Steroids 74 (2009) 322–328

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Identification of steroid isoxazole isomers marketed as designer supplement

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ARTICLE INFO

Article history:
Available online 17 November 2008

Keywords:
17β-Hydroxyandrostanoisoxazoles
Designer supplement
GC–MS
NMR
X-ray

ABSTRACT

The product Orastan-A from Gaspari Nutrition was analyzed for its steroid content. According to the labeling, it is supposed to contain "5a-Androstano[2,3-*c*]furazan-17b-tetrahydropyranol ether", also called furazadrol-THP ether. The GC–MS analyses of the liberated steroids (after extraction from the capsule matrix and cleavage of the THP ether, TMS-derivative and underivatized) revealed mass spectra of two components, both inconsistent with the labeling. Thus, the steroids were characterized by different analytical techniques such as mass spectrometry, nuclear magnetic resonance spectroscopy and X-ray crystal structure analysis. They were identified as 17β-hydroxyandrostano[3,2-*c*]isoxazole and -[2,3-*d*]isoxazole.

1. Introduction

During the last few years more and more products appeared on the market containing steroids that were never approved as therapeutic drugs mostly without proper labeling of the contents [1–7]. Recently, preparations containing stanozolol analogues, e.g. Prostanozol (17β-hydroxy-5α-androstano[3,2-*c*]pyrazole), were also advertised on the Internet market for sport supplements [5,8].

In this study, we report the detection of another unapproved steroid in a "dietary supplement". Two isomers of 17β-hydroxyandrostanoisoxazole (structure formula in Fig. 1) were identified by different analytical techniques. The synthesis of these steroids was already described in the 1960s [9]. The [2,3-*d*]-isomer was reported to show anabolic properties after *s.c.* application while the [3,2-*c*]-isomer has lower activity [9,10]. Following oral administration, both isomers were found to be inactive [10]. The 17-methylated Androisoxazol ([3,2-*c*]isoxazole) is listed under the therapeutic category "Anabolic" by the Merck Index and the only approved androstanoisoxazole, Danazol (17α-ethinyl-17β-hydroxyandrost-4-eno[2,3-*d*]isoxazole), is classified as antigonadotropin [11]. Danazol (e.g. Danatrol®, Cyclomen®, Danol®) is available in several countries, especially for the treatment of endometriosis [12]. According to the doping regulations of the World Anti-Doping Agency (WADA), anabolic androgenic steroids are prohibited substances for use in sports [13]. While Danazol is explicitly mentioned in the list of prohibited substances, the other androstanoisoxazoles are covered by the wording "and other substances with a similar chemical structure or similar biological effect(s)".

2. Experimental

2.1. Chemicals and reagents

Androisoxazol was obtained from Prof. Manfred Donike (Cologne, Germany), Danazol from Thinker Chemical (Hangzou, China). 5α-Dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) and hydroxylamine hydrochloride were purchased from Sigma–Aldrich GmbH (Steinheim, Germany) and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other reagents and solvents were of analytical grade and obtained from VWR (Darmstadt, Germany).

2.2. Instrumentation

2.2.1. GC–MS analyses

For GC–MS analyses the residues were either dissolved in cyclohexane or derivatized with trimethyliodosilane (TMIS) reagent (MSTFA/NH_4I/ethanethiol, 1000:2:3, v:w:v) by heating for 20 min at 60 °C and injected into the GC–MS.

The analyses of the underivatized compounds were carried out on a gas chromatograph (GC) Agilent 6890 coupled to a mass selective detector (MSD) Agilent 5973, injection volume: 2 μL, splitless, injection temperature: 220 °C, column: Macherey-Nagel Optima δ3 (20 m, 0.25 mm i.d., 0.25 μm film thickness), carrier gas: helium,

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doi:10.1016/j.steroids.2008.11.006

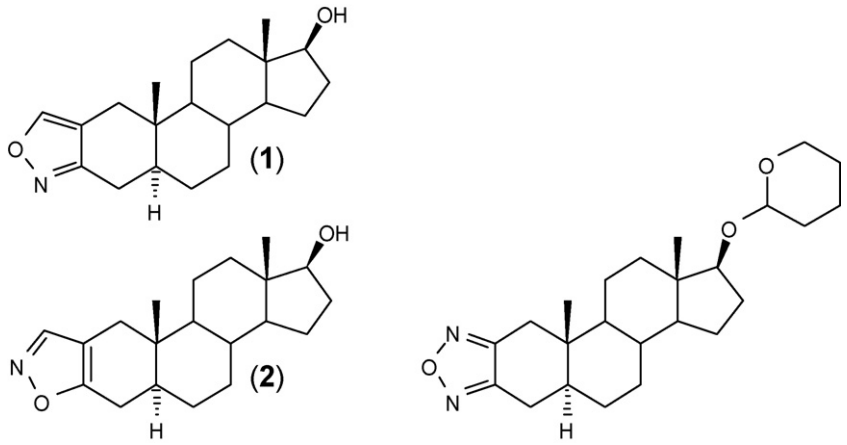


Fig. 1. Structures of the isolated steroids characterized as 17β-hydroxyandrostano[3,2-*c*]isoxazole (**1**) and -[2,3-*d*]isoxazole (**2**), and the steroid Furazadrol-THP ether labeled on the Orastan-A preparation but proven to be absent.

1.2 mL/min, oven temperature program: 1.5 min 60 °C, +40 °C/min, 0 min 240 °C, +2 °C/min, 0 min 260 °C, +40 °C/min, 1.5 min 300 °C, ionization: 70 eV, EI, data acquisition: full scan mode (m/z 40–800), sampling rate: 2^2.

The TMS-derivatives were analyzed on an Agilent 6890 GC coupled to an Agilent 5973 MSD using the following parameters: injection volume: 2 μL, split 1:10, injection temperature: 300 °C, column: Agilent HP5MS (16.5 m, 0.25 mm inner diameter (i.d.), 0.25 μm film thickness), carrier gas: helium, 0.8 mL/min, oven temperature program: 0 min 140 °C, +20 °C/min, 2 min 320 °C, ionization: 70 eV, electron ionization (EI), data acquisition: full scan mode (m/z 40–800), sampling rate: 2^2. For determination of the retention indices the GC oven was programmed as follows: 0 min 160 °C, +5 °C/min, 4 min 320 °C.

2.2.2. HPLC fractionation

The HPLC fractionation was performed on an Agilent 1090 equipped with an ODS Hypersil column (Thermo Electron, 10 mm × 250 mm, particle size 5 μm) using the following parameters: injection volume: 50 μL, mobile phase A: H_2O, B: acetonitrile, gradient: 0–25 min 50% B to 100% B, 15–32 min 100% B, 2 min re-equilibration, flow: 3 mL/min, detector wavelength: 228 nm.

The fractions were collected from 12.9 to 13.8 min (isomer **1**) and 14.1 to 15.0 min (isomer **2**). After evaporation of the solvent

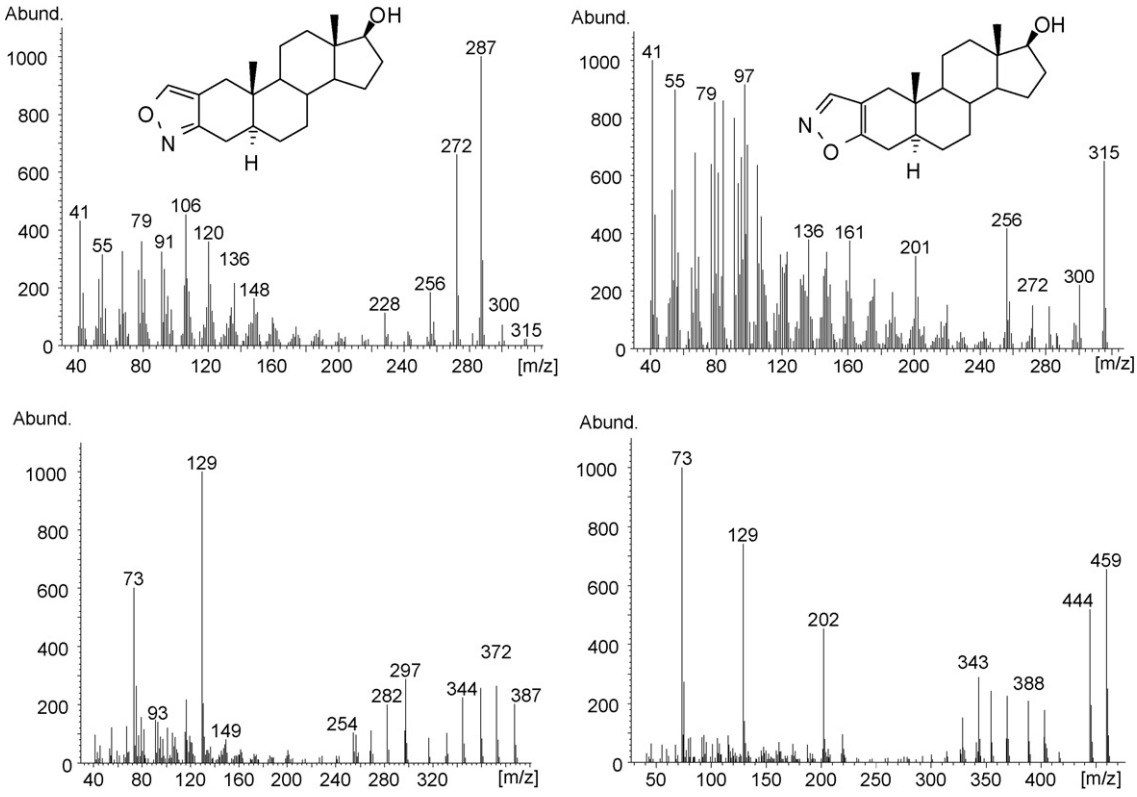


Fig. 2. Mass spectra (EI) of isomer **1** (upper left: underivatized, M^+ = 315, lower left: TMS-derivative, M^+ = 387) and isomer **2** (upper right: underivatized, M^+ = 315, lower right: TMS-derivative, M^+ = 459).

Table 1
Retention indices (methylene units) of androstanoisoxazoles (underivatized and as per-TMS-derivatives).

Analyte	Methylene units (Optima δ3) underivatized	Methylene units (HP5MS) after silylation
17β-Hydroxyandrostano[3,2-*c*]isoxazole (**1**)	3041	2888
17β-Hydroxyandrostano[2,3-*d*]isoxazole (**2**)	3071	3040
17β-Hydroxyandrostano[3,2-*c*]isoxazole (**1**)-acetate	3380	n.d.
17β-Hydroxyandrostano[2,3-*d*]isoxazole (**2**)-acetate	3410	n.d.
17β-Hydroxyandrostano[3,2-*c*]isoxazole (**1**)-THP	3571	n.d.
17β-Hydroxyandrostano[2,3-*d*]isoxazole (**2**)-THP	3579	n.d.
Androisoxazol ([3,2-*c*]isoxazole)	3077	3070
Danazol ([2,3-*d*]isoxazole)	3367	3137

n.d. value not determined.

under reduced pressure, the residues were crystallized from ethyl acetate (m.p. isomer **1**: 161–162 °C, Lit [3,2-*c*]: 156–158 °C, isomer **2**: 184–187 °C, Lit [2,3-*d*]: 184–186 °C [14]).

2.2.3. NMR spectroscopy

The NMR data were obtained using a Bruker DRX 500 instrument, equipped with a 5 mm inverse probehead with z-gradient coil. Chemical shifts were given in δ values (ppm) relative to tetramethylsilane. The spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) at 298 K using solutions of about 5 mg of each compound in deuterated DMSO. For confirmation of the assumed structures ^{1}H, H,H COSY, DEPT, APT, H,C HMQC, H,C HMBC and H,H NOESY spectra were recorded.

2.2.4. X-ray crystal structure analysis

Suitable single crystals of isomer **2** (17β-hydroxy-androstano[2,3-*d*]isoxazole, **2**) were obtained from ethyl acetate by evaporation. X-ray data were collected with a Nonius KappaCCD diffractometer [15,16] equipped with a low temperature device at 100 K by using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). The structure was solved by direct methods using SHELXS-97 [17] and the non-hydrogen atoms were refined by full-matrix least-squares methods using SHELXL-97 [18]. The positional parameters of the H atoms were obtained geometrically, with C–H distances fixed and refined as riding on their respective C atoms with U_{iso} H = 1.2 U_{eq} C.

Crystal data: $C_{20}H_{29}NO_2$, M = 315.46, colourless, crystal dimensions 0.46 mm × 0.31 mm × 0.11 mm, monoclinic, space group $P2_1$, Z = 4, a = 9.7180(7) Å, b = 7.5511(5) Å, c = 23.227(2) Å, β = 98.295(4)°, V = 1686.6(2) Å^3, D_X = 1.242 g cm^{-3}, μ (Mo Kα) = 0.079 mm^{-1}, T = 100(2) K, transmission factors (min/max) 0.9646/0.9914, analytical absorption correction based on the indexing of the crystal faces [19], $2\theta_{(max)}$ = 51°, number of unique data 4790, R_{int} 0.0558, R_1 [$I > 2\theta(I)$] = 0.0712, $w(R_2)$ all data 0.1669, final R = 0.0924, goodness of fit 1.062, $\Delta\rho$ (max/min) = 0.261/−0.363 e Å^{-3}.

2.2.5. High-resolution high-accuracy mass spectrometry

For the verification of the elemental composition high-resolution high-accuracy mass spectrometry was performed on a Thermo LTQ Orbitrap mass spectrometer (Bremen, Germany). The instrument was operated in positive ionization mode and calibrated using the manufacturer's calibration mixture (containing caffeine, MRFA, and ultramark that yield a total of 7 reference masses). Mass accuracies < 2 ppm (calculated from 30 averaged spectra) at a resolution of 30,000 (full width half maximum, FWHM) were determined before and after the period of analysis of target compounds. Working solutions were introduced into the mass spectrometer using a syringe pump at a flow rate of 5 μL/min. The ionization voltage was 3.0 kV, and the capillary temperature was set to 275 °C. Damping gas in the linear ion trap was helium (purity grade 5.0), and gas supplied to the curved linear ion trap (CLT) was nitrogen obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany).

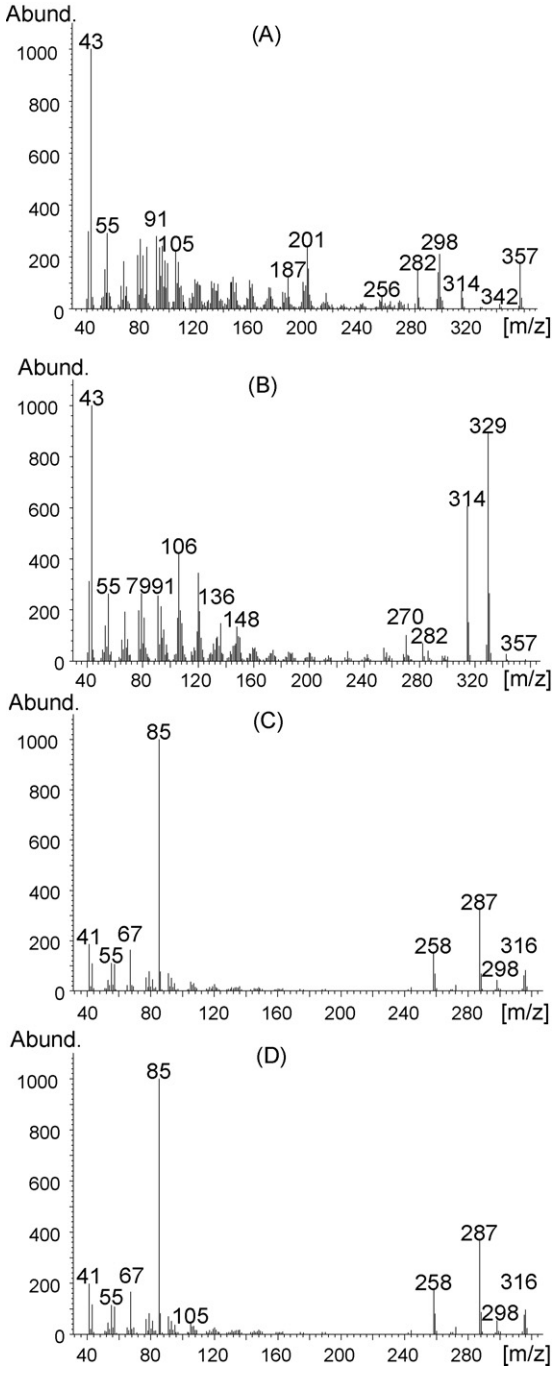


Fig. 3. Mass spectra (EI) of steroid compounds of Orastan-A, underivatized, (A) acetate of isomer **1**, (B) acetate of isomer **2**, (C) THP ether of isomer **1**, (D) THP ether of isomer **2**.

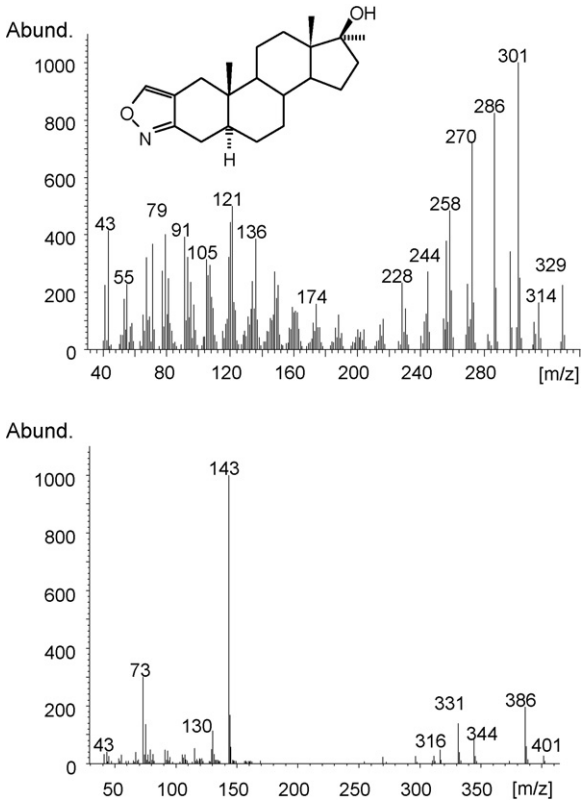


Fig. 4. Mass spectra (EI) of Androisoxazol (upper: underivatized, $M^+ = 329$, lower: TMS-derivative, $M^+ = 401$).

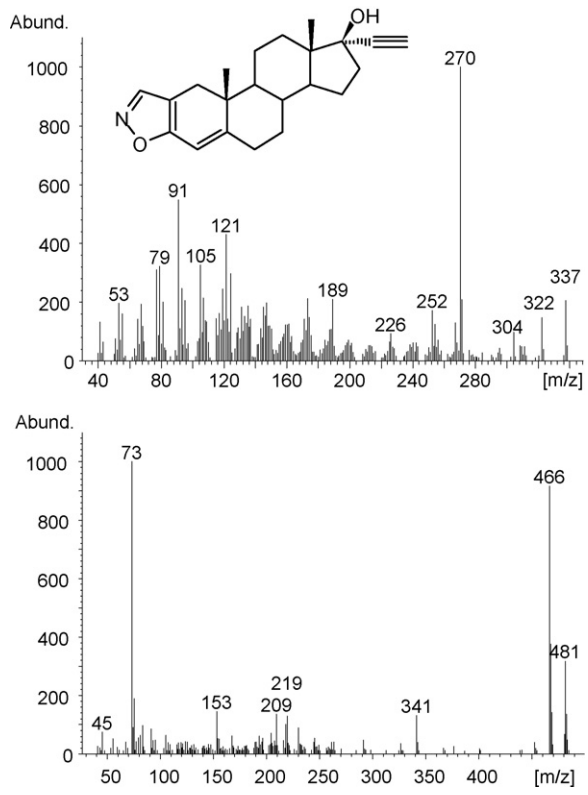


Fig. 5. Mass spectra (EI) of Danazol (upper: underivatized, $M^+ = 337$, lower: TMS-derivative, $M^+ = 481$).

2.3. Supplement analysis

The product Orastan-A from the company Gaspari Nutrition was obtained by Internet order from the sport supplement market, classified as "dietary supplement" according to its labeling. It was labeled to contain "5a-Androstano[2,3-*c*]furazan-17b-tetrahydropyranol ether", also called furazadrol-THP ether (structure formula in Fig. 1).

The homogenized content of one capsule was suspended in 5 mL of methanol. After shaking for 5 min and centrifugation for 5 min at 800 × *g*, the methanolic layer was separated. Aliquots were analyzed by GC–MS.

For characterization of the steroid moiety the content of 30 capsules was extracted with *n*-hexane in a Soxhlet apparatus. After evaporation of the solvent, the residue was hydrolyzed for 1 h in refluxing aqueous acetic acid (30%). Aliquots were analyzed by GC–MS (underivatized and as TMS-derivatives). The co-crystallizing (from ethyl acetate) steroids were separated by semi-preparative HPLC. After crystallization from ethyl acetate, the steroids were characterized by GC–MS, NMR, X-ray and high-resolution/high-accuracy mass spectrometry for the identification of the molecular structure.

2.4. Synthesis of reference material

2.4.1. 17β-Hydroxyandrostano[3,2-c]isoxazole (**1**)

For the synthesis of 17β-hydroxyandrostano[3,2-*c*]isoxazole (**1**), 2-hydroxymethylene-5α-dihydrotestosterone (**4**) was prepared by reaction of 5α-dihydrotestosterone (**3**) with ethyl formate and sodium methylate in absolute pyridine as described by Schänzer [20]. The isoxazole ring was formed by condensation of 33 mg of **4** with 27 mg of hydroxylamine hydrochloride using 3.5 mL of pyridine as solvent. After refluxing for 3 h the mixture was evaporated to dryness, re-dissolved in water and extracted with t-butyl methyl ether. Evaporation of the solvent resulted in 17β-hydroxyandrostano[3,2-*c*]isoxazole (**1**).

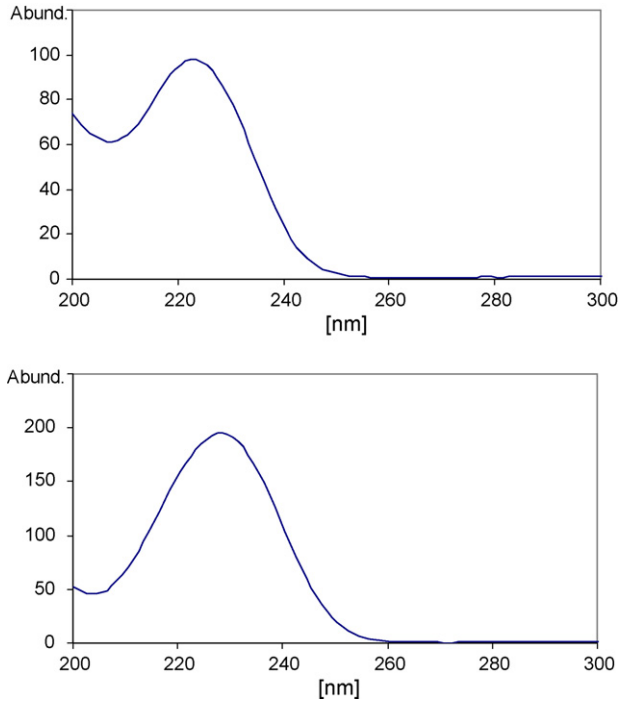


Fig. 6. UV spectra (HPLC-DAD) of isomer **1** (upper) and isomer **2** (lower).

Table 2
^{1}H and ^{13}C NMR spectral data (d_6-DMSO) of 17β-hydroxyandrostano[3,2-*c*]isoxazole (**1**) and 17β-hydroxyandrostano[2,3-*d*]isoxazole (**2**).

	17β-hydroxyandrostano[3,2-*c*]isoxazole (**1**)		17β-hydroxyandrostano[2,3-*d*]isoxazole (**2**)	
	δ_C	δ_H	δ_C	δ_H
1	30.951	2.011/2.652	33.567	2.034/2.419
2	114.136	–	111.176	–
3	158.672	–	165.617	–
4	24.581	2.222/2.674	26.069	2.230/2.651
5	41.333	1.504	41.860	1.564
6	28.738	1.319/1.535	28.360	1.328/1.515
7	32.088	0.852/1.646	30.931	0.854/1.642
8	35.456	1.316	35.378	1.312
9	53.159	0.816	53.174	0.822
10	35.672	–	36.120	–
11	20.477	1.337/1.554	20.584	1.355/1.534
12	36.678	0.984/1.758	36.665	0.986/1.757
13	42.508	–	42.533	–
14	50.585	0.885	50.525	0.887
15	23.249	1.160/1.500	23.226	1.161/1.496
16	29.998	1.333/1.826	29.972	1.334/1.823
17	80.175	3.436	80.151	3.432
18	11.324	0.639	11.320	0.641
19	11.382	0.661	11.463	0.669
isoxazole CH	154.812	8.511	150.315	8.298
OH	–	4.397	–	4.399

*2.4.2. 17β-Hydroxyandrostano[2,3-d]isoxazole (**2**)*

For the synthesis of 17β-hydroxyandrostano[2,3-*d*]isoxazole (**2**) the isoxazole ring was formed by condensation of 3.3 mg of 2-hydroxymethylene-5α-dihydrotestosterone (**4**) with 30 μL of an aqueous solution of hydroxylamine hydrochloride (90 mg/mL) using 1 mL of ethanol as solvent. After refluxing for 3 h the mixture was evaporated to dryness, redissolved in water and extracted with t-butyl methyl ether. 17β-Hydroxyandrostano[2,3-*d*]isoxazole (**2**) was obtained by evaporation of the solvent [14].

3. Results and discussion

3.1. Supplement analysis

The GC–MS analyses of the underivatized methanolic extract of Orastan-A revealed three pairs of steroidal compounds which were identified as two isomeric steroid alcohols, their corresponding acetates and tetrahydropyranyl (THP) ethers (mass spectra in Figs. 2 and 3, retention indices in Table 1). As the THP ethers represented the main ingredients, the steroids were released by acidic hydrolysis prior to further characterization, yielding both steroids in a ratio of about 1:2. The mass spectra of the liberated steroids (underivatized and as TMS-derivatives) are shown in Fig. 2. In disagreement with the product label the mass spectra of neither compound identified matched that of furazadrol. However, isomer **1** showed analogous mass spectra compared with Androisoxazol (17α-methyl-17β-hydroxy-5α-androstano[3,2-*c*]isoxazole, Fig. 4), lacking the 17-methyl group. High-resolution high-accuracy mass spectrometry ($[M+H]^+_{exp.} = 316.2273$, $[M+H]^+_{theor.} = 316.2271$, error 0.56 ppm) deduced the elemental composition $C_{20}H_{29}NO_2$ for both isomers. While isomer **1** yielded a mono-TMS-derivative, a bis-TMS-derivative was obtained from isomer **2** following derivatization with TMIS reagent. Analogous findings were obtained for Androisoxazol ([3,2-*c*]-) and Danazol ([2,3-*d*]-isoxazole) derivatized with TMIS reagent (mass spectra in Figs. 4 and 5). From these findings structures of 17β-hydroxyandrostano[3,2-*c*]isoxazole and -[2,3-*d*]isoxazole were assumed.

Unfortunately, both steroids co-crystallized and had to be separated by semi-preparative HPLC. The obtained UV spectra (HPLC-DAD, Fig. 6) showed maxima at 222 nm (isomer **1**) and 228 nm (isomer **2**). Comparing the melting points and UV maxima of the two isomers with data from the literature [9,14,21] supported the assignment of the [3,2-*c*]isoxazole for isomer **1** and of the [2,3-*d*]isoxazole for isomer **2**.

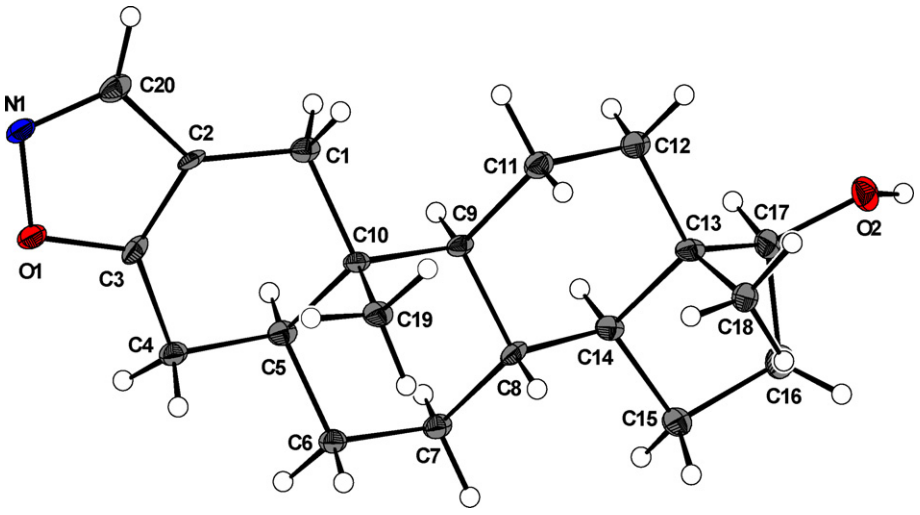
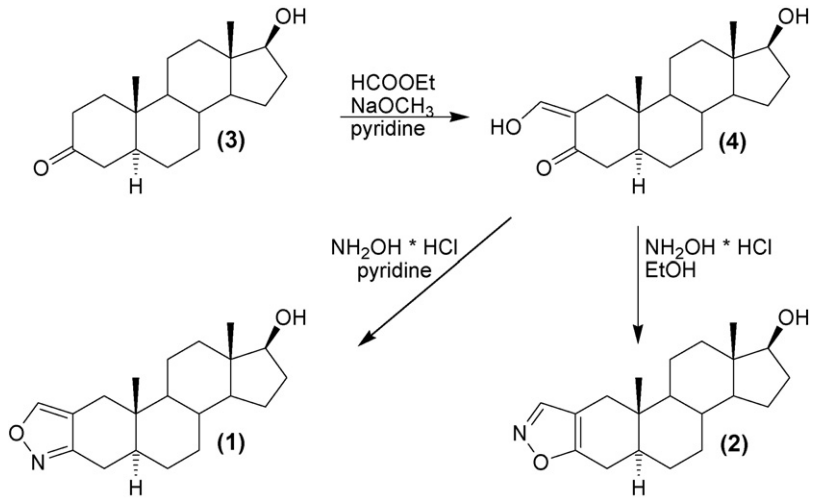


Fig. 7. Representation of the structure of 17β-hydroxyandrostano[2,3-*d*]isoxazole (isomer **2**) as determined by X-ray crystallography.



Scheme 1. Synthesis of 17β-hydroxyandrostano[3,2-*c*]isoxazole (**1**) and -[2,3-*d*]isoxazole (**2**).

The ^{1}H NMR spectra of the purified steroids (detailed data in Table 2) show a ^{1}H singlet at 8.51 ppm (isomer **1**) and 8.30 ppm (isomer **2**), representing the isoxazole CH. As described by Fahrenholtz et al. [22], these signals for [3,2-*c*]isoxazoles are shifted downfield compared to [2,3-*d*]isoxazoles. The corresponding ^{13}C chemical shift was determined at 154.8 and 150.3 ppm. Further significant differences occurred for C-2 (114.1 ppm vs. 111.1 ppm) and C-3 (158.7 ppm vs. 165.6 ppm). X-ray crystal structure analysis of isomer **2** (Fig. 7) finally confirmed the structures of the assignment 17β-hydroxyandrostano[3,2-*c*]isoxazole for isomer **1** and 17β-hydroxyandrostano[2,3-*d*]isoxazole for isomer **2**.

*3.2. Synthesis of 17β-hydroxyandrostano[3,2-c]isoxazole (**1**) and -[2,3-d]isoxazole (**2**)*

Both isomers were synthesized as displayed in Scheme 1. The reaction of 2-hydroxymethylene-5α-dihydrotestosterone (**4**) with hydroxylamine in refluxing pyridine yielded 17β-hydroxyandrostano[3,2-*c*]isoxazole (**1**) as almost single isomer while the reaction carried out in ethanol resulted in a mixture of both isomers (ratio (**1**):(**2**) ∼ 1:10). The synthesized material revealed the same analytical properties as the steroids liberated from the supplement, thus further confirming the identity of the ingredients.

4. Conclusion

In the product "Orastan-A" the two isomeric steroids, 17β-hydroxyandrostano[3,2-*c*]isoxazole (**1**) and -[2,3-*d*]isoxazole (**2**), were identified. They were mainly present as THP-ether. Following oral administration these THP ethers are expected to easily undergo hydrolysis by gastric acid [23].

The present study again shows that products, containing unapproved steroids that have already been synthesized in the 1960s, nowadays appear on the market of sport supplements, often labeled as "dietary supplement", even though the products have to be classified as non-licensed pharmaceuticals [24].

In addition to the enormous health risks, consumers should be aware of the doping risks connected with the use of such products. For the detection of the misuse of these steroids in doping control studies on the urinary metabolism are required in the future. Preliminary results of Sobolevsky et al. [25] and our working group [26] suggest the integration of hydroxylated metabolites into routine doping screening, preferably by LC–MS/MS.

Acknowledgements

The German Federal Ministry of the Interior and the Manfred Donike Institute for Doping Analyses e.V., Cologne, are acknowledged for their financial support.

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