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Sense or nonsense of prohormone designing: Reduced metandienone as supplement

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Abstract

Since the mid of the 1990s anabolic steroids are marketed as dietary supplements by labeling them as so-called "prohormones". The first prohormones on the market were derived from Testosterone and Nortestosterone by oxidation or reduction at C3 or C17 yielding (19-nor) androst-4- or -5-ene-3,17-dione or -diols.

Recently, products were found on the market stating to contain "17a-methyl-1,4-androstadiene-3,17-diol". These products were analyzed for their steroid content following soxhlett extraction with n-hexane using GC-MS of the underivatized compound and its per-TMS derivative. Additionally, NMR data were recorded to confirm the structure. Differing from the label, the derived structure was confirmed as 4,17-dimethylestra-1,3,5-trien-17β-ol.

Reproducing the reduction of the oxo-group on C3 of metandienone with lithium aluminium hydride or sodium borohydride under different conditions most likely provides the explanation: water was directly eliminated from the intermediate in a reaction called benzene-hydroxy dienol rearrangement.

To characterize the biological activity of 4,17-dimethylestra-1,3,5-trien-17β-ol it was tested in yeast androgen and estrogen receptor transactivation assays for agonistic and antagonistic effects.

None of the assays resulted in any transactivation. Additionally, it was studied in a rat animal model. Orchiectomized rats were treated s.c. for 12 days with 1 mg/kg BW/day. Tissue wet

weights were determined to indicate the anabolic (m. lev. ani) and androgenic (prostate) activity, which were confirming the inactivity of this substance.

Introduction

Since mid of the 1990s, a growing number of new dietary supplements became accessible on the market. The producers of dietary supplements promoted various hormonal compounds as so-called prohormones. Since lots of them were classified as schedule III controlled substances by the Anabolic Steroids Control Act [1] in the USA more and more products appeared on the market containing steroids that have never been marketed as approved drugs [2-8]. But still only very limited information is available on the characteristics and metabolism of these steroids.

The first prohormones being marketed were derived from Testosterone and Nortestosterone by either a reduction on C3 or an oxidation on C17 yielding (19-nor) androst-4- or -5-ene-3,17-dione or -diols. *In-vivo* they are converted to the respective active hormones by the metabolism and thereby foremost mediating anabolic and androgenic effects.

Lately, dietary supplements were available on the market stating to contain "17a-methyl-1,4-Androstadiene-3,17-diol" (M1,4ADD). The chemical structure derived from this name is depicted in Figure 1A. It may be interpreted as metandienone after reduction of the oxo-group on C3, and thereby it was claimed to be a prohormone as well.

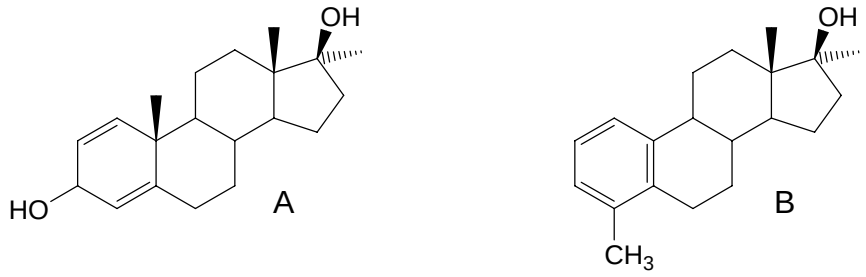

Figure 1: *Chemical structures of 17a-methyl-1,4-androstadiene-3,17-diol (A) and 4,17-dimethylestra-1,3,5-trien-17β-ol (B)*

Experimental

Chemicals and reagents

Metandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one), 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one, DHT), testosterone propionate (TP) 17β-estradiol (E2), lithium aluminium hydride ($LiAlH_4$) and sodium borohydride ($NaBH_4$) were obtained from Sigma-Aldrich GmbH (Steinheim, Germany). All other reagents and solvents were of analytical grade and obtained from VWR International (Darmstadt, Germany).

Synthesis of reference material

Metandienone (301.6 mg, 1 mmol) was reduced with 10-fold excess of $NaBH_4$ or $LiAlH_4$ at different conditions. After extraction of the products with t-butyl methyl ether the resulting mixture was analyzed by GC-MS.

Supplement

The "supplements" M1,4ADD™ from Competitive Edge Labs and M14-e™ from Purus Labs were purchased on the Internet. Both products were labeled to contain "Methyl-1,4-androsta-diene-3,17-diol". After soxhlett extraction with n-hexane the products were analyzed for their steroid content by GC-MS as both the underivatized compound and its per-TMS derivative as well as by NMR. The steroid extracted is further referred to as M1,4ADD.

GC-MS analyses

Gas chromatography mass spectrometry (GC-MS) was performed on an Agilent 6890N gas chromatograph coupled to an Agilent 5973 mass selective detector (MSD) applying the following parameters: column: Agilent HP5MS (17 m; 0.25 mm i.d.; 0.25 µm film thickness), carrier gas: helium, head pressure: 1 bar, oven temperature program: 0 min 100°C, +40°C/min, 0 min 190°C, +5°C/min, 0 min 245°C, +40°C/min, 3 min 320°C, injection volume: 2 µL, splitless, injection temperature: 300°C, ionization: 70 eV, EI, full scan mode, 40-800 Da.

Prior to injection the samples were either redissolved in acetone or derivatized with TMIS reagent (MSTFA/ NH_4I/ ethanethiol, 1000:2:3, v:w:v) by heating for 20 min at 60°C.

NMR analyses

The nuclear magnetic resonance (NMR) analyses were performed at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR) at 298 K on a Bruker (Rheinstetten, Germany) Avance II 600 instrument equipped with a 5 mm inverse probehead with actively shielded z-gradient coil. Chemical shifts are reported in δ values (ppm) relative to tetramethylsilane. Solutions of about 5 mg of each compound in deuterated chloroform were used for conducting ^{1}H; H,H COSY; APT; H,C HMQC; H,C HMBC and NOESY experiments.

Yeast transactivation assay

Yeast cells of the yeast androgen receptor reporter gene system also called yeast androgen screen (YAS) were cultured as described previously [9]. For the assessment of the androgenity or antiandrogenity, respectively, M1,4ADD was dissolved and diluted alone or with 2.94 ng/ml DHT in DMSO and used in a concentration dependent assay from 10^{-5} to 10^{-11} M. DHT served as reference.

The YAS contained both a stably transfected AR construct and an expression plasmid carrying androgen-responsive sequences controlling the expression of the reporter gene LacZ encoding the enzyme β-galactosidase. The β-galactosidase activity, corresponding to androgenic activity, catalyzed the enzymatic hydrolysis of chlorophenol red β-D-galactopyranoside, which was read at 565 nm using a colorimetric assay. Similarly yeast cells of the yeast estrogen receptor reporter gene system also called yeast estrogen screen (YES) were cultured as also described previously [10]. For the assessment of the estrogenity or antiestrogenity M1,4ADD was dissolved and diluted alone or with 2.72 ng/ml E2 in DMSO and used in a concentration dependent assay from 10^{-6} to 10^{-12} M. E2 served as reference.

Hershberger assay

Male Wistar rats (130 g, age 7 weeks) were obtained from Janvier Laboratories (Le Genest St. Isle, France) and were maintained under controlled conditions of temperature (20°C ± 1, relative humidity 50-80%) and illumination (12 h light, 12 h dark). All rats had free access to a standard rat diet (SSniff R10-Diet, SSniff GmbH, Soest, Germany) and water. The animals were maintained according to the Institutional Animal Care and Use guidelines, regulated by the German federal law for animal welfare which follows the European Union guidelines for the care and use of laboratory animals. The study was undertaken with the approval of the regional administration of the governmental body.

The Hershberger assay was performed according to the guidelines of the rodent Hershberger assay [11]. The rats were orchiectomized under anesthesia (Xylazine / Ketamine). After 7 days of endogenous hormone decline, the animals were randomly allocated to treatment and vehicle groups (n=6). For s.c. administration, M1,4ADD was dissolved in ethanol and diluted in corn oil, for p.o. administration it was dissolved in propandiol. The animals were treated once a day with M1,4ADD (s.c. 1 mg/kg BW/day), TP (1 mg/kg BW/day) or vehicle only for 12 days. The doses were chosen on the basis of dosage recommendation on the supplement.
After necropsy, wet weights of the prostate, seminal vesicle and levator ani muscle (m. lev ani) were determined.

Statistical analysis

To determine statistical significance, a two way Mann-Whitney U-test was applied. If not indicated differently, significance was established at $p \leq 0.05$.

Results and Discussion

Supplement analysis

Recently, so-called "prohormone" products were found on the market stating to contain "17a-methyl-1,4-Androstadiene-3,17-diol". The chemical structure derived from this is shown in **Figure 1**A. The "supplements" M1,4ADD™ and M14-e™, labeled like this, were found to contain a steroid yielding mass spectra differing from the labeled one. Following soxhlett extraction with n-hexane the mass spectra of the underivatized compound as well as its TMS derivative are shown in **Figure 2**.

From the fragmentation pattern an elimination of H_2O from the reduced metandienone is very likely. The structure derived from a benzol-hydroxydienol rearrangement was confirmed by one and two dimensional NMR as 4,17-dimethylestra-1,3,5-trien-17β-ol (**Figure 1**B). By reducing metandienone with either $LiAlH_4$ or $NaBH_4$ the same steroid was obtained as main product besides 17α-methylandrost-4-ene-3β,17β-diol. This reaction called benzene-hydroxydienol rearrangement was analogously described by Numazawa et al. [12] for the reduction of androsta-1,4-diene-3,17-dione.

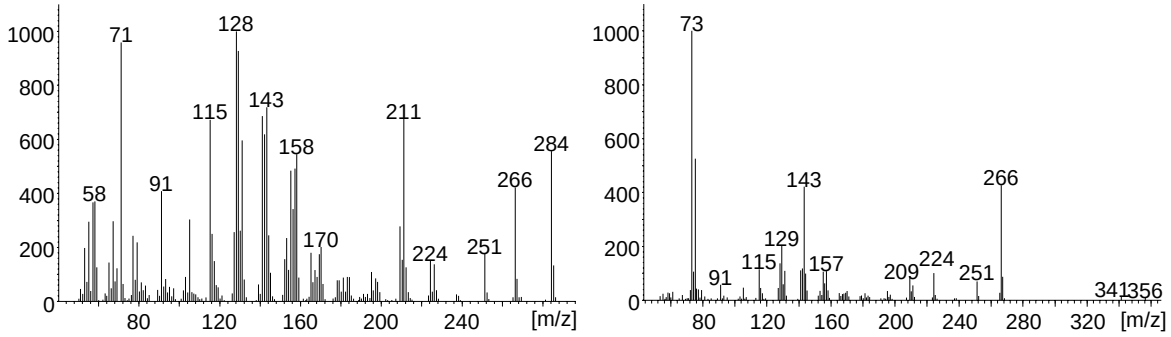

***Figure 2**: Mass spectra (EI) of main extracted steroid from "M1,4ADD", left: underivatized (M^+=284), right: per-TMS derivative (M^+=356)*

Biological in-vitro activity

To further characterize the effects of 4,17-dimethylestra-1,3,5-trien-17β-ol (M1,4ADD) it was tested in two independent yeast assays, one androgen and one estrogen receptor transactivation assay for potential agonistic and antagonistic effects of the substance. M1,4ADD induces neither relevant AR nor ER dependent gene activity in a dose dependent manner (**Figure 3**).

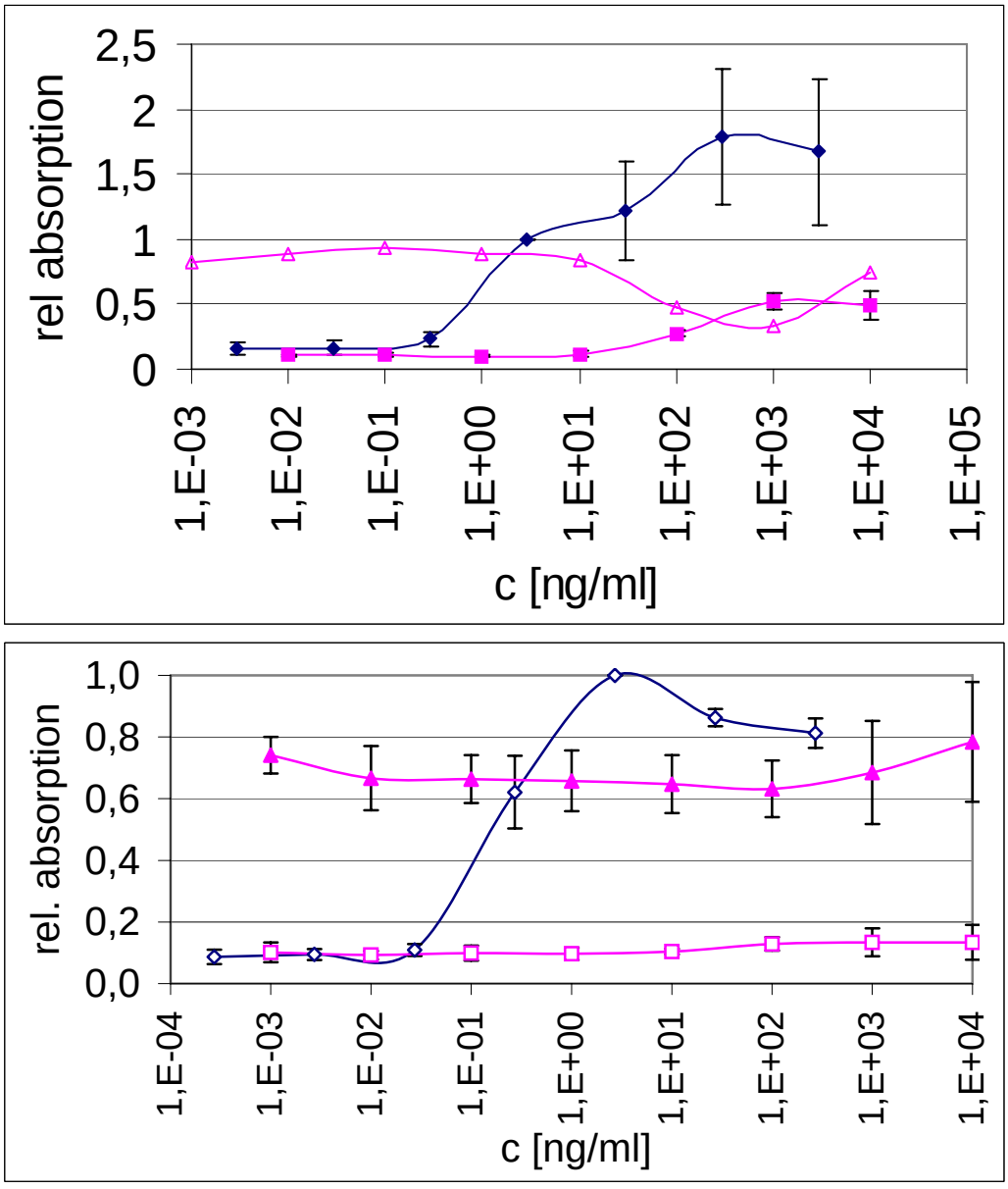

Figure 3: *Dose dependent transactivation of an androgen (upper) and estrogen (lower) dependent reporter gene in yeast stably transfected with the human AR e.g. ER of M1,4ADD in comparison to DHT and E2 (M1,4ADD ■, M1,4ADD+2.94 ng/mL DHT Δ, DHT ◆, M1,4ADD □, M1,4ADD + 2.72 ng/mL E2 ▲, E2 ◊)*

In-vivo activity

Additionally, 4,17-dimethylestra-1,3,5-trien-17β-ol was studied in the classical animal model for androgenic effects, the rodent Hershberger assay. To investigate the dose dependent anabolic and androgenic potency of M1,4ADD™ orchiectomized rats (ORCHI) were treated with the substance for 12 days s.c. with 1 mg/kg BW/day of M1,4ADD. Non-treated and TP treated orchiectomized as well as intact rats served as reference groups. The organ weights of prostate, seminal vesicles and m. lev. ani were determined and are shown in **Figure 4**. The s.c. administration of M1,4ADD resulted in no increase of prostate, seminal vesicles nor m. lev. ani weights. Taken together these results were confirming the inactivity of this substance.

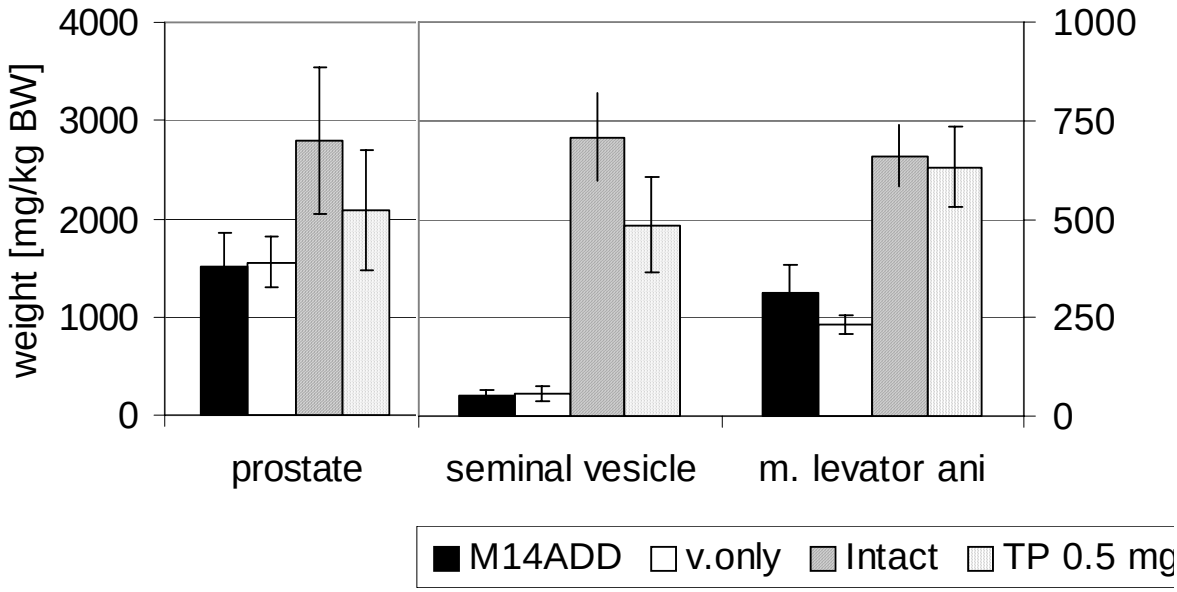

Figure 4: *Dose dependent effects of M1,4ADD on tissue wet weight of the prostate, seminal vesicle and levator ani muscle in orchiectomized rats. M1,4ADD and TP subcutaneously treated animals in the indicated doses (1 mg/kg BW/day); v. only = vehicle treated and intact animals. Animals were treated as described in material and methods.*

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