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Synthesis of Reference Compounds for the Identification of Metabolites of 4-Hydroxytestosterone

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Introduction

Recently, preparations containing 4-hydroxytestosterone (4,17β-dihydroxyandrost-4-en-3-one) have become available by Internet purchase. 4-Hydroxytestosterone is promoted as a substance with high anabolic index and as a prohormone of the aromatase inhibitor formestane (4-hydroxyandrost-4-ene-3,17-dione), its 17-oxo analogue.

Due to the list of WADA and IOC 4-hydroxytestosterone is classified as anabolic androgenic steroid and is therefore prohibited in sports.

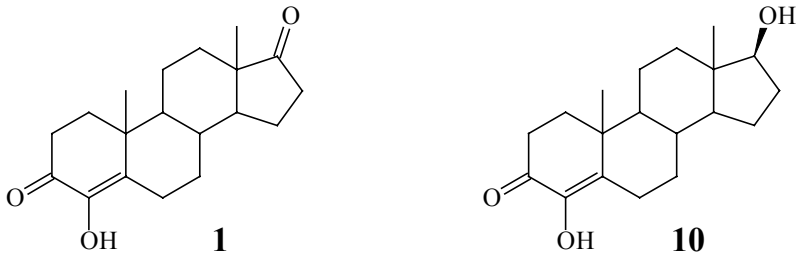

*Fig. 1: Structure formula of formestane (**1**) and 4-hydroxytestosterone (**10**)*

Only little information is available on its urinary excretion. Its main metabolite was identified as the glucuronidated formestane (Fig. 1, (**1**)) by gas chromatography / mass spectrometry [1]. Additionally, 4-hydroxytestosterone (Fig. 1, (**10**)) itself was detected in the glucuronide fraction in post-administration (p.a.) urines besides several other metabolites. During the entire study different reference substances are synthesised and some of those metabolites are identified by comparing mass spectra and retention times.

Experimental

Synthesis of reference compounds

Synthesis of trans-3,4-dihydroxyandrostan-17-ones

The synthesis of 3α,4β-dihydroxy-5α-androstan-17-one (**8**), 3β,4α-dihydroxy-5β-androstan-17-one (**7**) and 3α,4β-dihydroxy-5β-androstan-17-one (**6**) from androst-4-ene-3,17-dione (Fig. 2) is performed as described by da Silva et al. [2]. For the assignment of the products the intermediate 3α,4α-epoxy-5α-androstan-17-one (1 mg) is reduced with borane-tetrahydrofurane (BH_3-THF, 0.5 ml, 1 M in THF). The mass spectrum and the retention time of the product are compared with the commercially available 5α-androstan-3α,17β-diol (as bis-TMS derivative).

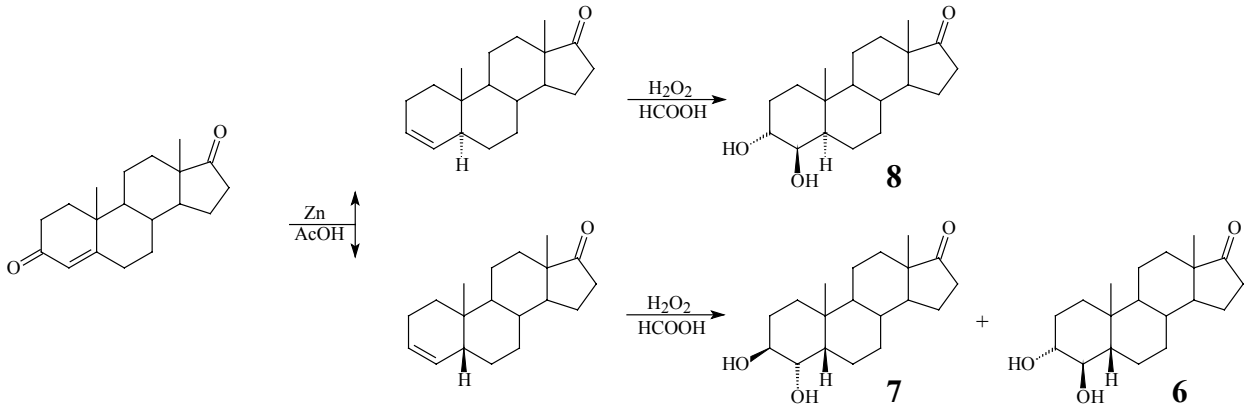

*Fig. 2: Synthesis of 3α,4β-dihydroxy-5β-androstan-17-one (**6**), 3β,4α-dihydroxy-5β-androstan-17-one (**7**) and 3α,4β-dihydroxy-5α-androstan-17-one (**8**)*

Catalytic hydrogenation of formestane, 4-hydroxytestosterone and dihydroformestane

Formestane ((**1**), 300 mg) is reduced with hydrogen in 20 ml of methanol using either 5 mg of palladium on charcoal (Pd/C) or platinum dioxide (PtO_2) as catalyst. After separation of the solid, the filtrate is evaporated to dryness, the products are separated by crystallisation from acetonitrile followed by chromatography on silica gel with n-hexane/ethyl acetate (60:40, v:v). Finally the products are recrystallised from acetonitrile/water.

The hydrogenation of 4-hydroxytestosterone (**10**) is only performed in µg-amounts as described above using Pd/C as catalyst. Also µg-amounts of the obtained dihydroformestane isomers (3β-hydroxy-5α-androstane-4,17-dione (**2**) and 3α-hydroxy-5β-androstane-4,17-dione (**3**)) are hydrogenated in the same way with PtO_2 as catalyst.

Deuterated analogues are synthesised in the same way by using deuterium gas instead of hydrogen. Additionally formestane is deuterated by H/D-exchange with d_1-methanol/ D_2O/NaOD (125:15:1, v:v:w) followed by catalytic hydrogenation with deuterium.

Selective reduction of oxo groups with K-selectride

3α-Hydroxy-5β-androstane-4,17-dione ((**3**), 1 mg) is dissolved in absolute diethylether (1 ml) and K-selectride (5 µl, 1 M) is added. After 75 min at ambient temperature 1 ml of water is added. The mixture is extracted with 5 ml of t-butyl methyl ether (TBME) and the products are hydrolysed in 1 ml of 1 M HCl/MeOH (1:1, v:v) and extracted with 5 ml of TBME.

Synthesis of 6,7-dehydroformestane from androstenedione

The synthesis of 6,7-dehydroformestane (4-hydroxyandrosta-4,6-diene-3,17-dione) was performed as described by Marsh et al [3]. In brief, androst-4-ene-3,17-dione is stirred with potassium t-butylate in t-butanol at 45°C. After 7 h the mixture is neutralised and the products are extracted with TBME.

Dehydrogenation of formestane

The dehydrogenation of formestane is performed only in µg-amounts for GC/MS characterisation. Formestane and 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ), are refluxed in dioxane. After 7 h water is added (1 ml) and the mixture is extracted with TBME.

Metabolism studies

Excretion study

The p.a. urine is provided by the World Association of Anti-Doping Scientists (WAADS) as educational sample within its proficiency testing programme. Two tablets of Testobol™ (each containing 100 mg of 4-hydroxytestosterone) were taken orally by a male volunteer and a 27 hour pooled urine was collected.

Isolation of steroids for GC/MS analysis

To analyse the glucuronide fraction the p.a. urine is treated as shown in Fig. 3.

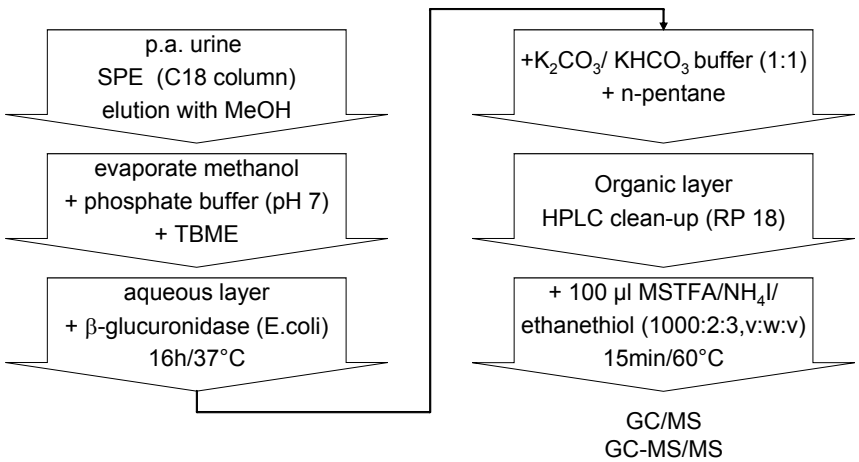

Fig. 3: Sample preparation for p.a. urines, glucuronide fraction

GC/MS analyses

Derivatisation for GC/MS analyses

After removal of the solvent the dry residue is redissolved in 100 µl of TMIS reagent (MSTFA/NH_4I/ethanethiol (1000:2:3, v:w:v)) and heated for 15 min at 60°C to obtain the enol-TMS ethers.

Instrumentation

The GC/MS analyses are performed with the following parameters:

GC/MS system:	GC: Hewlett Packard (HP) 6890 coupled to mass selective detector HP 5973
Injection parameters:	Volume: 2 µl, Temp.: 300°C
Column:	HP 5 MS; 16.5 m; 0.25 mm i.d.; 0.25 µm film thickness
Carrier gas:	Helium, splitless, head pressure 13 psi
Oven temp.:	0 min 100°C, 40°C/min, 0 min 190°C, 5°C/min, 0 min 240°C, 40°C/min, 3 min 320°C
Ionisation:	70 eV, electron impact (EI)
Data aquisition:	SCAN, 40-800 amu, sampling rate 2^2

Additionally GC-MS/MS analyses are performed using the following conditions:

GC/MS/MS system:	GC Finnigan, GCQ
Injection parameters:	Volume: 2 µl, Temp.: 325°C
Column:	HP Ultra-1 (OV 1); 14 m; 0.25 mm i.d.; 0.11 µm film thickness
Carrier gas:	Helium, split 1:10, head pressure 10 psi
Oven temp.:	0 min 100°C, 40°C/min, 0 min 190°C, 5°C/min, 0 min 240°C, 40°C/min, 3 min 320°C
Ionisation:	70 eV, electron impact (EI)
Data aquisition:	Product Ion Scan
Collision Energy:	1.2 volts

For HPLC clean-up of the p.a. urines the following conditions are used:

HPLC system:	Agilent 1100
Injection volume:	50 µl
Column:	Merck LiChroCart 250-4 LiChrospher 100 RP_{18} EC (5 µm)
Pre-column:	Merck LiChroCart 25-4 LiChrospher 100 RP_{18} (5 µm)
Mobile phase:	A: H_2O, B: acetonitrile at 24°C
Flow parameter:	1 ml/min, 0-20 min 30%B to 100%B, reequil. 5 min 30%B

Chemicals and solvents

Formestane was purchased from Thinker Chemical (Hangzhou, China), 4-hydroxy-testosterone from Steraloids (Wilton, USA), androst-4-ene-3,17-dione, 5α-androstane-3α,17β-diol, palladium on charcoal (Pd/C, 10 %), platinum dioxide, zinc dust (<10 µm particle size), 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ), borane-tetrahydrofurane (BH_3-THF, 1 M in THF) and K-selectride (potassium tri-2-butylborohydride, 1 M in THF), from Sigma-Aldrich GmbH (Steinheim, Germany). N-Methyl-N-trimethylsilyl-trifluoro-acetamide (MSTFA) was obtained from Chem. Fabrik Karl Bucher (Waldstetten, Germany). Other reagents and solvents were of analytical grade and provided by Merck (Darmstadt, Germany).

Results and Discussion

Synthesis of reference compounds

Synthesis of trans-3,4-dihydroxyandrostan-17-ones

The reactions described by da Silva et al. [2] yield three different trans-3,4-dihydroxy-androstan-17-ones (**6**-**8**) with similar mass spectra of the enol-TMS derivatives. The mass spectra of 3β,4α-dihydroxy-5β-androstan-17-one (**7**) and 3α,4β-dihydroxy-5α-androstan-17-one (**8**) are shown in Fig. 4. The retention times of the derivatives are listed in Tab. 1. The reduction of the intermediate epoxide (**9**) with BH_3-THF results in 5α-androstan-3α,17β-diol (Fig. 5) which is identified by comparison with the reference compound. Therefore the configuration of the intermediate (**9**) is recognised as 3α,4α-epoxy-5α-androstan-17-one. The reductive opening of the corresponding 5β isomer with BH_3-THF is not successful.

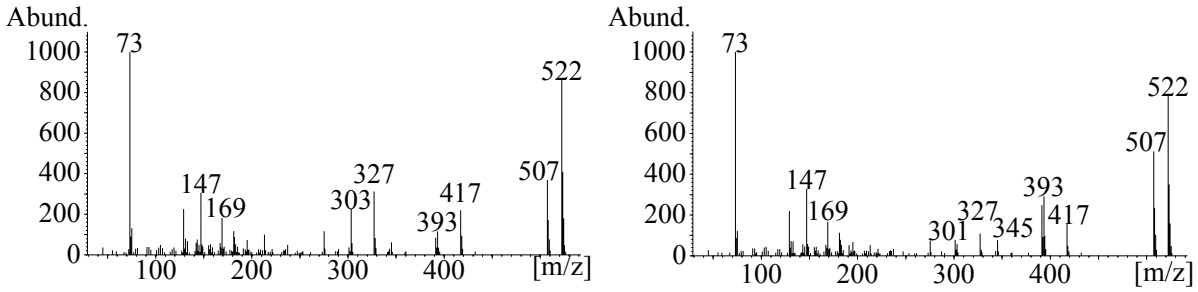

*Fig. 4: Mass spectra (EI) of 3,4-dihydroxyandrostan-17-one, per-TMS (M^+=522), left: 3β,4α,5β (**7**), right: 3α,4β,5α (**8**)*

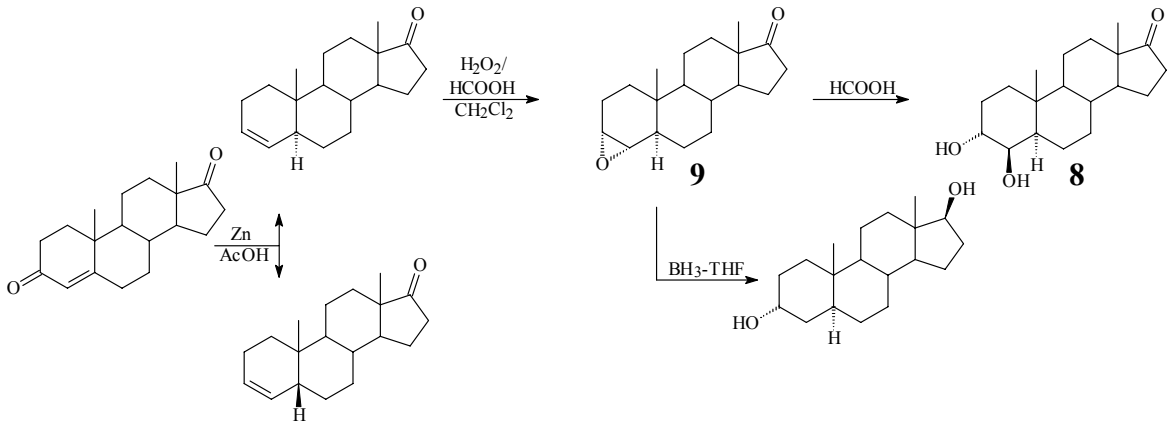

*Fig. 5: Identification of the intermediate epoxide (**9**) by reduction with borane*

Catalytic hydrogenation of formestane, dihydroformestane and 4-hydroxytestosterone

The hydrogenation of formestane using Pd/C as catalyst (Fig. 9) yields the two isomeric 3-hydroxyandrostane-4,17-diones (3β,5α (**2**) and 3α,5β (**3**)). By derivatisation with TMIS reagent, the products yield two derivatives each, the 3- and the 4-enol-TMS respectively (Fig. 6). Their mass spectra are shown in Fig. 7, the retention times in Tab. 1. The obtained derivatives show 4 different retention times which can only be explained by different configurations (α and β) at position 3 and 5 in the two products of the hydrogenation.

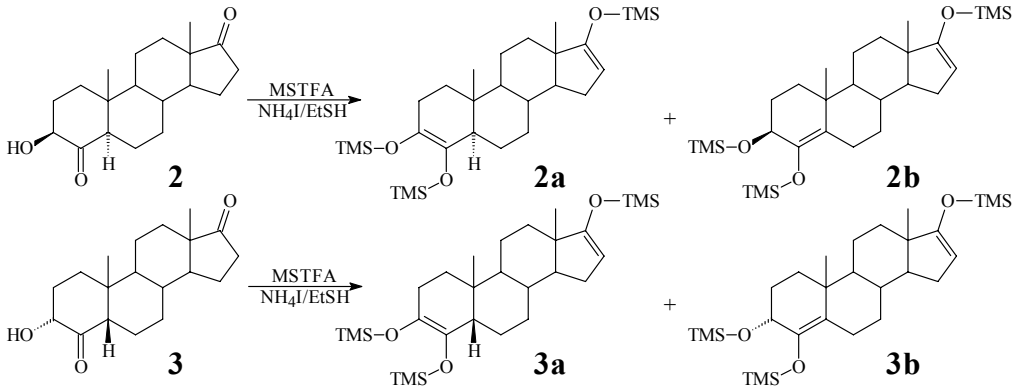

Fig. 6: Isomers of hydrogenated formestane obtained by derivatisation

Using PtO_2 as catalyst only 3α-hydroxy-5β-androstane-4,17-dione ((**3**), yield 15%) is obtained in addition to two 3,4-dihydroxyandrostan-17-ones (3β,4β,5α (**4**) yield 72% and 3α,4α,5β (**5**) yield 11%; the 3α,4β,5β isomer (**6**) was not detectable under these conditions).

This result can be explained by additional hydrogenation of the 4-oxo group using PtO_2. The hydrogenation of 3β-hydroxy-5α-androstane-4,17-dione (**2**) results in 3β,4β-dihydroxy-5α-androstan-17-one ((**4**) yield 85%), while the 4-oxo group of the 5β isomer reacts very slowly. 3α-Hydroxy-5β-androstane-4,17-dione (**3**) yields two isomeric 3,4-dihydroxyandrostan-17-ones. However after 20 min of hydrogenation 90% still remain unchanged. One of the isomers (~0.6% yield) is identified as 3α,4β,5β isomer (**6**) by comparison with the product of trans-bishydroxylation of 5β-androst-3-en-17-one (Fig. 2, da Silva et al. [2]). The products of the hydrogenation of the 3,5-bis-deuterated analogue still contains two deuterium atoms, hence direct hydrogenation of the oxo group is presumed. Thus, the second dihydroxy-product (6% yield) is identified as 3α,4α-dihydroxy-5β-androstan-17-one (**5**).

4-Hydroxytestosterone (**10**) is hydrogenated with Pd/C as catalyst as described above yielding two different isomers of 3,17β-dihydroxyandrostan-4-one (presumably also 3β,5α (**11**) and 3α,5β (**12**)). The mass spectra of their per-TMS derivatives are shown in Fig. 8, their retention times are listed in Tab. 1. The confirmation of the structures with NMR is pending.

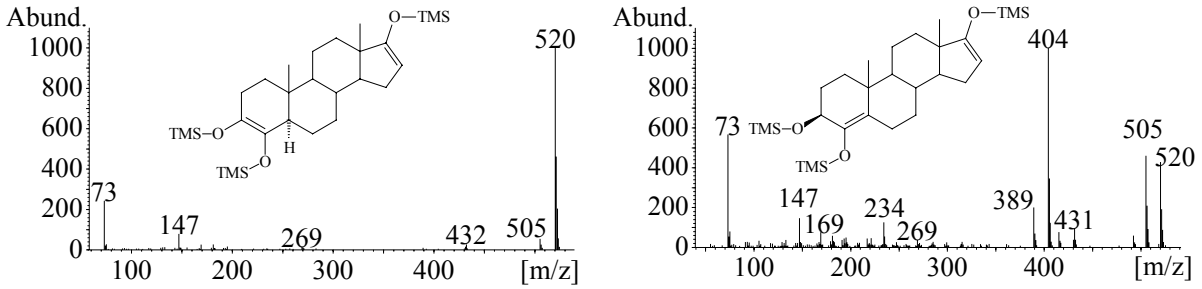

Fig. 7: Mass spectra (EI) of 3ξ-hydroxy-5ξ-androstane-4,17-dione, per-TMS (M^+=520), left: 3-enol, right: 4-enol

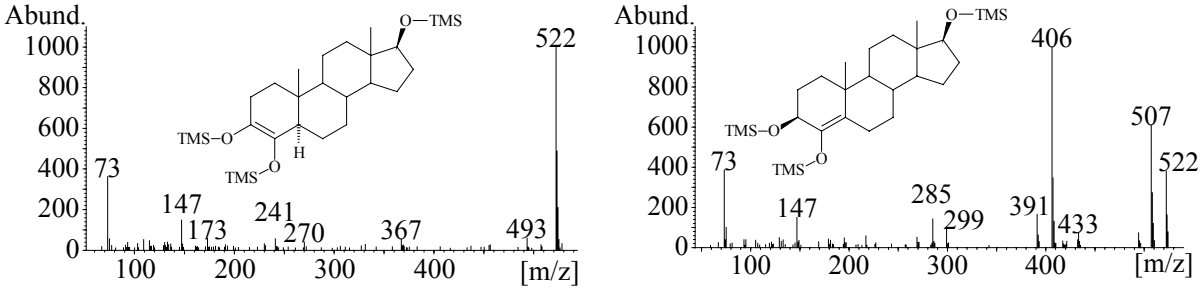

Fig. 8: Mass spectra (EI) of 3ξ,17β-dihydroxy-5ξ-androstan-4-one, per-TMS (M^+=522) left: 3-enol, right: 4-enol

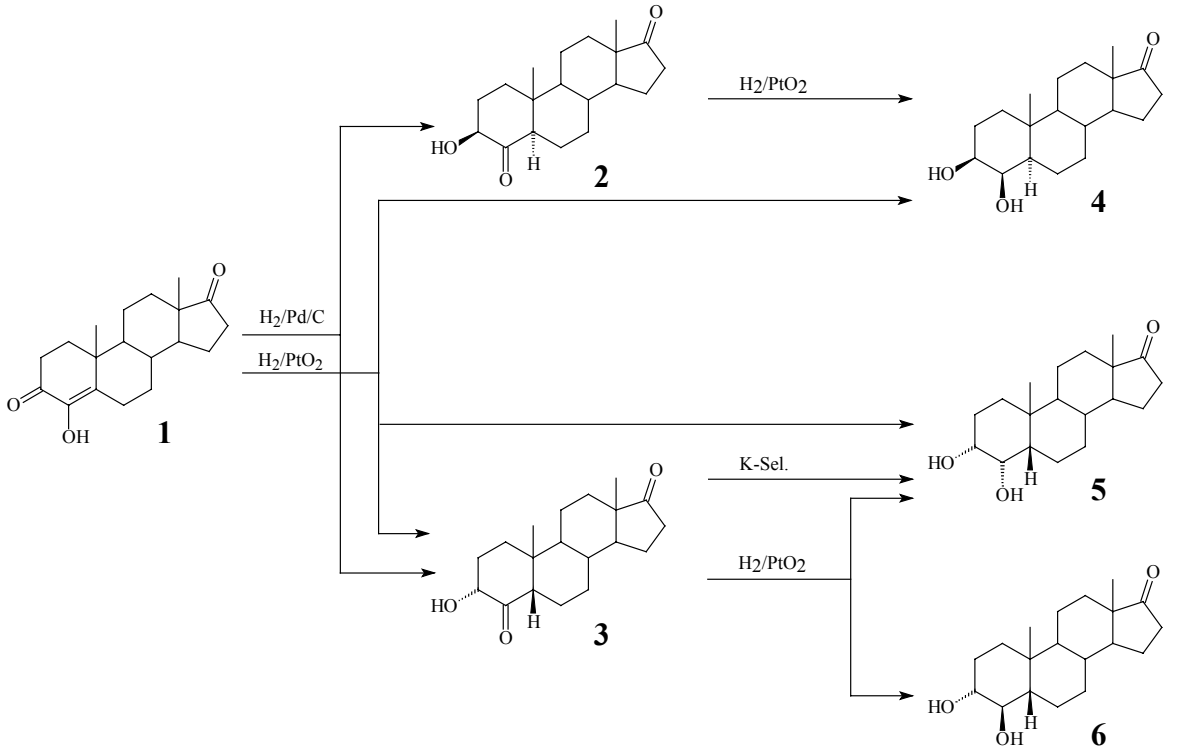

Fig. 9: Reduction of formestane and identification of the products

Selective reduction of oxo groups with K-selectride

3α-Hydroxy-5β-androstane-4,17-dione (**3**) is reduced with K-selectride yielding 3α,4α-dihydroxy-5β-androstan-17-one ((**5**), ~50% yield).

Deuteration experiments and proposal of fragmentation pathways

Several deuteration reactions are performed. Two 3,5-$^2H_2$-3ξ-hydroxy-5ξ-androstane-4,17-diones are obtained by catalytic hydrogenation of formestane with deuterium gas and Pd/C as catalyst. The mass spectra of the corresponding derivatisation isomers (both show M^+=521, standing for a loss of one of the deuterium atoms during derivatisation) confirm that the oxo group is located at C-4. 3,5-$^2H_2$-3,4-dihydroxyandrostan-17-one (M^+=524 as per-TMS) is achieved by hydrogenation of these products applying PtO_2. Catalytic deuteration of formestane with PtO_2 results in 3,4,5-$^2H_3$-3,4-dihydroxyandrostan-17-one (M^+=525 as per-TMS) and the 2,2,3,4,5,16,16-$^2H_7$-analogue (M^+=528 as per-TMS, with one deuterium on C-16 lost during derivatisation) is derived by deuterium exchange of formestane in alkaline CH_3OD followed by catalytic hydrogenation with deuterium gas as described above.

Fragmentation pathways, also with respect to these deuterated products, are shown in Fig. 10. The formation of m/z 391 (391 for 3,5-$^2H_2$, 392 for 3,4,5-$^2H_3$, 393 for 2,2,3,4,5,16-$^2H_6$) can be explained by a loss of the atoms C-1, C-2 and C-3 with additional loss of the hydrogen at C-5, while m/z 393 (394 for 3,5-$^2H_2$, 394 for 3,4,5-$^2H_3$, 397 for 2,2,3,4,5,16-$^2H_6$) is formed in the same way but with hydrogen shift from C-2 into the fragment. A loss of TMS-OH from these fragments yields 301 and 303 respectively. The fragment m/z 507 is generated by loss of a methyl radical and an additional loss of TMS-OH (once or twice) results in m/z 417 and 327. M/z 169 can be addressed to a D-ring fragment.

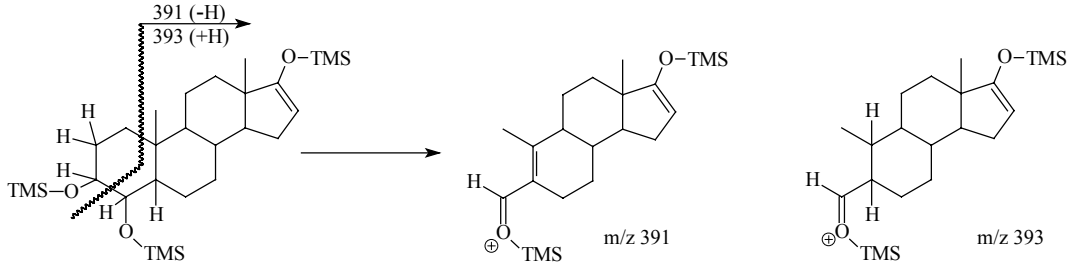

Fig. 10: Generation of the fragments m/z 391 and 393 of 3,4-dihydroxyandrostan-17-one, tris-TMS (explicitly shown H-atoms can be substituted by deuterium for substantiation)

Synthesis of 6,7-and 1,2-dehydroformestane

The reaction of androst-4-ene-3,17-dione with t-butylate results in 6,7-dehydroformestane (4-hydroxyandrosta-4,6-diene-3,17-dione, (**13**)) as described by Marsh et al [3]. Dehydrogenation with DDQ yields the same product and 1,2-dehydroformestane (4-hydroxyandrosta-1,4-diene-3,17-dione, (**14**)) additionally.

The mass spectra of the enol-TMS derivatives are shown in Fig. 11 and the retention times are presented in Tab. 1.

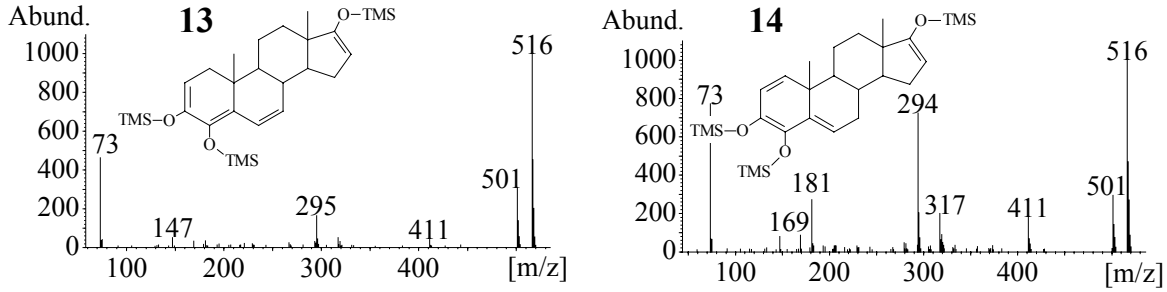

*Fig. 11: Mass spectra (EI) of 6,7- (**13**) and 1,2-deydroformestane (**14**), per-TMS (M^+=516)*

Identification of metabolites of 4-hydroxytestosterone in p.a. urine

The p.a. urine of 4-hydroxytestosterone is cleaned by SPE (C_{18}) followed by extraction of the free fraction with TBME at pH 7. After hydrolysis of the glucuronides the metabolites are extracted with n-pentane and analysed with GC-MS and GC-MS/MS as per-TMS derivatives. By comparing mass spectra, product ion spectra and retention times the following metabolites of 4-hydroxytestosterone are identified: 4-hydroxyandrost-4-ene-3,17-dione (**1**), 3β-hydroxy-5α-androstane-4,17-dione (**2**), 3α-hydroxy-5β-androstane-4,17-dione (**3**), 3α,4β-dihydroxy-5α-androstan-17-one (**8**), 3ξ,17β-dihydroxy-5ξ-androstan-4-one, 4-hydroxytestosterone (**10**), 4-hydroxyandrosta-4,6-diene-3,17-dione (**13**) and 4-hydroxyandrosta-1,4-diene-3,17-dione (**14**).

Tab. 1: Retention times (RT) of the reference compounds as per-TMS derivatives (conditions as described for GC-MS/MS analyses, NMR confirmation of the configuration is pending)

Compound	**RT [min], per-TMS**
4-hydroxyandrost-4-ene-3,17-dione (**1**)	14.83 (2,4-diene) 16.00 (3,5-diene)
3β-hydroxy-5α-androstane-4,17-dione (**2**)	15.72 (3-ene) 15.52 (4-ene)
3α-hydroxy-5β-androstane-4,17-dione (**3**)	11.24 (3-ene) 13.96 (4-ene)
3β,4β-dihydroxy-5α-androstan-17-one (**4**)	15.58
3α,4α-dihydroxy-5β-androstan-17-one (**5**)	12.39
3α,4β-dihydroxy-5β-androstan-17-one (**6**)	14.29
3β,4α-dihydroxy-5β-androstan-17-one (**7**)	11.03
3α,4β-dihydroxy-5α-androstan-17-one (**8**)	13.26
4-hydroxytestosterone (**10**)	13.80 (2,4-diene) 16.39 (3,5-diene)
3β,17β-dihydroxy-5α-androstan-4-one (**11**)	11.41 (3-ene) 14.24 (4-ene)
3α,17β-dihydroxy-5β-androstan-4-one (**12**)	16.12 (3-ene) 15.92 (4-ene)
4-hydroxyandrosta-4,6-diene-3,17-dione (**13**)	15.30
4-hydroxyandrosta-1,4-diene-3,17-dione (**14**)	15.46

Summary

For the identification of metabolites of 4-hydroxytestosterone reference substances are synthesised:

Different isomers of 3,4-dihydroxyandrostan-17-one (3α,4β,5β, 3β,4α,5β and 3α,4β,5α, (**6-8**)) are obtained by the reaction of androst-4-ene-3,17-dione with zinc dust in acetic acid, epoxidation of the products (5α- and 5β-androst-3-en-17-one) and hydrolysis of the epoxides.

Hydrogenation of formestane with PtO_2 results in two more isomeric 3,4-dihydroxyandrostan-17-ones (3β,4β,5α (**4**) and 3α,4α,5β (**5**)) in addition to 3α-hydroxy-5β-androstane-4,17-dione (**3**). Using Pd/C as catalyst 3β-hydroxy-5α-androstane-4,17-dione (**2**) is obtained as main product besides the 3α,5β isomer (**3**).

The 4-oxo group of 3α-hydroxy-5β-androstane-4,17-dione is also reduced to the above mentioned 3α,4α-dihydroxy-5β-androstan-17-ones with high selectivity using K-selectride. Catalytic hydrogenation of 4-hydroxytestosterone (**10**) leads to two 3,17β-dihydroxy-androstan-4-ones (3β,5α (**11**) and 3α,5β (**12**)).

The reaction of androst-4-ene-3,17-dione with t-butylate yields 6,7-dehydroformestane (4-hydroxyandrost-4,6-diene-3,17-dione (**13**)), which is also obtained by dehydrogenation of formestane with DDQ in addition to 1,2-dehydroformestane (**14**).

In the glucuronide fraction of the p.a. urine of 4-hydroxytestosterone, 4-hydroxyandrost-4-ene-3,17-dione (**1**), 3β-hydroxy-5α-androstane-4,17-dione (**2**), 3α-hydroxy-5β-androstane-4,17-dione (**3**), 3α,4β-dihydroxy-5α-androstan-17-one (**8**), 3ξ,17β-dihydroxy-5ξ-androstan-4-one, 4-hydroxyandrosta-4,6-diene-3,17-dione (**13**), 4-hydroxyandrosta-1,4-diene-3,17-dione (**14**) and 4-hydroxytestosterone itself (**10**) are identified by comparing mass spectra and retention times (GC-MS and GC-MS/MS) with reference substances data. As the main metabolite 4-hydroxyandrost-4-ene-3,17-dione should be used for screening purpose in doping control.

Acknowledgements

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