC-MSMS

Rate [°C/min]	Temperature [°C]	Hold time [min]
-	170	0
3	210	1
25	305	3

The mass spectrometer conditions were positive EI mode with collision energies ranging from 6 to 23 eV. It was measured in SRM mode.

4. Literature

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ANNEX

3α-chloro-5αH-androstane-17-one

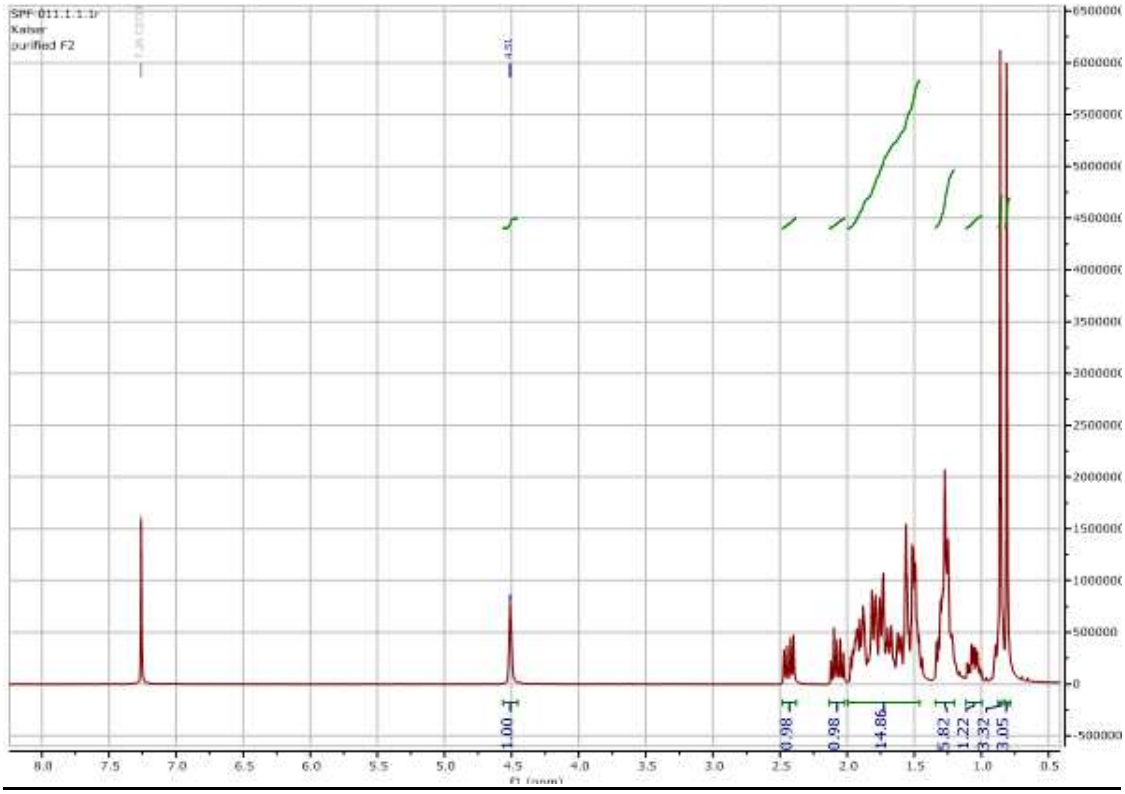

*Figure 19: ^{1}H-NMR of **12***

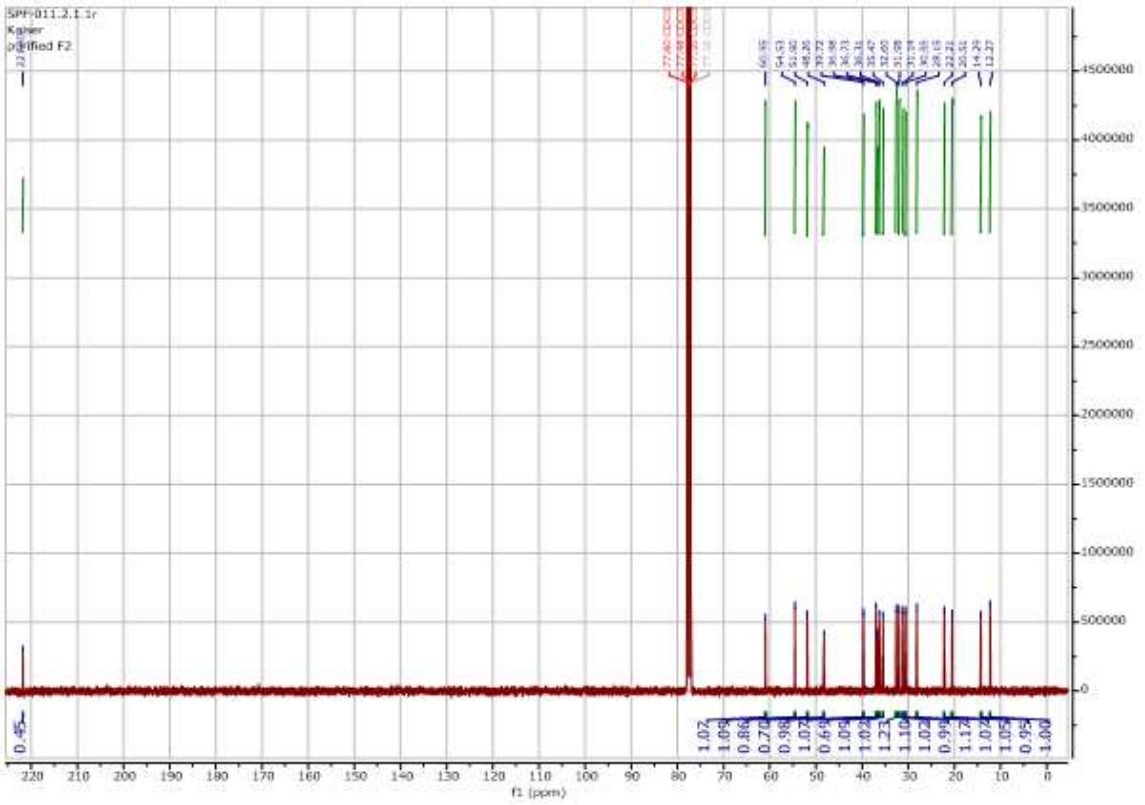

*Figure 20: ^{13}C-NMR of **12***

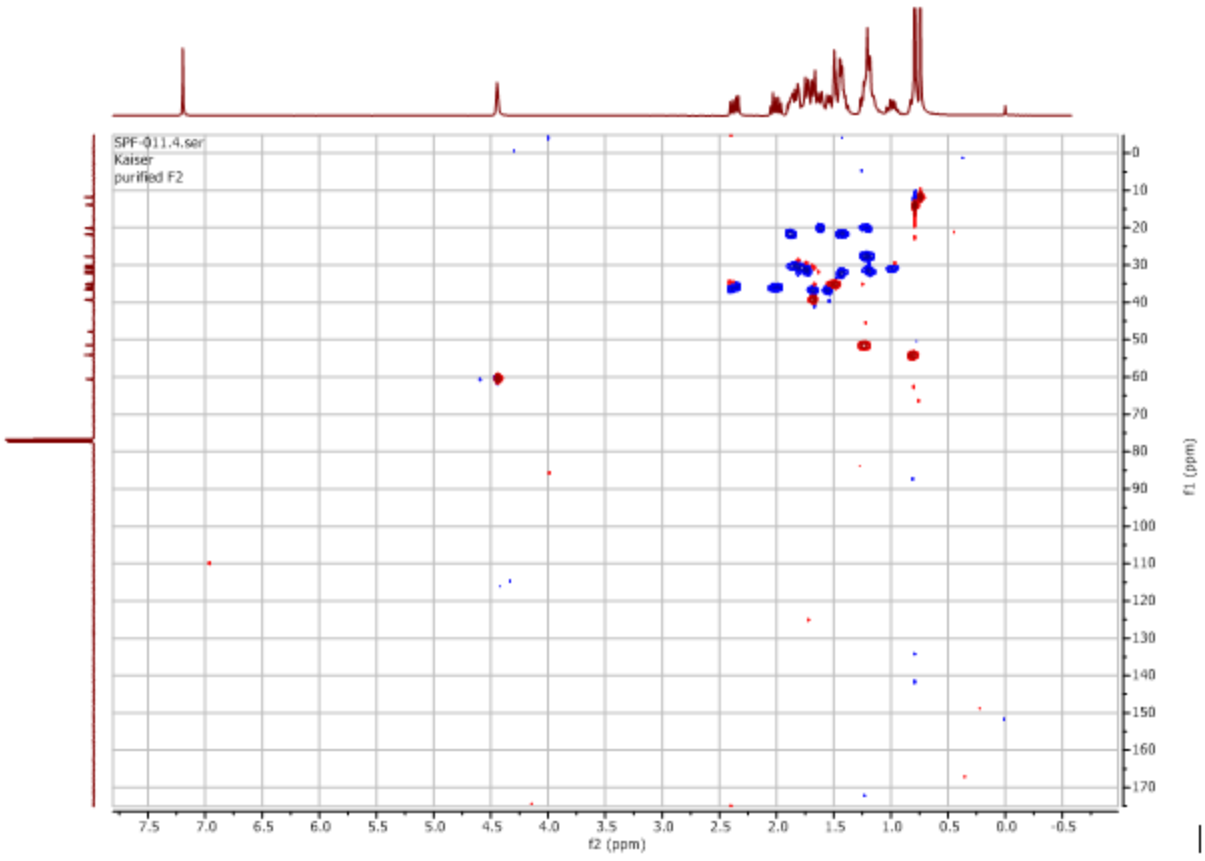

*Figure 21: HSQC of **12***

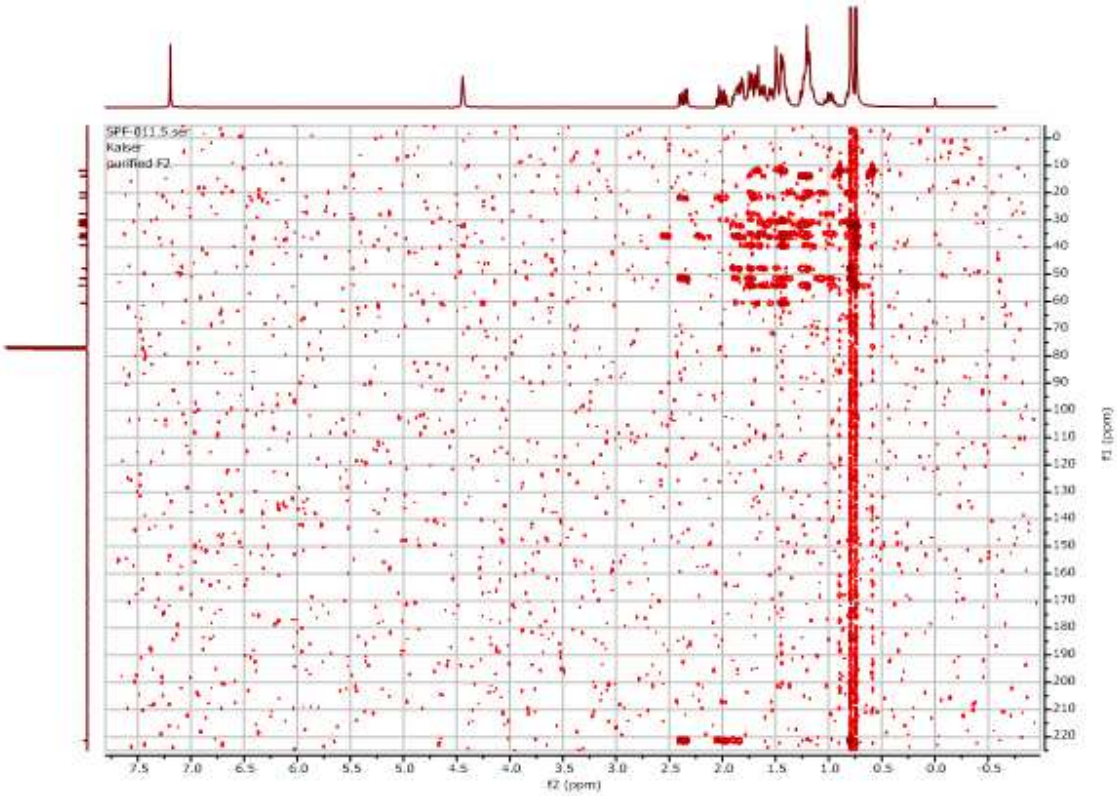

*Figure 22: HMBC of **12***

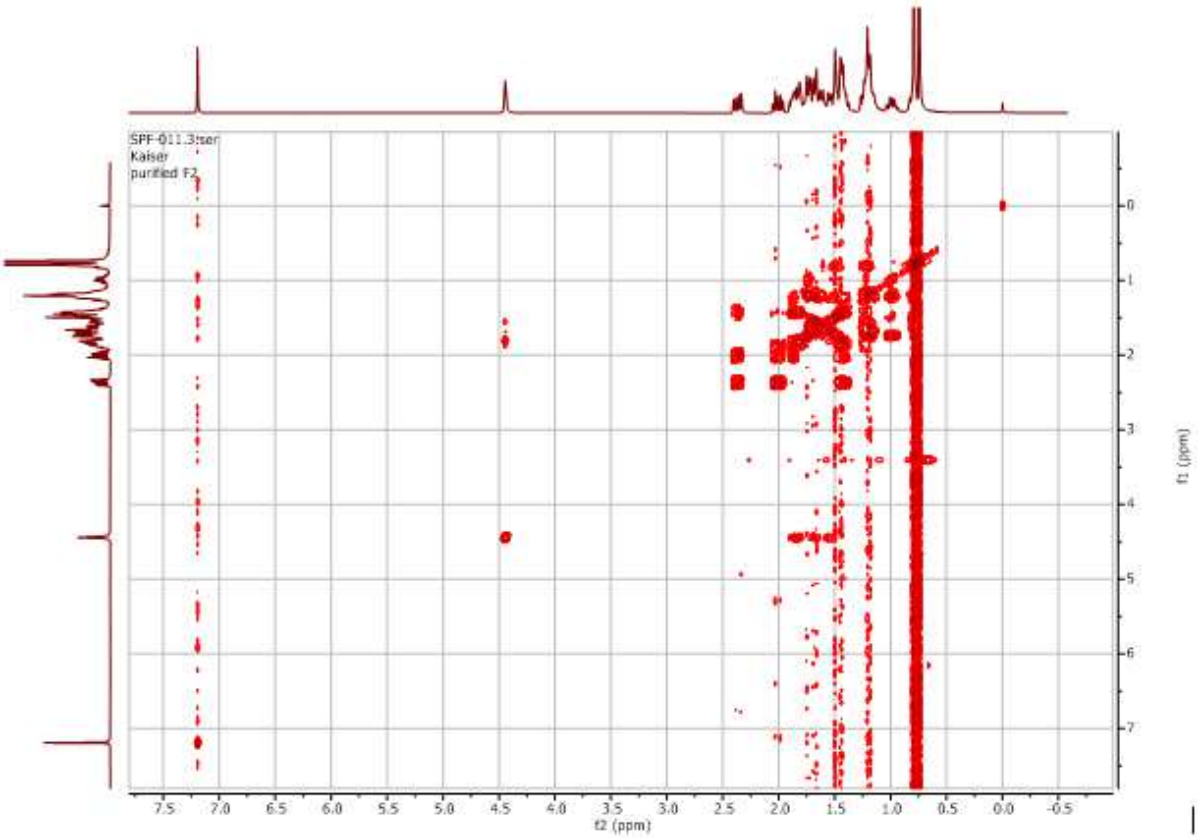

*Figure 23: COSY of **12***

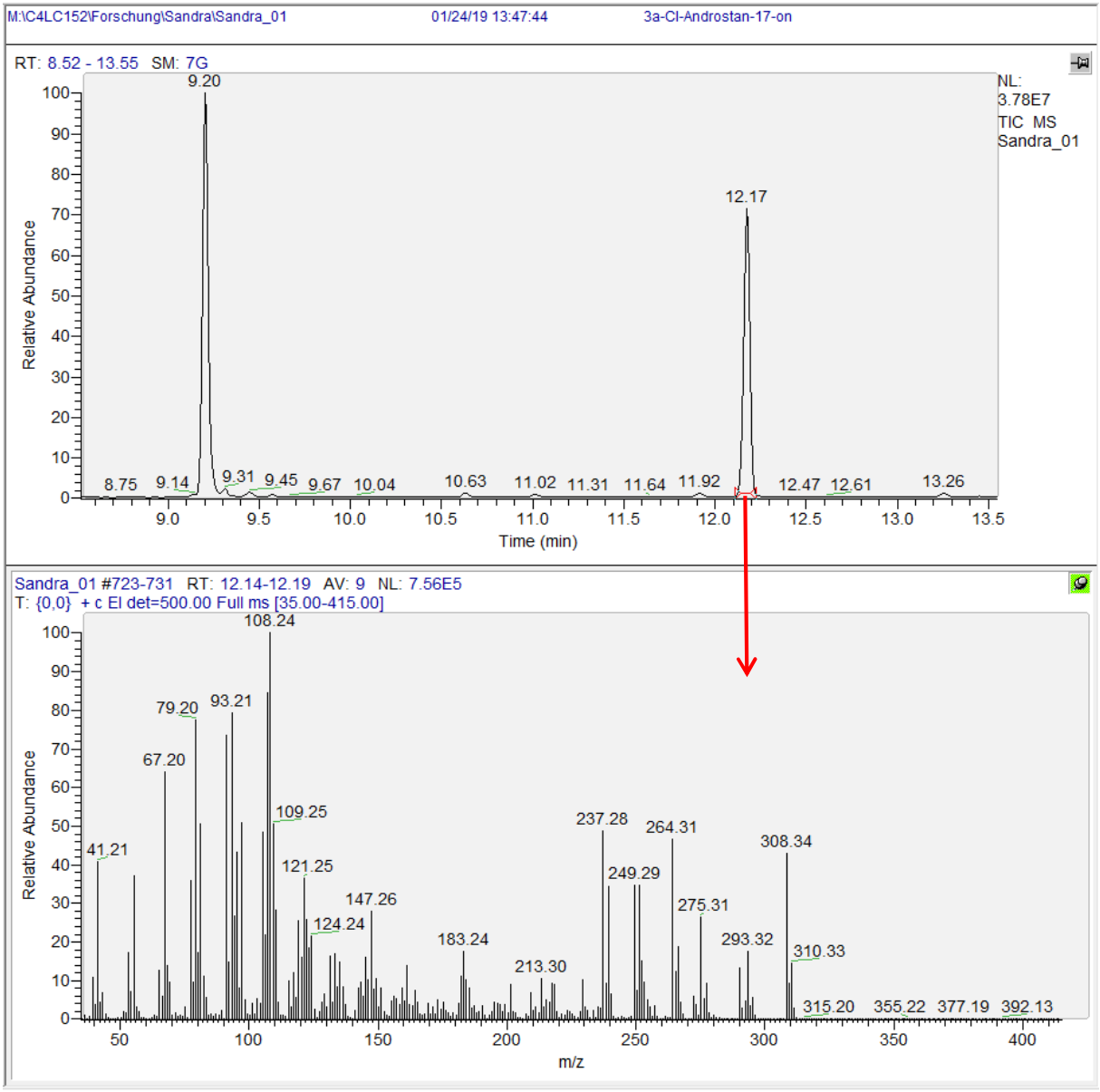

*Figure 24: GC-MS chromatogram and full scan spectra of **12** without derivatization*

3α-chloro-5αH-androstane-17ß-ol

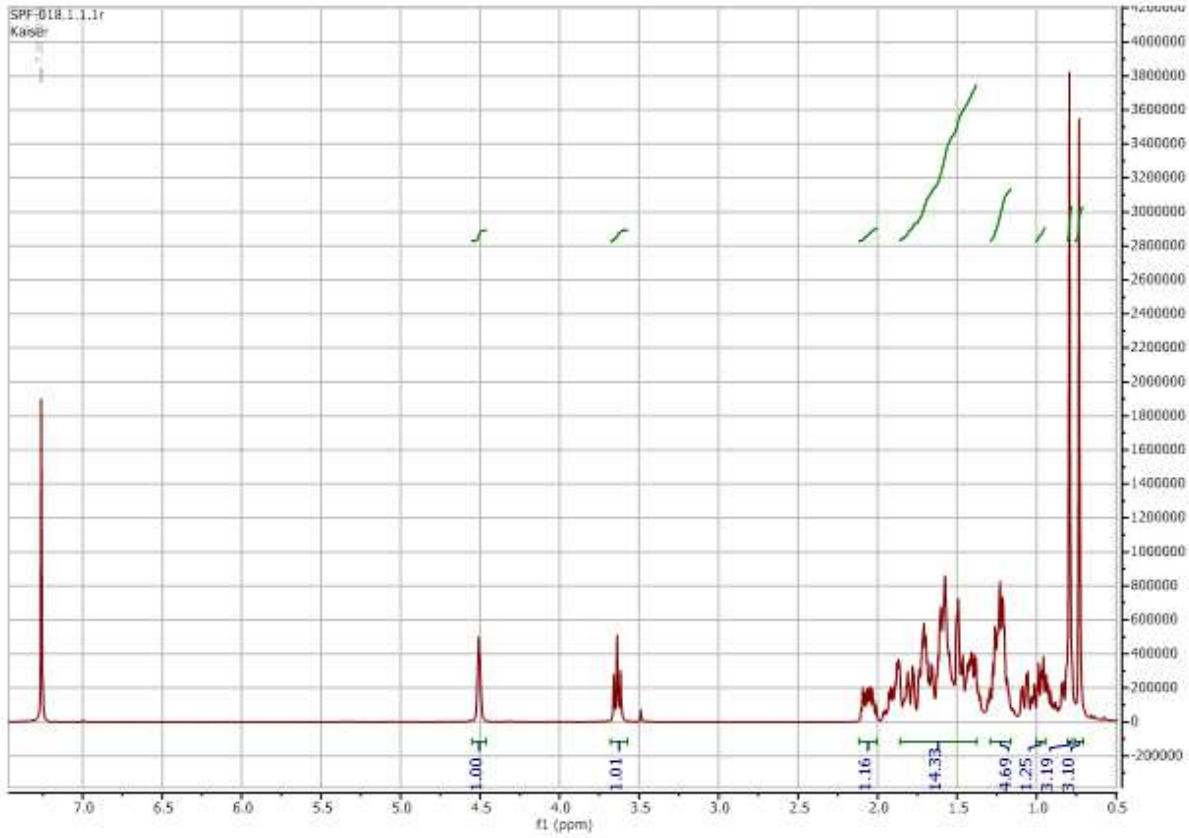

*Figure 25: [1]H-NMR of **13***

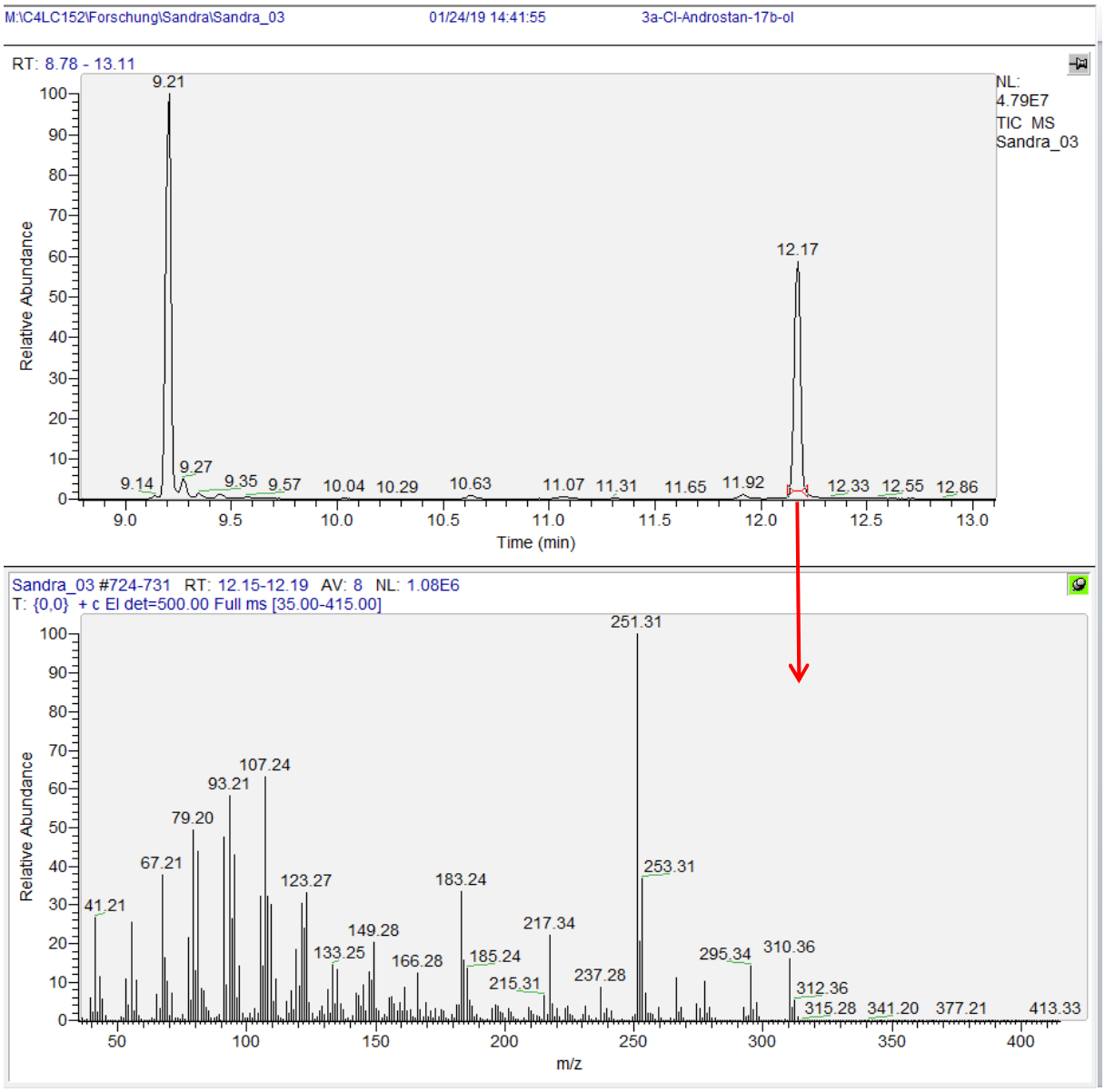

*Figure 26: GC-MS chromatogram and full scan spectra of **13** without derivatization*

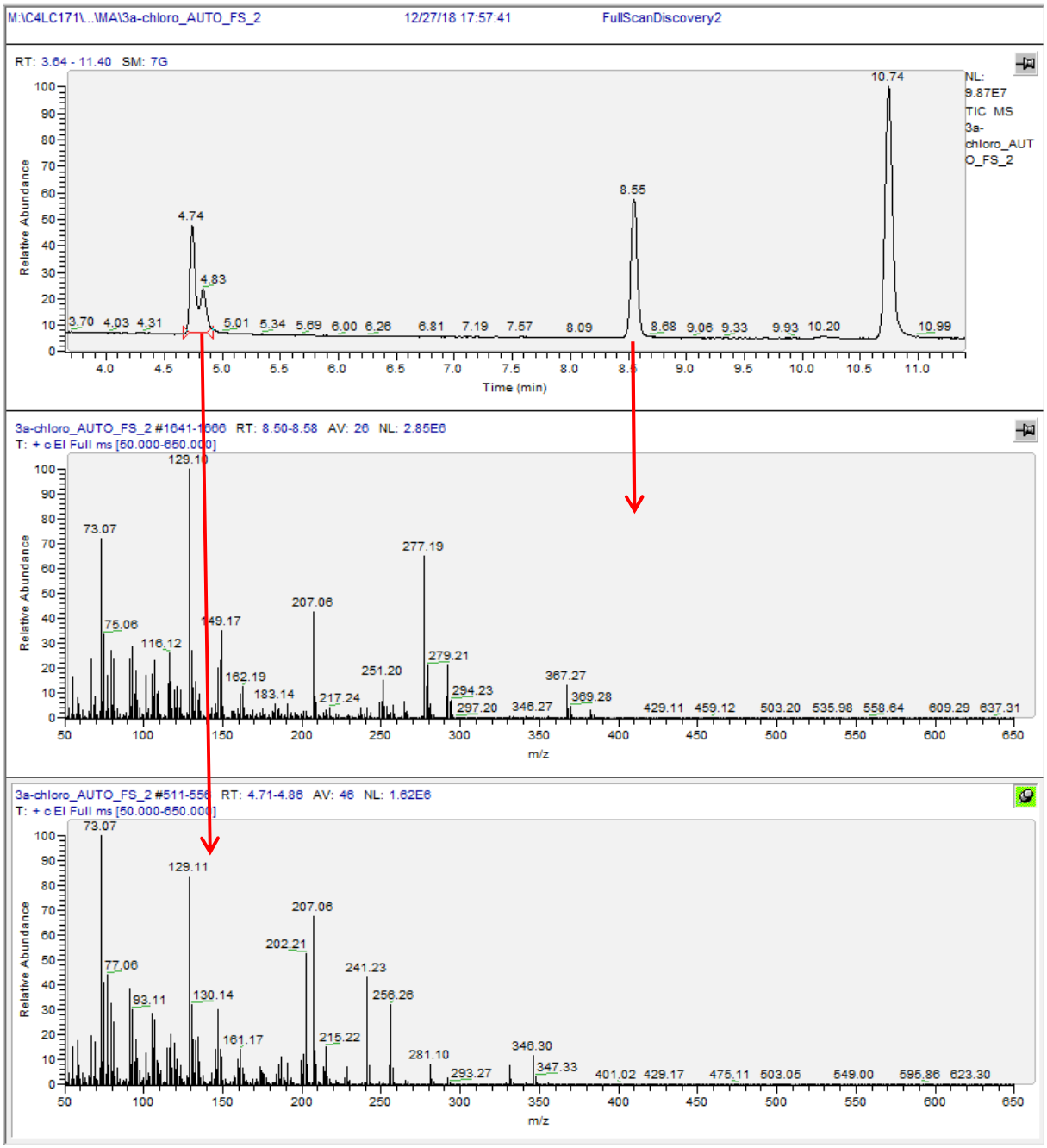

*Figure 27: full scan chromatogram of internal standard d3-testosterone (RT 10.74), **13** (RT 8.55) and its elimination product (RT 4.74). The corresponding mass spectra for **13** and its elimination product are shown below.*

3α-fluoro-5αH-androstane-17-one

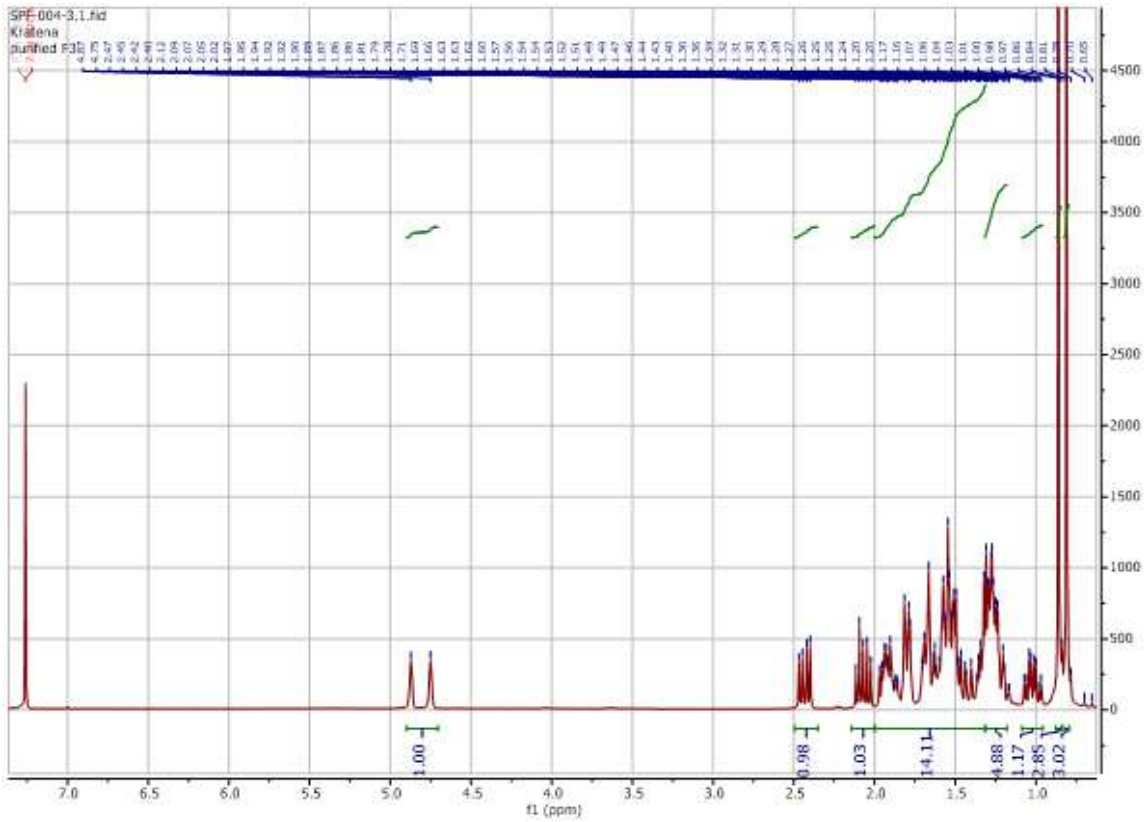

*Figure 28: [1]H-NMR of **14***

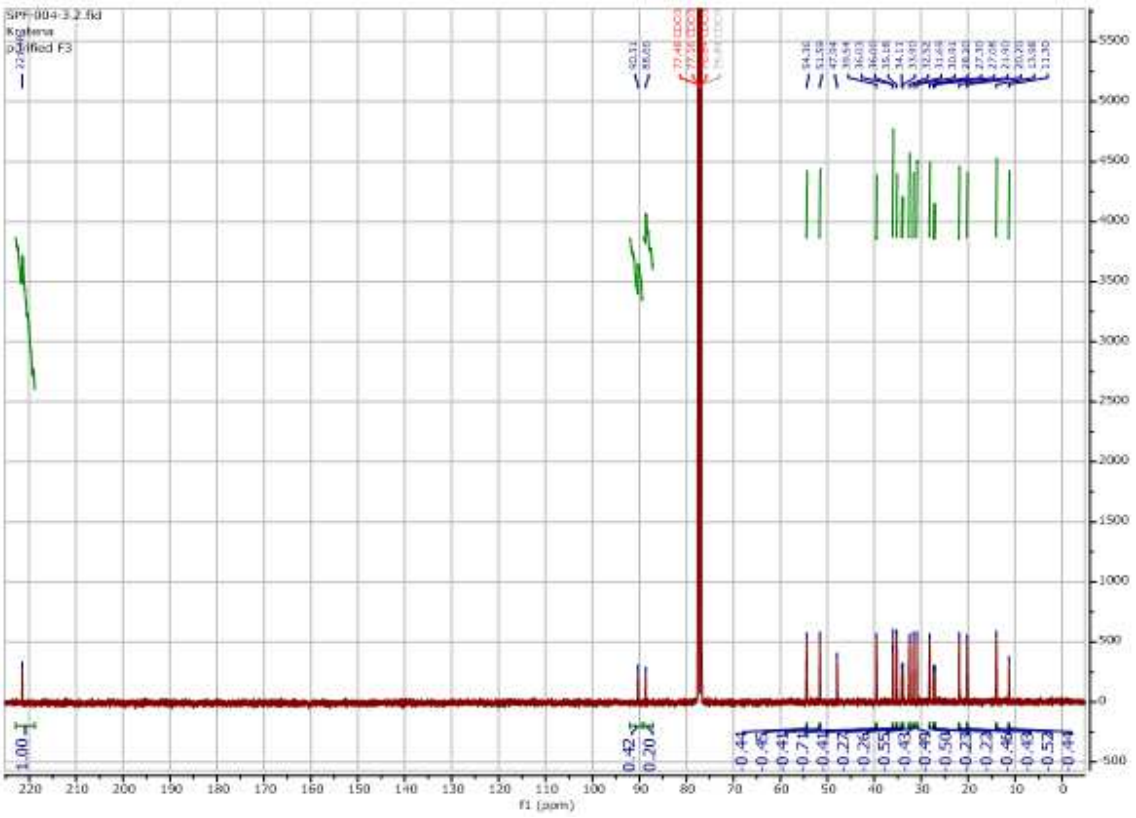

*Figure 29: ^{13}C-NMR of **14***

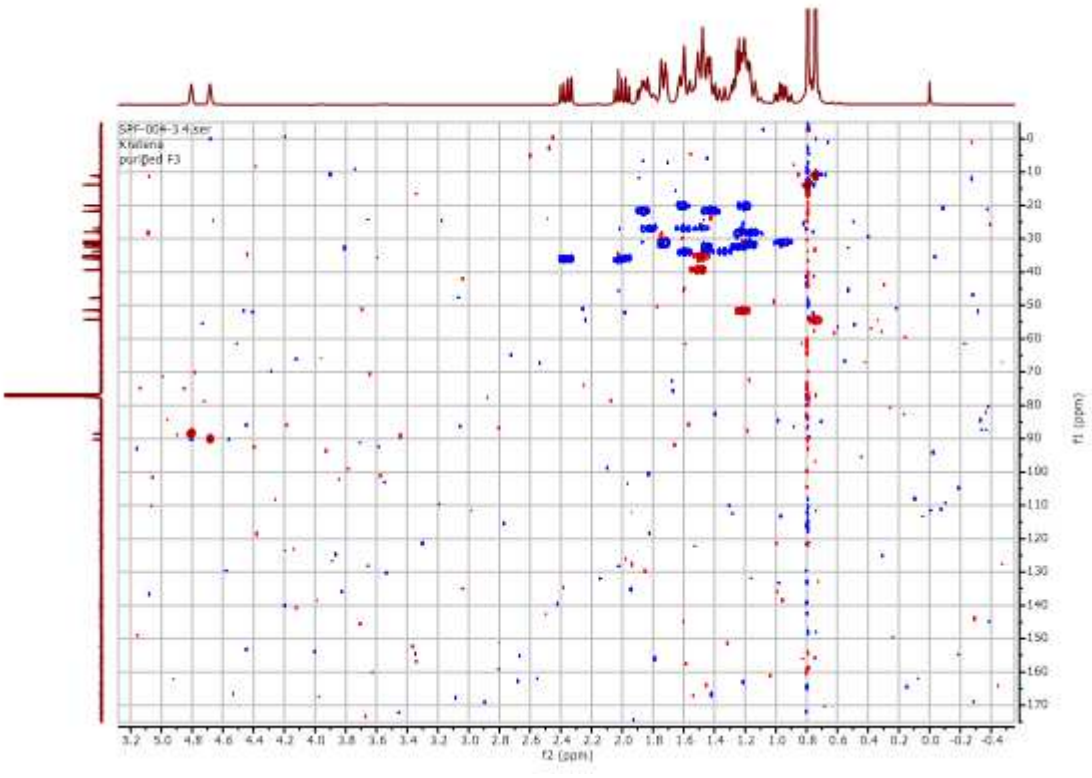

*Figure 30: HSQC of **14***

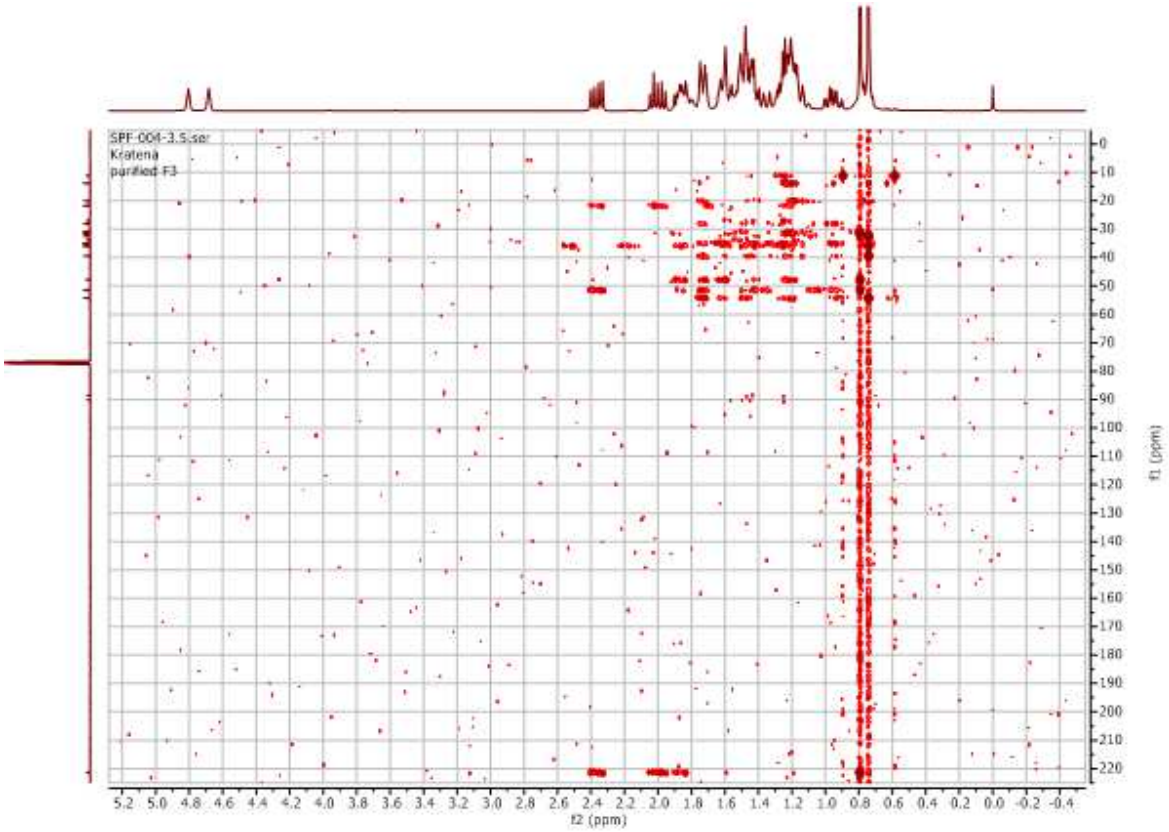

*Figure 31: HMBC of **14***

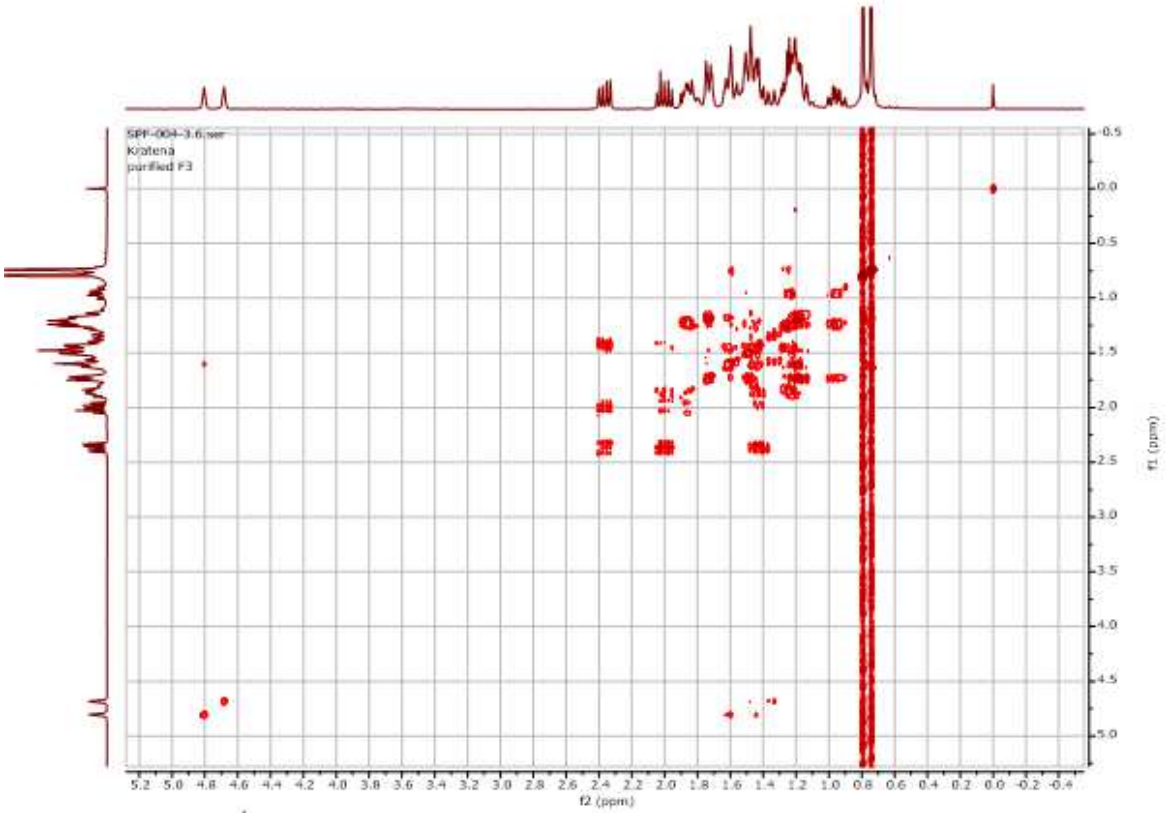

*Figure 32: COSY of **14***

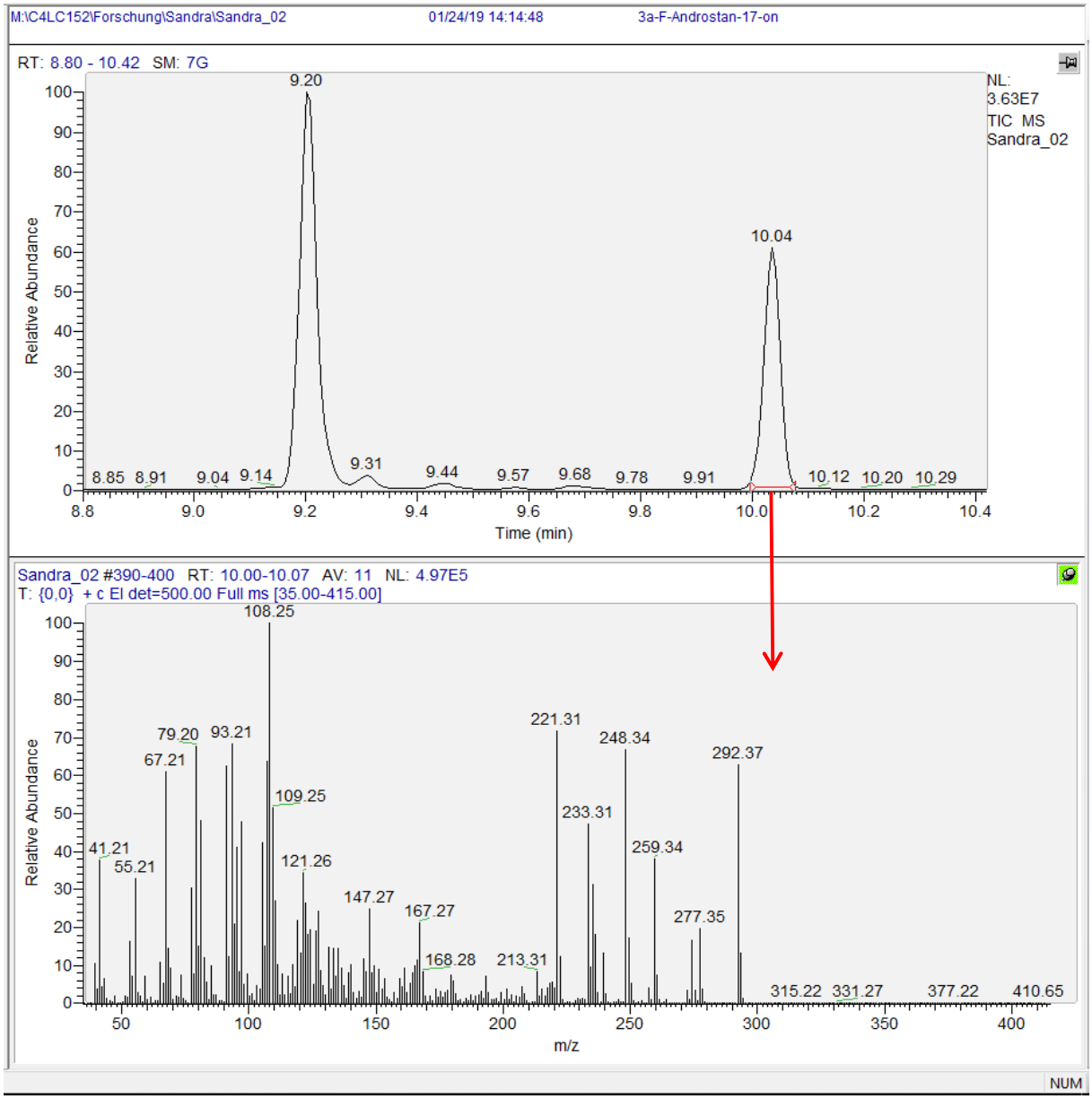

*Figure 33: GC-MS chromatogram and full scan spectra of **14** without derivatization*

3α-fluoro-5αH-androstane-17ß-ol

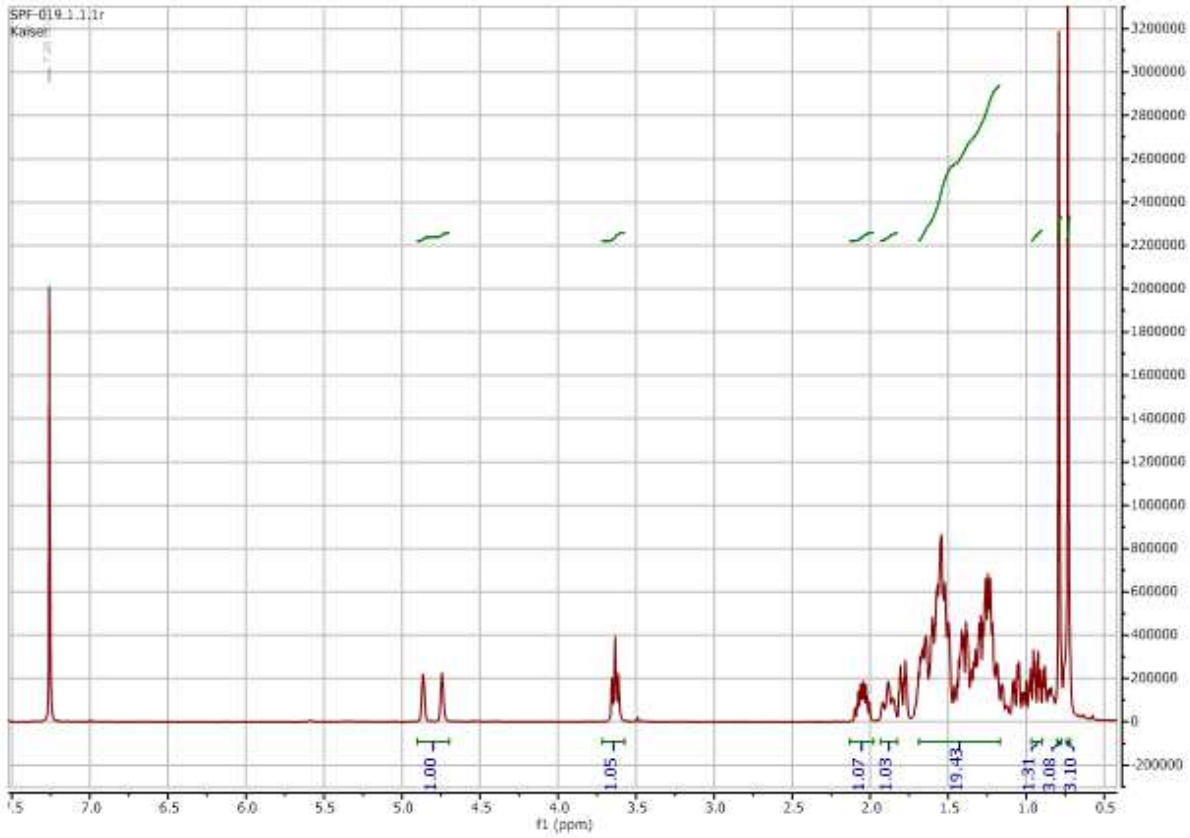

*Figure 34: ^{1}H-NMR of **15***

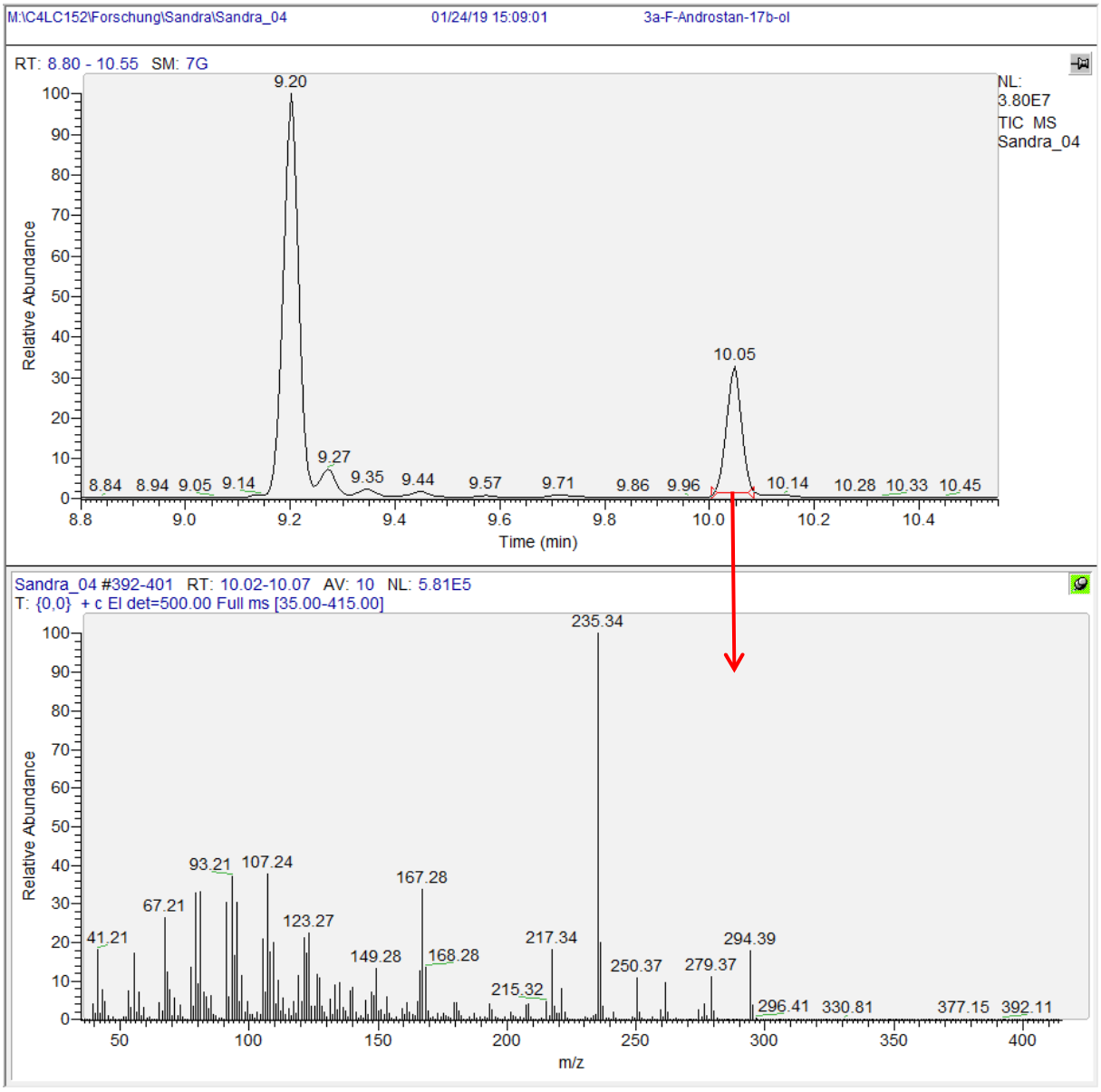

*Figure 35: GC-MS chromatogram and full scan spectra of **15** without derivatization*

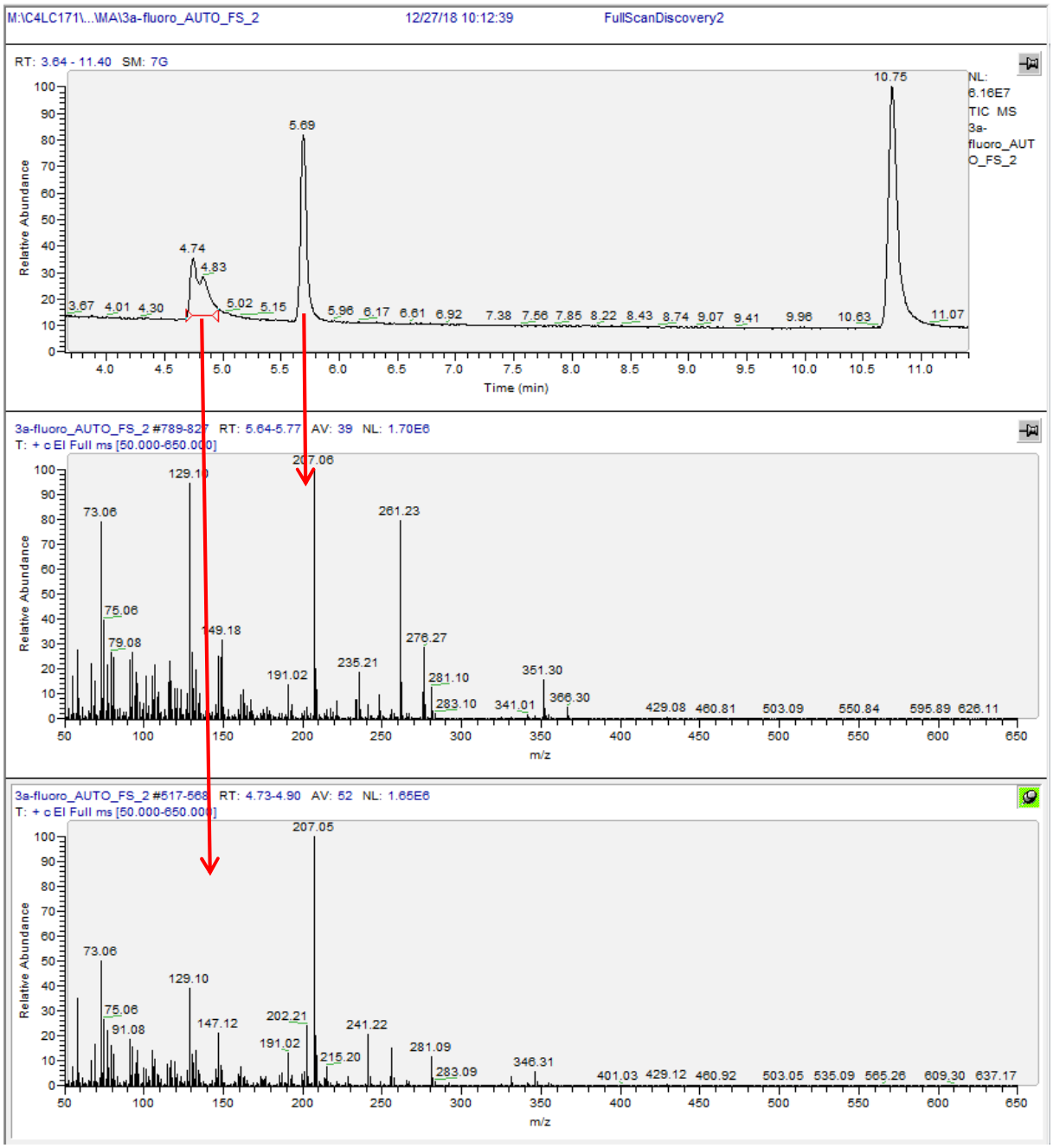

*Figure 36: full scan chromatogram of internal standard d3-testosterone (RT 10.75), **15** (RT 5.69) and its elimination product (RT 4.74). The corresponding mass spectra for **15** and its elimination product are shown below.*

3ß-ethoxy-5αH-androstane-17-one

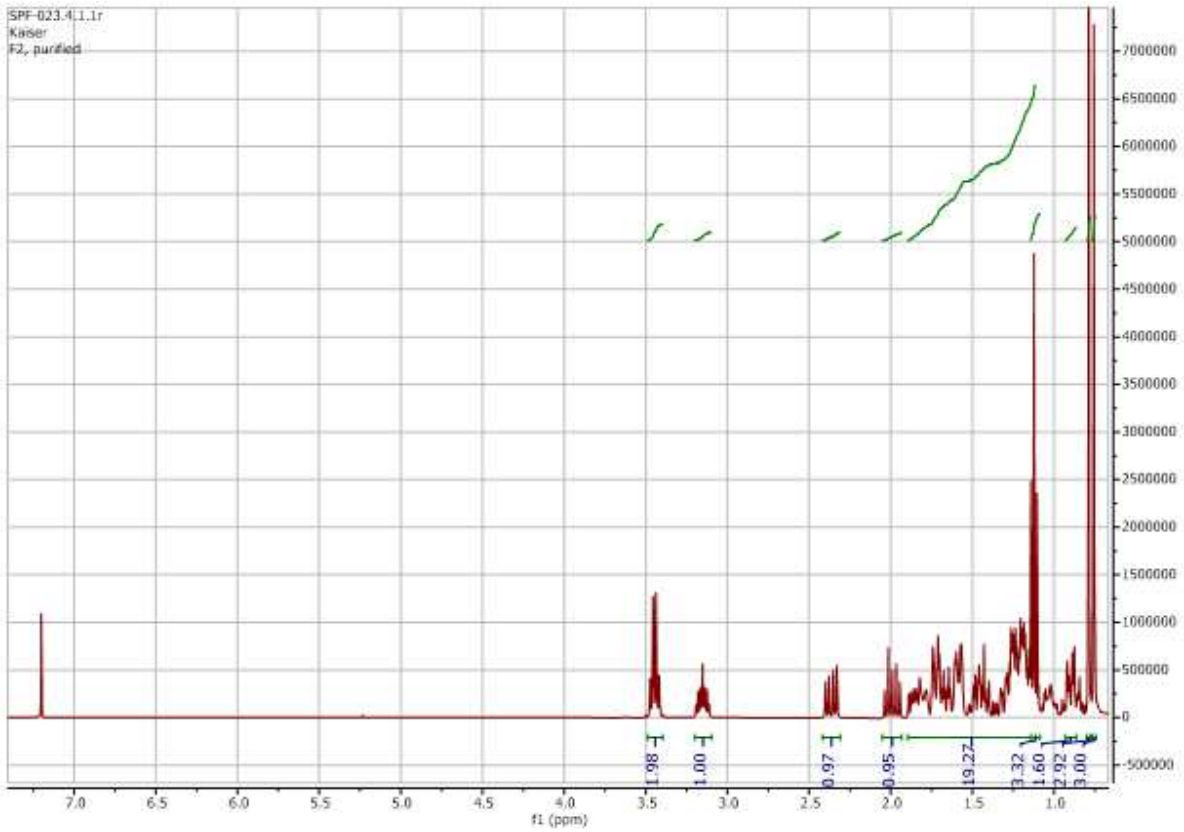

*Figure 37: [1]H-NMR of **16***

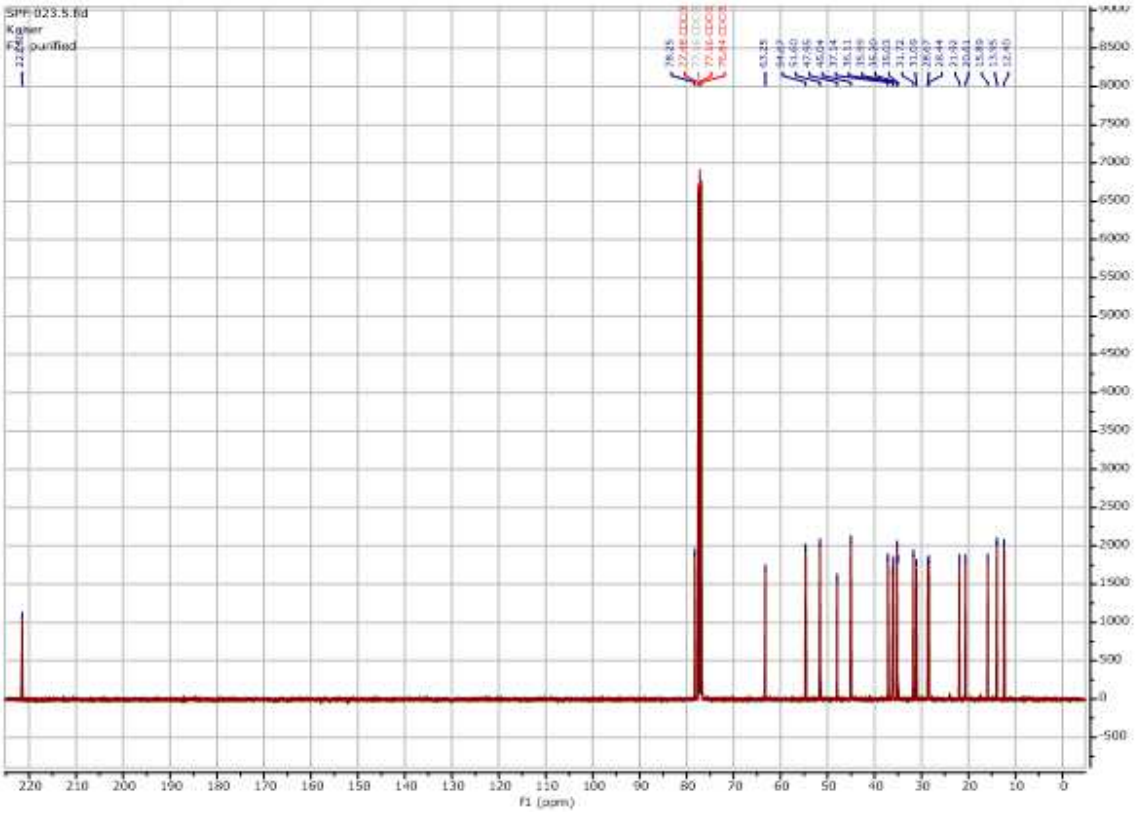

*Figure 38: ^{13}C-NMR of **16***

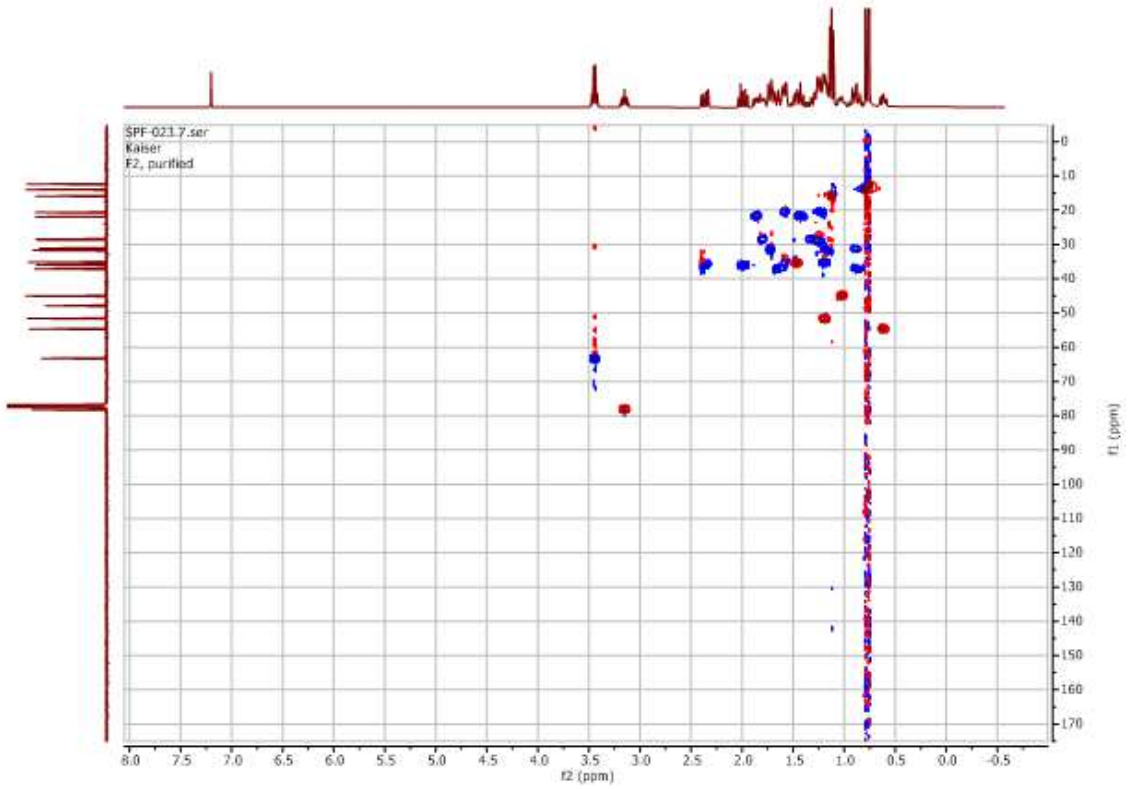

*Figure 39: HSQC of **16***

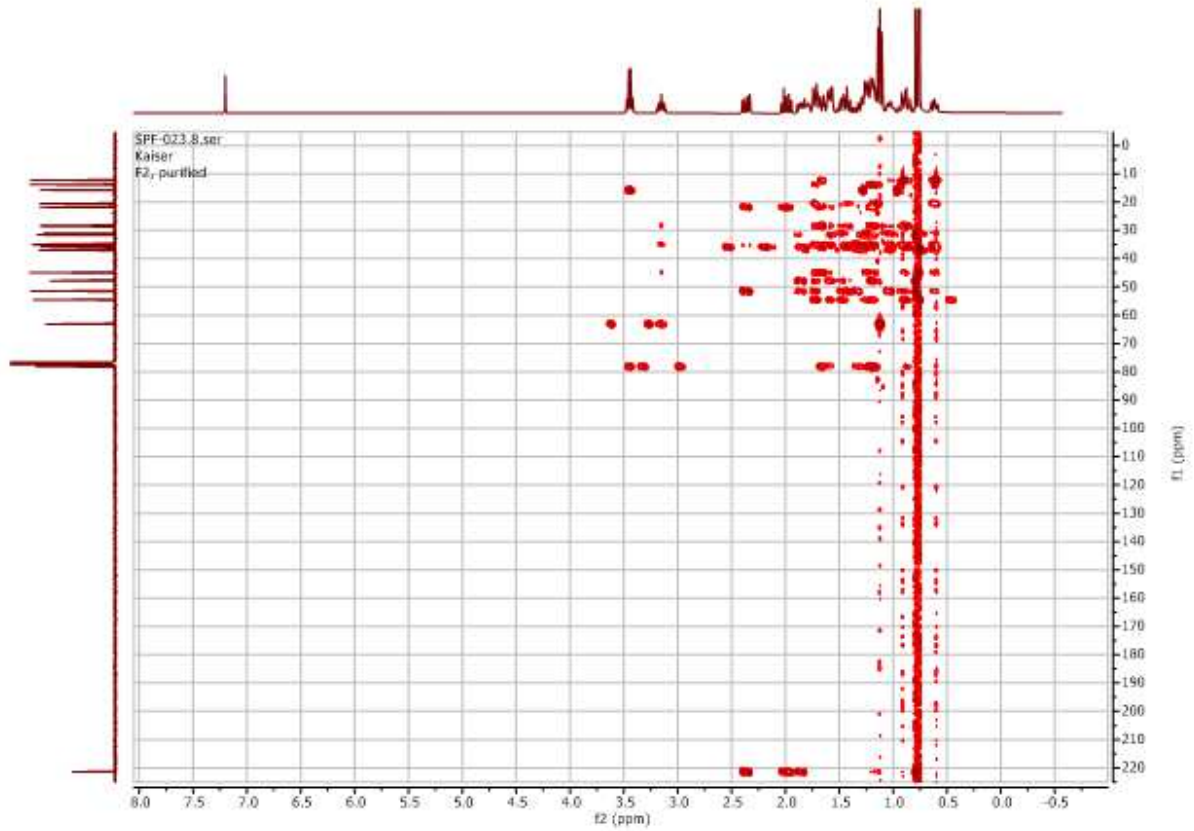

*Figure 40: HMBC of **16***

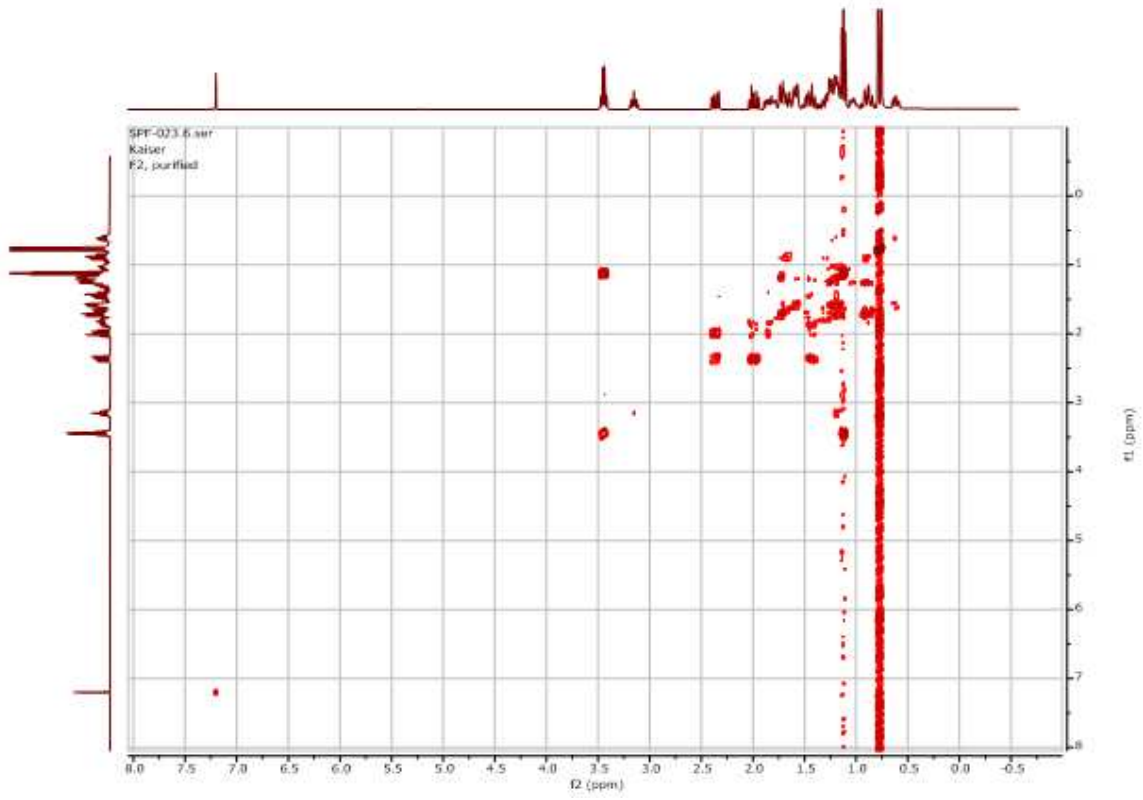

*Figure 41: COSY of **16***

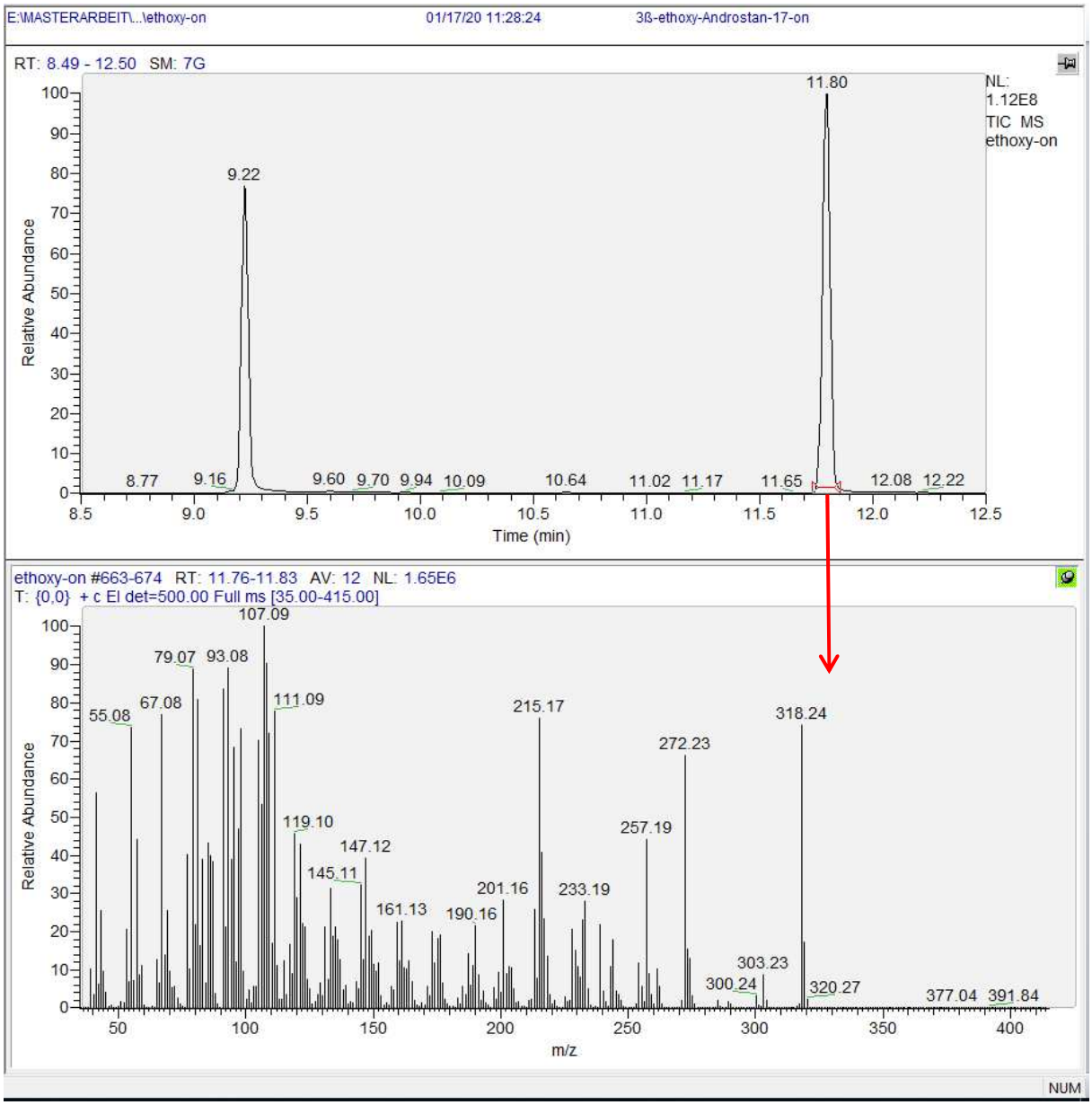

*Figure 42: GC-MS chromatogram and full scan spectra of **16** without derivatization*

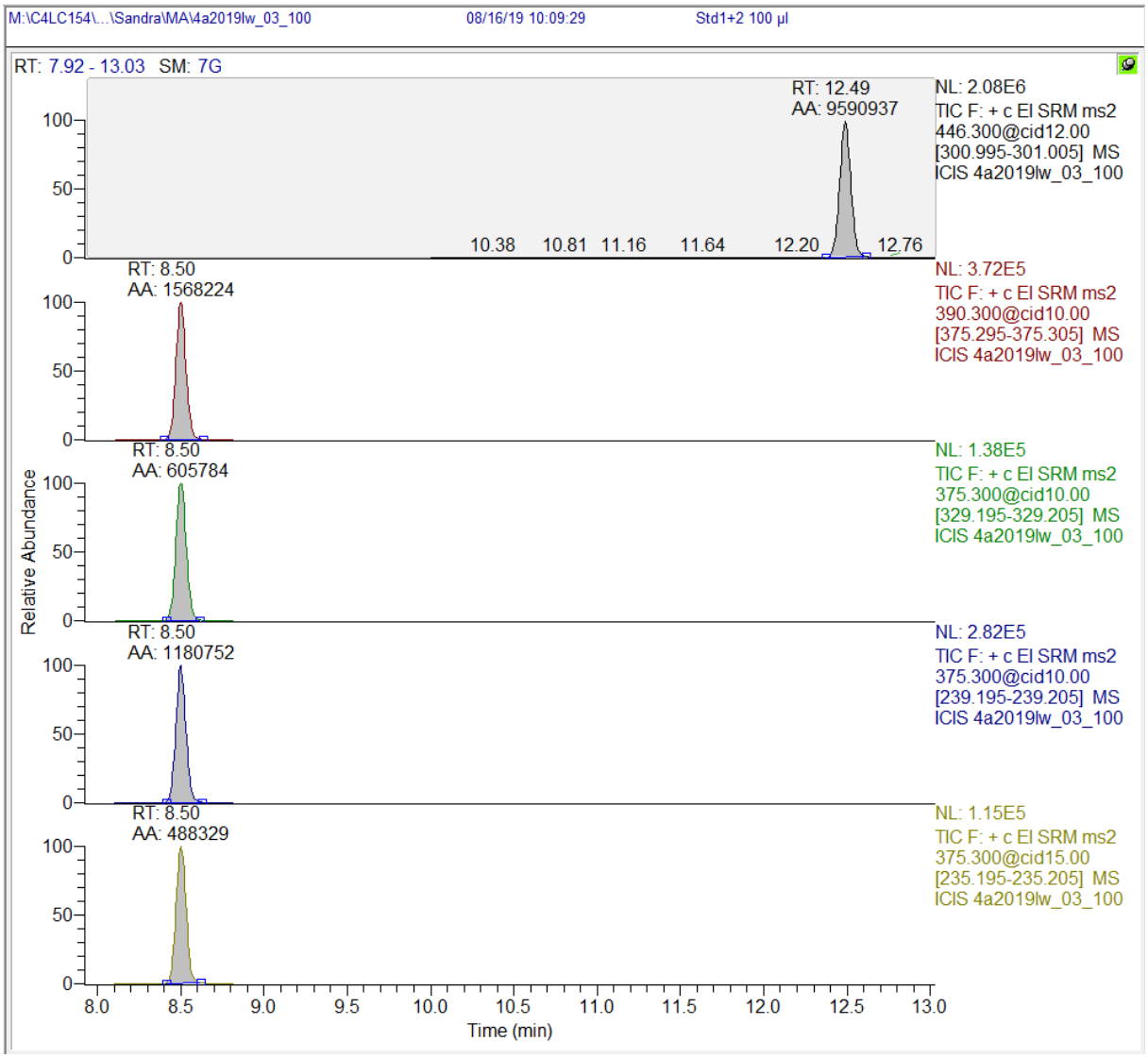

*Figure 43: transition of IS methyltestosterone (RT 12.49) and four transitions for **16** (RT 8.50)*

3ß-ethoxy-5αH-androstane-17ß-ol

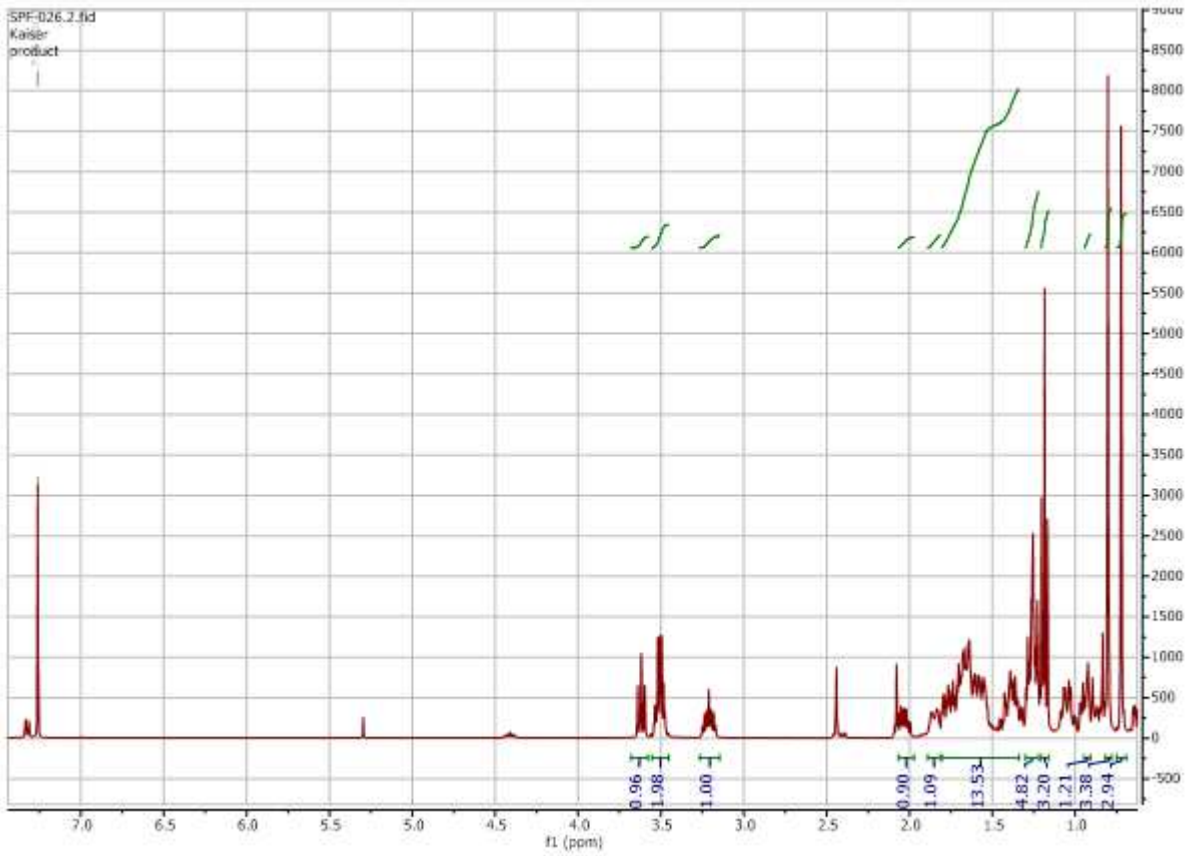

*Figure 44: [1]H-NMR of **17***

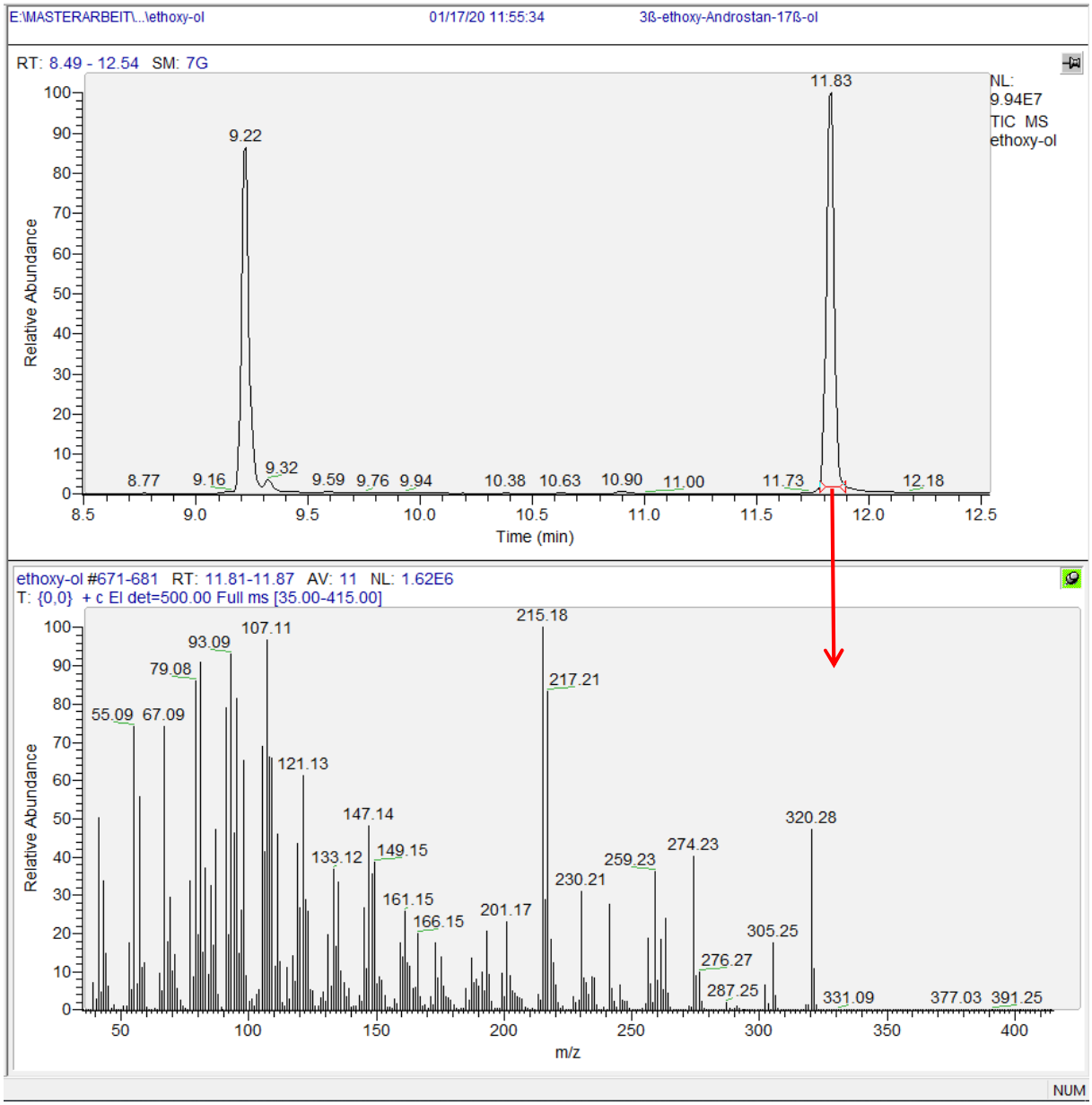

*Figure 45: GC-MS chromatogram and full scan spectra of **17** without derivatization*

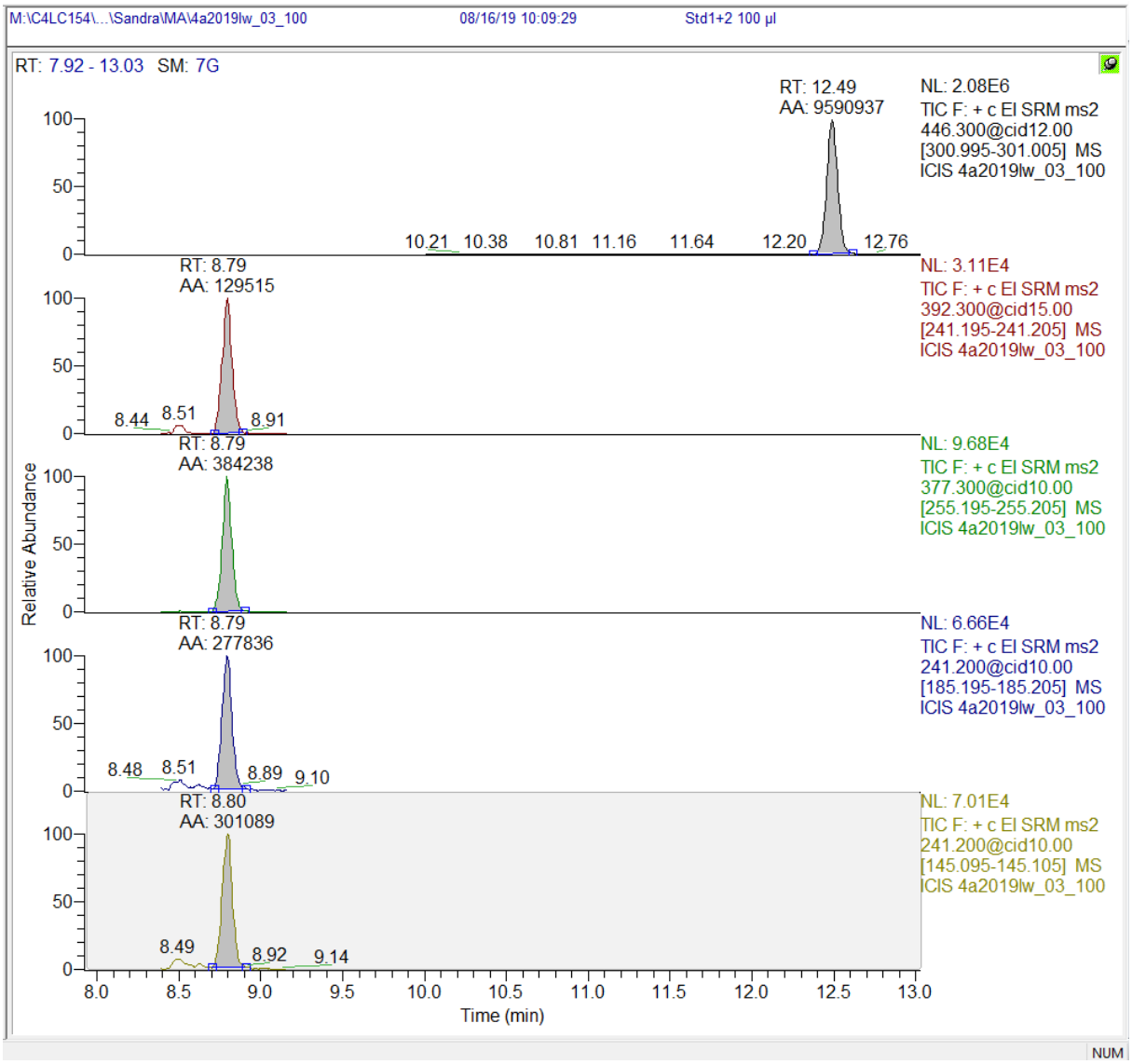

*Figure 46: transition of IS methyltestosterone (RT 12.49) and four transitions for **17** (RT 8.79)*