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Structure proof of tibolone 3-hydroxy metabolite

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Abstract

Following the WAADS discussions on the stereochemistry of the 3-hydroxy metabolite of tibolone and confusing assignments of the stereochemistry of reference substances provided by different suppliers, we have studied the urinary excretion of tibolone with special respect to the stereochemistry of hydrogenation on C3. As already reported in previous studies, only the early eluting epimer is excreted in the glucuronide fraction.
The chemical reduction of tibolone with lithium tri-t-butoxyaluminum hydride (LiR_3AlH) yielded one isomer with excellent enantiomeric excess, namely the later eluting one. It was characterized by GC-MS and NMR and its structure was confirmed as 3α product by X-ray analysis. Interestingly this product elutes later than the 3β isomer, which is in contrast to the findings for 5α- and 4-ene steroids. This was confirmed by reduction of the structure analogue norethisterone within this investigation. The urinary excretion of the 3-hydroxy metabolite of tibolone in humans was reinvestigated and the metabolite detected in glucuronide fraction was proven to have 3β-OH configuration, while the urinary metabolism of 4-ene steroids follows 3α pathway.

Introduction

Since January 2006 the WADA list of prohibited substances and methods explicitly references tibolone (7α-methyl-17α-ethinyl-17β-hydroxyestr-5(10)-en-3-one) under the category 'other anabolic agents'. It is therapeutically used as approved drug in pharmaceuticals such as Liviella® or Livial® for the treatment of menopausal disorders or in endometriosis. According to literature it binds in almost non-selective way to all type-I steroid hormone receptors [1]. Thus, anabolic-androgenic effects are well known besides the main estrogenic activity [2]. In contrast to its 7α-nor analogue norethisterone (17α-ethinyl-17β-hydroxyestr-4-en-3-one) the double bond in tibolone is located between C5 and C10.
As metabolites, 3α- and 3β-reduction products and the 4-ene analogue are described in literature [1-3], with only the early eluting isomer excreted in the human urinary glucuronide fraction. In 2011 discussions on the WAADS website on the stereochemistry were triggered by confusing assignments by different suppliers on their reference substances. Depending on the supplier either the isomer labelled as 3α or as 3β was found to elute earlier or later following persilylation with MSTFA. Thus, inhouse synthesis and subsequent structural identification was carried out.

Experimental

Chemicals and reagents
Reference material of 3α-OH-tibolone was obtained from WAADS (Zwijnaarde, Belgium, Organon material), TRC (Toronto, Canada) and Atlanchimpharma (Nantes, France). 3β-OH-Tibolone was provided from Atlanchimpharma (Nantes, France), norethisterone and tri-t-butoxyaliuminium hydride (1M in tetrahydrofurane, THF) from Sigma (Steinheim, Germany). Liviella® tablets were purchased in a German pharmacy. β-Glucuronidase from *E.coli* was obtained from Roche Diagnostics (Mannheim, Germany), and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other reagents and chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

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Instrumentation
NMR spectroscopy
NMR data were obtained using a Bruker Avance II$^+$ 600 instrument, equipped with a 5 mm inverse probehead with actively shielded z-gradient coil. Chemical shifts were given in δ values (ppm) relative to tetramethylsilane. The spectra were recorded at 600 MHz (^{1}H) and 150MHz (^{13}C) at 298 K using solutions of about 5 mg of each compound in deuterated pyridine. For confirmation of the assumed structures ^{1}H and ^{13}C spectra were measured together with 2D gradient-selected HH-COSY, NOESY, HSQC and HMBC experiments.

GC-MS analyses
GC-MS analyses of the TMS derivatives were performed on an Agilent 6890N gas chromatograph coupled to an Agilent 5973 inert mass selective detector (MSD) applying the following parameters: column: Agilent Ultra-1 (polysiloxane, 17m; 0.20mm i.d.; 0.11µm film thickness), carrier gas: helium, head pressure: 1 bar, oven temperature program: 0 min 183°C, +3°C/min, 0 min 232°C, +40°C/min, 2 min 310°C, injection volume: 3µL, split 1:16, injection temperature: 300°C, ionization: 70 eV, EI, full scan mode, 40-800 Da or in case of the urine extract analysis selected ion monitoring. Derivatization was achieved with TMIS reagent (MSTFA/ NH_4I/ ethanethiol, 1000:2:3, v:w:v) by heating for 20 min at 60°C as introduced by Donike and Geyer [4,5].

X-ray crysal structure analysis
X-ray data of suitable single crystals were collected with a Nonius KappaCCD diffractometer [6,7] equipped with a low temperature device at 100K by using graphite-monochromated MoKa radiaton (λ = 0.71073 Å). The structure was solved by direct methods using SHELXS-97[8] and the non-hydrogen atoms were refined by full-matrix least-squares methods using SHELXL-97[9].

3-Reduction of tibolone and norethisterone
To a stirred solution of 150mg of tibolone (extracted by soxhlett from Liviella® tablets) in 10mL absolute tetrahydrofuran 1.5mL tri-t-butoxyaliuminium hydride solution were added at –70°C. After 3h 1mL of an aqueous ammonium chloride solution (10% in H_2O) was added, pH~10 was adjusted by adding solid sodium hydrogencarbonate/potassium carbonate (2:1, w:w) and the mixture was extracted with 5mL of t-butyl methyl ether (TBME). The major product precipitated from methylenchloride and was crystallized several times from hexane/acetone. Norethisterone was reduced under analogous conditions.

Administration study
Urine samples were collected from two female patients using Liviella on a once per day regimen. Additionally an excretion urine provided by WAADS was analyzed within this study. Urine samples were prepared according to the routine steroid screening procedure used in our laboratory [5].

Results and Discussion

Reference substances from different commercial sources labelled as 3α-OH-tibolone were analyzed as their per-TMS derivatives by GC-MS. Even if labelled identical only two of them displayed the same retention time (RT = 13.03min), but the one from TRC showed up at RT = 12.00min.
Thus, within this investigation tibolone (**1**) was reduced with lithium tri-t-butoxyaluminum hydride (LiR_3AlH) yielding the two epimers, (**2**) and (**3**), in a ratio of 5:95. The retention times of the bis-TMS derivatives were found at $RT_{(2)}$ = 12.00min and $RT_{(3)}$ = 13.04min with the internal standard methyltestosterone (MT) at RT_{MT} = 14.96min. As common, the mass spectra of both epimers are quite similar (spectra of bis TMS derivatives in Figure1). To confirm the stereochemistry the major product (**3**) was purified by sequential crystallization steps and analyzed by NMR (Figure2) and X-ray (Figure3). Its structure was confirmed as 3α-product by X-ray analysis. This is in analogy with the findings of Levine et al. for 5(10)-ene-nortestosterone [10] and Colombo et al. [11]. Thus, the products provided by WAADS (Organon) and Atlanchimpharma are labelled correctly. For additional proof 3β-OH-tibolone (**2**) from Atlanchimpharma was also analyzed by NMR.

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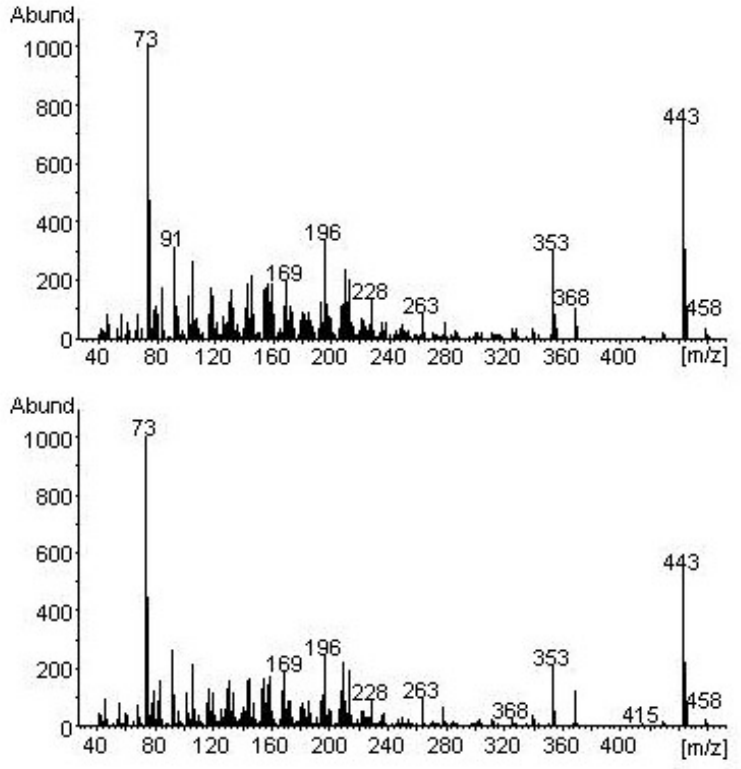

FIGURE1: Mass spectrum of 3β-OH-tibolone (2, RT = 12.00 min, upper) and 3α-OH-tibolone (3, RT = 13.04 min, lower)

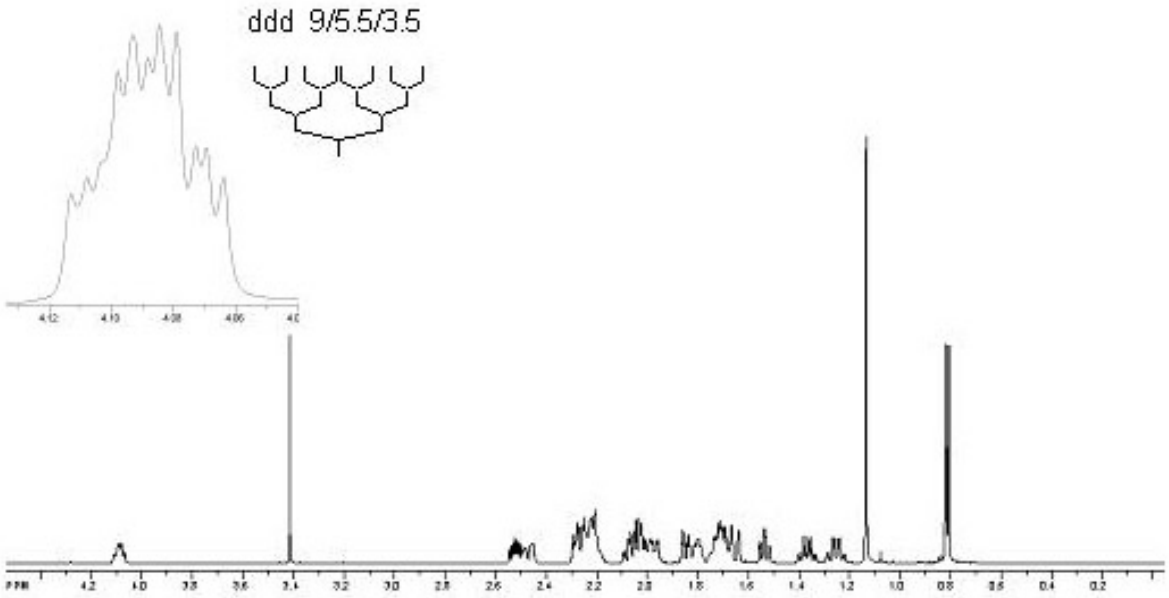

FIGURE2: 1H NMR of 3α-OH-tibolone (3)

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Interestingly the 3α-product ((**3**), $RT_{3\alpha}$= 13.04min) elutes later than the 3β-isomer ((**2**), $RT_{3\beta}$= 12.00min), which is in contrast to the findings for 5α- and 4-ene steroids [12,13]. Additionally, the reduction of 5α- and 4-ene steroids with LiR_3AlH mainly results in 3β-products. This was confirmed by reduction of norethisterone (**4**) within this investigation ($RT_{3\alpha}$= 11.75min, $RT_{3\beta}$ = 13.24min, yield (3β) : (3α) = 85 : 15, mass spectra in Figure 5, ^{1}H NMR of 3β in Figure 6).
The different behaviours between the 5-ene and 4-ene steroids may be explained by the differences in the molecules geometry: While 4-enes generate a pseudochair conformation the 5-ene double bond results in a pseudoboat conformation of the A-ring.
The urinary excretion of 3ξ-OH-tibolone was reinvestigated in two female volunteers and in the WAADS quality control excretion urine. The metabolite found in the glucuronide fraction could be confirmed as 3β-OH-tibolone (**2**) by GC-MS comparison with the synthesized reference material in all samples.

FIGURE3: Representation of the structure of 3α-OH-tibolone (3) as determined by X-Ray crystallography

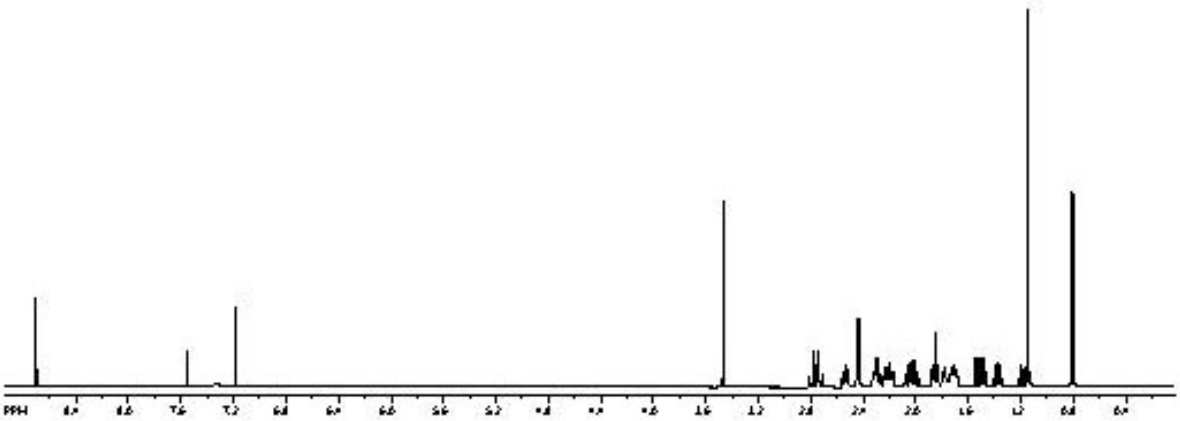

FIGURE4: 1H NMR

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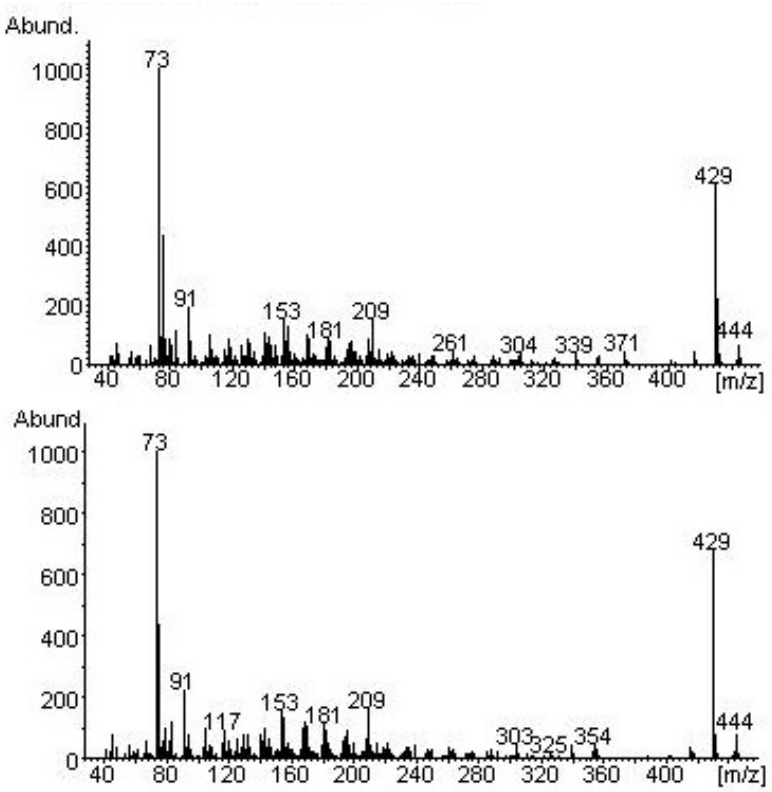

FIGURE5: Mass spectrum of 3α-OH- (upper, RT = 11.75 min) and 3β-OH-norethisteronene (lower, RT = 13.24 min)

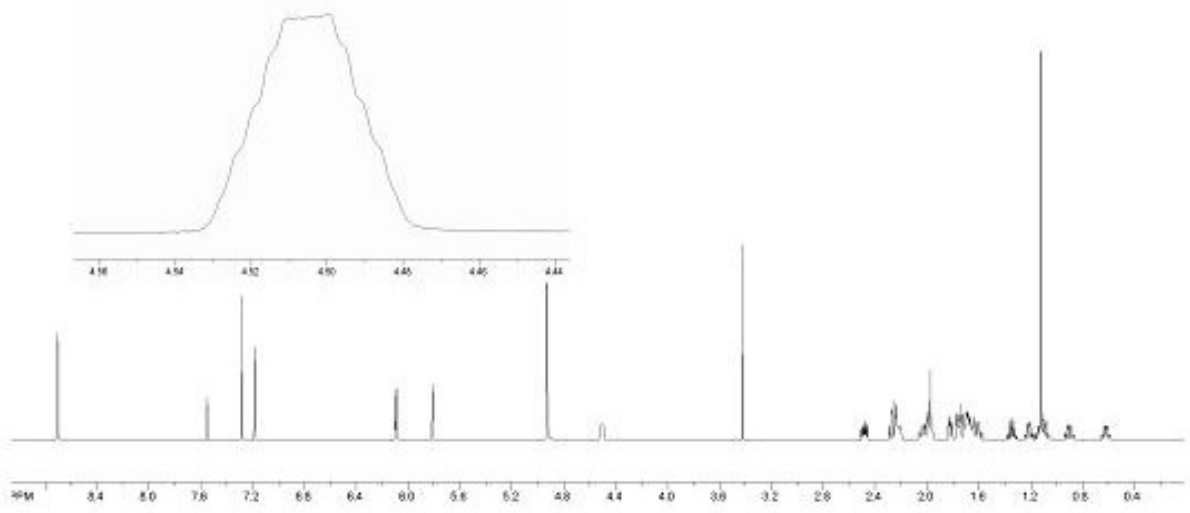

FIGURE6: 1H NMR of 3β-OH-norethisteronene

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Conclusions

The chemical reduction of 3-oxo-5(10)-ene steroids mainly yields 3α-OH, while 3-oxo-4(5)-ene steroids mainly yield 3β-OH products. The reason is an untypical pseudoboat conformation of the A-ring in 5-enes.
In 3OH-5(10)-ene steroids the retention times of the per-TMS derivatives in GC-MS are $RT_{3\beta\text{-}5\text{-}ene} < RT_{3\alpha\text{-}5\text{-}ene}$, while in 3OH-4(5)-ene steroids $RT_{3\alpha\text{-}4\text{-}ene} < RT_{3\beta\text{-}4\text{-}ene}$.
The tibolone 3-OH metabolite in glucuronide fraction has 3β-OH configuration, while the urinary metabolism of 4-ene steroids follows 3α pathway.
The reference substances provided by WAADS (Organon) and Atlanchimpharma are labelled correctly.

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Acknowledgements

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