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17α- and 17β-boldenone 17-glucuronides: Synthesis and complete characterization by ^{1}H and ^{13}C NMR

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ABSTRACT

Boldenone is an androgenic anabolic steroid intensively used for growth promoting purposes in animals destined for meat production and as a performance enhancer in athletics. Therefore its use is officially banned either in animals intended for consumption or in humans. Because most anabolic steroids are completely metabolized and usually no parent steroid is excreted, metabolite identification is crucial to detect the illegal use of anabolic steroids either in humans or in livestock. 17α- and 17β-boldenone 17-glucuronides were synthesized, purified and characterized in order to provide suitable standards for the identification and quantification of these metabolites.

1. Introduction

17β-Boldenone (androsta-1,4-dien-17β-ol-3-one, **1**) is a synthetic androgenic steroid hormone with anabolic properties, obtained by dehydrogenation of the male hormone testosterone [1]. As for other anabolic steroids, its illegal use to increase body mass and enhance physical conditioning is widely demonstrated.

17β-Boldenone is included in the 2008 list of banned substances in sports by the World Anti-Doping Agency (WADA) and at the same time its illegal administration for growth promotion purposes in animals destined for meat production (cattle fattening) is be coming particularly widespread. Its use is forbidden by European directives 96/22/EC and 03/74/EC [2,3] to protect the consumer's health.

The forensic chemistry of natural and/or synthetic steroids as illegal abuse substances calls for the development of analytical methods fit for the identification of both the parent drugs and their metabolites, in order to demonstrate its effective administration.

Biotransformation reactions of drugs are generally divided in two classes. In phase I reactions, polar functional groups are inserted in a drug molecule via typical oxidation, reduction and hydrolysis. In phase II reactions, the drug and/or its phase I metabolites are conjugated with endogenous species such as glucuronic acid, sulphate, amino acids, biliary acids and so on, forming soluble conjugate products which are readily excreted from the body via urine or faeces. Conjugation with glucuronic and sulphate acid is a common route of metabolism for steroids and may make up to 90% of the excreted metabolites. They are therefore an important class of compounds for drug screening in sports [4].

The human metabolism of 17β-boldenone was investigated in 1971 [5] and it has been reported that 17β-boldenone is excreted in human urine as free and conjugate. Its main metabolites are 5β-androst-1-en-17β-ol-3-one, 5β-androst-1-en-3α-ol-17-one and 5β-androst-1-en-3α,17β-diol [6] while 17α-boldenone (androsta-1,4-dien-17α-ol-3-one, **2**) is considered to be the main metabolite in human and equine urine [7,8]. The detection of 17α-boldenone would become meaningful evidence for the abuse of 17β-boldenone if at the same time additional metabolites, such as glucuronide of 17β-boldenone (**3**) and 17α-boldenone (**4**), were identified (Fig. 1).

At present, the analytical methods for boldenone glucuronide-conjugated detection are based on application of liquid chromatographic (LC) separation interfaced by soft ionization techniques, such as electrospray ionization (ESI), with tandem mass spectrometry (MS/MS) [4,9]. However, a significant drawback of the conjugate analysis is the lack of reference material, essential for the development and application of analytical methods. In addition to doping control analytical purposes, the conjugated reference standards are also needed for steroid metabolism research in pharmaceutical industry and research institutes.

A complete characterization of boldenone glucuronides-conjugated is apparently not reported in literature. In this paper, the chemical synthesis of 17α- and 17β-boldenone 17-glucuronides are reported together with the complete assignment of ^{1}H and ^{13}C data

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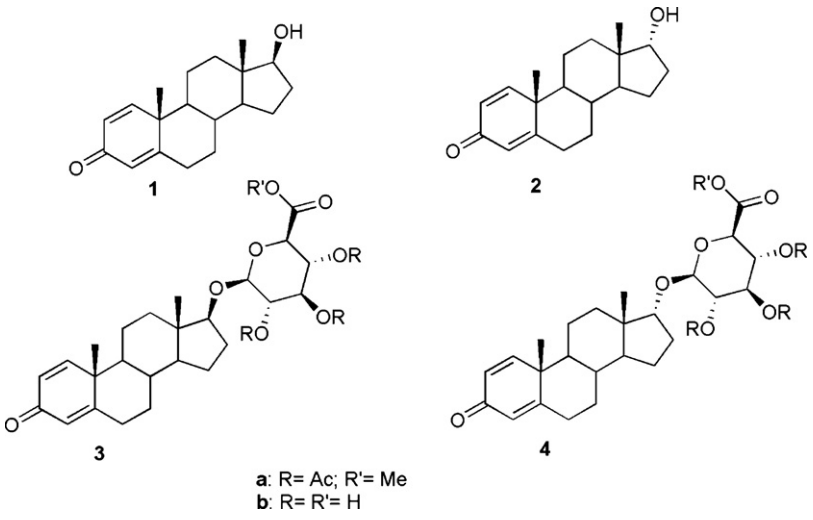

Fig. 1. Structural formula of 17α- and 17β-boldenone, and corresponding 17-glucuronides.

and the anomericity of the glucuronic acid moiety in the steroid glucuronides of these compounds by 1D and 2D NMR experiments. However, new synthesis improving the yield of 17α-boldenone17-β-glucuronide (**4a**) has been reported.

2. Experimental

2.1. General

17β-boldenone (**1**) was obtained from Steraloids (Newport, RI, USA). 17α-Boldenone (**2**) was synthesized from 17β-boldenone by a modified Mitsunobu reaction [10].

Acetobromo-α-D-glucuronic acid methyl ester (**5**) was purchased from Sigma–Aldrich.

Melting points were recorded on a Stuart Scientific SMP3 instrument and were uncorrected. All reagents were purchased from Sigma–Aldrich.

Optical rotations were measured on a PerkinElmer 241 polarimeter (sodium D line at 25 °C). Mass spectra were recorded on a Finnigan LCQdeca (TermoQuest) in ESI positive-ion mode, kV 5.00, 220 °C, 15 V. Mass spectral data are given as *m*/*z* (relative abundance).

All reactions were monitored by TLC on silica gel 60 F_{254} precoated plates with a fluorescent indicator (Merck). Flash chromatography was performed using silica gel 60 (230–400 mesh, Merck).

2.2. NMR experiments

NMR spectra were done on a Bruker AVANCE 500 spectrometer equipped with a 5-mm broadband reverse probe with field *z*-gradient operating at 500.13 and 125.76 MHz for ^{1}H and ^{13}C, respectively. All NMR spectra were recorded at 298 K in DMSO-d_6 (isotopic enrichment 99.95%) or $CDCl_3$ (isotopic enrichment 99.95%) solution and the chemical shifts were reported on a δ (ppm) scale. The central peak of DMSO-d_6 signals (2.50 ppm for ^{1}H and 39.50 ppm for ^{13}C) and of $CDCl_3$ signals (7.26 ppm for ^{1}H and 77.7 ppm for ^{13}C) were used as internal reference standard. Acquisition parameters for 1D were as follows: ^{1}H spectral width of 5000 Hz and 32 K data points providing a digital resolution of ca. 0.305 Hz per point, relaxation delay 2 s; ^{13}C spectral width of 29412 Hz and 64 K data points providing a digital resolution of ca. 0.898 Hz per point, relaxation delay 2.5 s. The experimental error in the measured ^{1}H–^{1}H coupling constants was ±0.5 Hz. The experiments were performed through standard pulse sequences. gCOSY-45 experiments were acquired with 512 t_1 increments; 2048 t_2 points; spectral/spectrum width 10.0 ppm. The acquisition data for gHSQC and gHMBC experiments were obtained with 512 t_1 increments; 2048 t_2 points; spectral/spectrum width 10.0 ppm for ^{1}H and 220 ppm for ^{13}C. Delay values were optimized for $^1J_{C,H}$ 140.0 Hz and $^nJ_{C,H}$ 3.0 Hz. Zero filling in F_1 to 1 K, $\pi/2$ shifted sine-bell squared (for gHSQC) or sinebell (for gHMBC) apodization functions were used for processing. For overlapped signals of hydrogen atoms important for the identification of compounds, the 1D HOHAHA technique [11] was used to obtain the chemical shifts and coupling constants.

2.3. Chemistry

2.3.1. Methyl (androsta-1,4-dien-17β-ol-3-one)-17β-O-2,3,4-tri-O-acetyl-β-D-glucuronate (**3a**)

$CdCO_3$ (0.76 mmol; 130 mg) was added to a solution of β-boldenone (**1**) (0.35 mmol, 100 mg) in anhydrous toluene (5 ml) under vigorous stirring. A portion of the toluene (0.5–1 ml) was distilled off under azeotropic conditions to remove all traces of water from the system. A solution of acetobromo-α-D-glucuronic acid methyl ester (**5**) (0.43 mmol, 170 mg) in toluene (2 ml) was then added over 5 min to the reaction mixture, further stirred and heated under azeotropic conditions for an additional 6 h. A mixture of inorganic salts was removed by filtration, and the solvent was evaporated from the filtrate, affording a dry residue. The product was purified by column chromatography on silica gel: petroleum ether/ethyl acetate (60:40, v/v) to give the methyl (androsta-1,4-dien-17β-ol-3-one)-17β-*O*-2,3,4-tri-*O*-acetyl-β-D-glucuronate (**3a**, white solid, 168 mg, 80%): mp 116–118 °C; $[\alpha]_D^{20}$ + 32.2 (*c* = 1.00, $CHCl_3$); ESI-MS (positive) *m*/*z*: 625 (M + 23). $C_{32}H_{42}O_{11}$: calcd. C, 63.77; H, 7.02. Found. C, 63.84; H, 7.08.

2.3.2. Methyl (androsta-1,4-dien-17α-ol-3-one)-17β-O-2,3,4-tri-O-acetyl-β-D-glucuronate (**4a**)

*2.3.2.1. Synthesis of the glucuronide donor: methyl-(2,3,4-tri-O-acetyl-α-D-glucopyranosyl-trichloroacetimidate)-glucuronate (**7**).* H_2O (50 μl) and acetobromo-α-D-glucuronic acid methyl ester (**5**) (1.5 mmol, 600 mg) were added in sequence to a suspension of $CdCO_3$ (1.5 mmol, 500 mg) in acetonitrile. The reaction mixture was stirred and heated to 70 °C for 1 h under nitrogen, filtered through Celite, washed with 5 ml of acetonitrile, and concentrated in vacuo.

Purification of the crude residue by flash chromatography on silica gel: ethyl acetate/hexane (50:50, v/v) afforded the hydrolyzed product (white solid, 393 mg, 78%), 1-hydroxy-methyl 2,3,4-tri-*O*-acetyl-α/β-D-glucopyranuronate that was subsequently dissolved in dichloromethane (12 ml); trichloroacetonitrile (8 mmol, 1.1 g, 0.8 ml) was then added under nitrogen. After cooling to 0 °C, 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU, 0.2 mmol, 36 μl) was added. The reaction mixture was allowed to stir for 1 h and then concentrated to afford a sticky, dark-brown residue. The product was purified by flash chromatography on florisil: petroleum ether/ethyl acetate (70:30, v/v) supplying compound **7** (off-white solid, 359 mg, 64%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.71 (s, 1H, NH), 6.66 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1), 5.65 (dd, 1H, $J_{3,2}$ 9.8 Hz, $J_{3,4}$ 9.8 Hz, H-3), 5.29 (dd, 1H, $J_{4,3}$ 9.8 Hz, $J_{4,5}$ 9.8 Hz, H-4), 5.17 (dd, 1H, $J_{2,1}$ 3.6 Hz, $J_{2,1}$ 9.8 Hz, H-2), 4.52 (d, 1H, $J_{5,4}$ 9.8 Hz, H-5), 3.77 (s, 3H, COOMe), 2.08 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.04 (s, 3H, OAc). ^{13}C NMR δ 169.78 (CO), 169.73 (CO), 169.48 (CO), 167.15 (CO), 160.59 (CN), 92.63 (C(Cl_3)), 70.49 (C-5), 69.47 (C-1), 69.10 (C-3), 68.96 (C-4), 53.06 ($COOCH_3$), 20.68 ($OCOCH_3$), 20.50 ($OCOCH_3$), 20.42 ($OCOCH_3$).

*2.3.2.2. Synthesis of 17α-boldenone-17-βglucuronide (**4a**).* A solution of methyl-(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl-trichloroacetimidate)-glucuronate (**7**) (0.9 mmol, 400 mg) in dry dichloromethane (3 ml) was slowly added to a solution of 17α-boldenone (**2**) (0.35 mmol, 100 mg) in dry dichloromethane (3 ml) and trimethylsilyl trifluoromethanesulfonate (TMSOTf, 100 μl of a solution 0.06 M) under nitrogen at −15 °C. After 48 h the reaction was quenched with triethylamine. Solvent evaporation under reduced pressure, and purification by flash chromatography on silica gel: dichloromethane/acetone (95:5, v/v) afforded the methyl (androsta-1,4-dien-17α-ol-3-one)-17β-*O*-2,3,4-tri-*O*-acetyl-β-D-glucuronate (**4a**, white solid, 143 mg, 68%): mp 124–125 °C; $[\alpha]_D^{20}$ − 0.5 (*c* = 1.00, $CHCl_3$); ESI-MS (positive) *m*/*z*: 625 (M + 23). $C_{32}H_{42}O_{11}$: calcd. C, 63.77; H, 7.02. Found. C, 63.64; H, 7.18.

*2.3.3. Methyl (androsta-1,4-dien-17α-ol-3-one)-17β-O-cyclic-1,2-(hydrogen-orthoacetyl-O-3,4-di-acetyl)-β-D-glucuronate (**8**)*

100 μl of a solution 0,06 M of trimethylsilyl trifluoromethanesulfonate (TMSOTf) in dry dichloromethane were added in three portions to a solution of 17α-boldenone (**2**) (0.35 mmol, 100 mg) and methyl-(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl-trichloroacetimidate)-glucuronate (**7**) (0.9 mmol, 400 mg) in dry dichloromethane (5 ml) under nitrogen at 0 °C. After usual work-up, methyl(androsta-1,4-dien-17α-ol-3-one)-17β-*O*-cyclic1,2-(hydrogen-orthoacetyl-*O*-3,4-di-acetyl)-β-D-glucuronate (**8**, white solid, 100 mg; 48%) was obtained. mp 103–105 °C; $[\alpha]_D^{20}$ + 18.5 (*c* = 1.00, $CHCl_3$).

2.3.4. General procedure for hydrolysis of acetylated glucuronides

NaOH 1% in methanol was slowly added to a solution of glucuronides **3a** or **4a** in methanol (150 mg, 0.25 mmol). After 24 h the solution was neutralized with dowex 50WX8-200, filtered and evaporated.

*2.3.4.1. Androsta-1,4-dien-17β-ol-3-one-17β-O-glucuronide (**3b**).* The product was purified by chromatography on silica gel column: dichloromethane/methanol/water (70:27:3, v/v/v) (**3b**, white solid, 110 mg, 97%): mp 173–174 °C; $[\alpha]_D^{20}$ + 6.2 (*c* = 1.00, $CHCl_3$); ESI-MS (negative) *m*/*z*: 461 (M-1), 923 (M + M-1). $C_{25}H_{34}O_8$: calcd. C, 64.92; H, 7.41. Found. C, 65.04; H, 7.28.

*2.3.4.2. Androsta-1,4-dien-17α-ol-3-one-17β-O-glucuronide (**4b**).* The product was purified by chromatography on silica gel: dichloromethane/methanol/water (70:27:3, v/v/v) (**4b**, white solid, 109 mg, 95%): mp 188–190 °C; $[\alpha]_D^{20}$ − 6.3 (*c* = 1.00, $CHCl_3$); ESI-MS (negative) *m*/*z*: 461 (M-1), 923 (M + M-1). $C_{25}H_{34}O_8$ calcd. C, 64.92; H, 7.41. Found. C, 64.81; H, 7.59.

3. Results and discussion

3.1. Chemistry

In the synthesis of 17α- and 17β-boldenone 17-glucopyranosiduronic acid derivatives (referred to as glucuronides) (**3b**, **4b**), the starting compounds were 17β-boldenone (**1**) and its 17α-derivative **2**, which had been synthesized earlier from 17β-boldenone by a modified Mitsunobu reaction [10].

The synthesis of glucuronides is most frequently carried out via the Koenigs–Knorr reaction or its modifications [12] in which the electrophilic character of the anomeric bromide **5** is enhanced by a halophilic agent such as cadmium carbonate. The main drawbacks of these procedures are the instability of the bromo derivatives [13], the need for elevated reaction temperatures and frequently prolonged reaction times. These events, moreover, could lead to undesirable modifications of aglycones.

When this reaction was applied to 17β-boldenone (**1**) the corresponding glucuronide **3a** was obtained in 80% yields. Instead when the same reaction was applied to the glucuronidation of 17α-boldenone (**2**) byproducts were mainly obtained. A TLC separation of the reaction mixture revealed a lot of spots, one of which corresponded to the unreacted sterol and one more was found to be glucuronide **4a**. The same difficulties, probably due to steric hindrance of 18-methyl group on the 17α position, have been also reported by Bowers and Bowers [14] in the synthesis of epitestosterone glucuronide.

These difficulties were circumvented by modifying the leaving group. The conversion of bromide **5** to the imidate **7**, followed by the activation through TMSOTf, allowed stereospecific reactions to obtain the corresponding β-glucuronides (Fig. 2).

The glucuronic acid imidate donor **7** was synthesized in 50% yield in two steps from the commercially available acetobromo-α-D-glucuronic acid methyl ester (**5**). In turn, **5** was converted to its corresponding free emiacetal **6** by using $CdCO_3/H_2O$, subsequently converted to the α-imidate derivative **7** through trichloroacetonitrile and 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) [15]. Reaction of 17α-boldenone (**2**) and imidate **7** under stan-

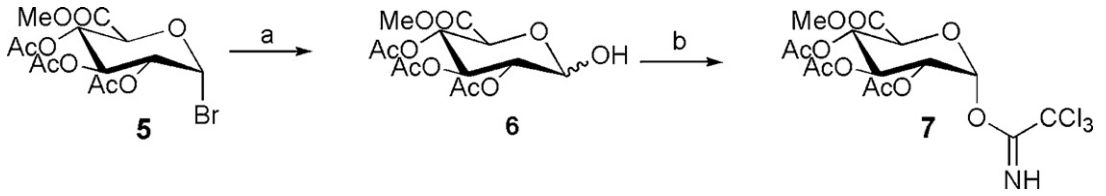

Fig. 2. Synthesis of imidate donor **7**. Reagents and conditions: (a) $CdCO_3$, H_2O (2 equiv.), CH_3CN, 70 °C, 1 h, yield = 78% and (b) CCl_3CN, 1,2-dichloroethane, 1,8-DBU, 0 °C, 1 h, yield = 64%.

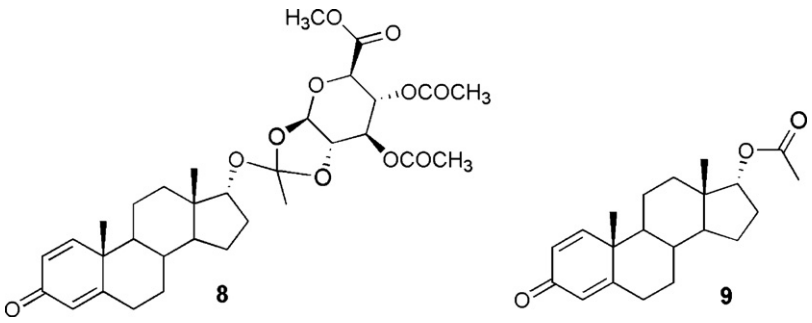

Fig. 3. Structural formula of the orthoester **8** and the transacylation derivative **9**.

dard conditions (dichloromethane, TMSOTf, 0 °C) afforded almost exclusively the orthoester **8** together with the transacylation derivative **9** (Fig. 3).

Structure of the orthoester **8** was unequivocally established by NMR techniques (Table 1).

However, we observed that when imidate **7** was added slowly at −15 °C to a mixture of α-boldenone **2** and TMSOTf, rather than adding TMSOTf to premixed **2** and **7** in dichloromethane, the conjugate **4a** was isolated in 68% yield after chromatography.

Our findings agree with other authors [16,17] who demonstrated that the reaction was subjected to a profound order-of-addition effects.

Schmidt and Toepfer [18] considered that, in the inverse-addition mode, prior complexation of acceptor component and catalyst led to minimal contact between the imidate donor and the catalyst, thereby reducing decomposition and side reactions of the imidate.

Our findings confirm that both the use of imidate and the inverse addition of the reagent gave advantages for the formation of glucuronide **4a**.

The following deprotection of glucuronides **3a** and **4a** with 1% NaOH in methanol afforded the target compounds **3b** (97%) and **4b** (95%).

3.2. NMR spectroscopy

As the resulting steroid substrates 17α- and 17β-boldenone contain only one possible site for glucuronidation there was no

Table 1
^{1}H NMR chemical shifts (ppm) assignment for compounds **3a**, **3b**, **4a**, **4b** and **8**.

	3a $CDCl_3$	**3b** DMSO	**4a** $CDCl_3$	**4b** DMSO	**8** $CDCl_3$
Aglycon moiety					
1	7.06	7.20	7.09	7.26	7.08
2	6.25	6.12	6.26	6.11	6.25
4	6.09	5.89	6.09	5.98	6.08
6 α	2.38	2.32	2.37	2.33	2.38
6 β	2.48	2.47	2.48	2.49	2.48
7 α	0.95	0.91	1.11	1.48	1.50
7 β	1.96	1.85	1.55	1.65	1.61
8 (β)	1.68	1.70	1.64	1.71	1.59
9 (α)	1.04	0.95	1.09	0.99	1.14
11 α	1.73	1.69	1.78	1.75	1.78
11 β	1.61	1.62	1.66	1.63	1.68
12 α	1.17	1.13	1.11	1.00	1.13
12 β	1.83	1.92	1.96	1.98	1.98
14 (α)	0.91	0.93	1.11	1.43	1.36
15 α	1.55	1.66	1.54	1.63	1.63
15 β	1.31	1.25	1.31	1.24	1.20
16 α	2.09	1.87	1.98	1.90	1.61
16 β	1.65	1.49	1.57	1.63	1.47
17 α	3.58	3.57	–	–	–
17 β	–	–	3.81	3.76	3.68
18-CH_3	0.81	0.82	0.78	0.73	0.78
19-CH_3	1.25	1.20	1.25	1.21	1.25
Sugar moiety					
1′	4.58	4.23	4.54	4.03	5.80
2′	5.03	2.97	4.95	2.86	4.36
3′	5.26	3.15	5.28–5.22	3.05	5.23
4′	5.22	3.30		3.11	5.16
5′	4.01	3.55	4.01	3.08	4.31
$COOCH_3$	3.77		3.77		3.78
$OCOCH_3$	2.07		2.04		2.12
	2.04		2.04		2.11
	2.03		2.02		
$OCOCH_3$[a]					1.71

[a] Orthoester.

Table 2
^{13}C NMR chemical shifts (ppm) assignment for compounds **3a**, **3b**, **4a**, **4b** and **8**.

	3a $CDCl_3$	**3b** DMSO	**4a** $CDCl_3$	**4b** DMSO	**8** $CDCl_3$
Aglycon moiety					
1	155.70	157.00	155.82	157.10	155.76
2	127.55	127.18	127.52	127.13	127.54
3	186.33	185.46	186.39	185.44	186.28
4	124.00	123.51	123.96	123.44	123.88
5	167.20	170.00	169.38	170.15	169.34
6	32.72	32.40	32.89	32.59	32.87
7	33.08	33.30	31.21	31.66	31.69
8	35.29	35.18	35.79	35.66	35.88
9	52.41	52.51	51.76	52.64	51.86
10	43.55	43.71	45.14	44.89	45.20
11	22.45	22.38	22.30	22.44	22.64
12	36.99	36.79	33.84	34.44	33.79
13	43.18	43.28	43.57	43.78	43.56
14	49.80	49.66	48.68	48.59	48.74
15	23.51	23.44	24.77	24.96	24.90
16	28.56	28.89	28.86	28.73	31.34
17	89.74	87.93	85.83	83.90	81.01
18	11.66	11.89	16.91	17.40	17.30
19	18.69	18.95	18.83	18.95	18.74
Sugar moiety					
1′	101.41	104.05	98.51	100.52	96.03
2′	71.43	73.84	71.32	73.86	73.40
3′	72.87	76.11	72.07	74.35	69.09
4′	69.47	71.99	69.50	72.68	68.67
5′	72.50	76.57	72.64	77.38	68.25
COOH	–	170.92	–	173.15	–
$COOCH_3$	170.20	–	170.23	–	169.29
$COOCH_3$	52.84	–	52.81	–	52.72
$OCOCH_3$	169.36	–	169.38	–	169.02
	169.03		168.95		168.89
	169.03		168.88		–
$OCOCH_3$	20.72		20.68		20.79
	20.64		20.58		20.70
	20.51		20.53		
$OCOCH_3$[a]					122.61
$OCOCH_3$[a]					22.37

[a] Orthoester.

ambiguity about the position of the glycosidic linkage in the corresponding glucuronides.

Confirmation of the anomericity of the glucuronic acid moiety in the steroid glucuronides and of the stereochemistry of the 17-hydroxyl function was obtained by one-dimensional ^{1}H NMR. Unambiguous assignments of the carbohydrate and steroid protons of the compounds **3a**, **3b**, **4a**, **4b** and **8** were obtained using 1D and 2D NMR spectra (Tables 1 and 2). Table 3 reports the coupling constants of the sugar moiety since the coupling constants of the steroid moiety have already been shown [19].

Each glucuronide spectrum contained the five methine signals arising from the glucuronic acid. Assignment of the configuration at the anomeric centre of the carbohydrate conjugate is typically based on the magnitude of the vicinal $J_{1,2}$ coupling constants for the anomeric proton. The magnitude of the coupling constant (Table 3) is characteristic of a diaxial configuration for 1′-H and 2′-H protons on the Karplus plot. Thus, a β-conformation can be assigned to the anomeric centre on the glucuronic acid for each glucuronide.

Integration of the signals provided a 1:1 glucuronic acid/aglycone ratio indicating the correct stoichiometry.

The ^{13}C NMR spectral data also showed that glucuronide **3a** contained one anomeric carbon at δ 101.41 (C-1) that correlated to the proton signal at 4.58 (1H, d, J = 7.7 Hz) and that the compound **4a** contained one anomeric carbon at δ 98.51 (C-1) that correlated to the proton signal at 4.54 (1H, d, J = 7.7 Hz) indicating the presence of one sugar unit in the β-form.

In 17α- and 17β-boldenone 17-glucuronides (**3a**, **3b** and **4a**, **4b**), 17-H resonates downfield to the steroid bulk region and can be distinguished from each other protons due also to their different coupling patterns. In compounds of β series 17α-H signal of steroid aglycone (δ 3.58 for **3a** and δ 3.57 for **3b**) was a double doublet of the same coupling constant with H-16α and H-16β (8.6 Hz). Otherwise, in compounds of α series (**4a** and **4b**) the 17β-H signal at δ 3.81 for **4a** and at δ 3.76 for **4b** was a broad doublet resulting from a coupling constant of 6.0 Hz with H-16β and a small coupling constant (<1 Hz) between H-17β and H-16α, due to dihedral angle close to 90° between them. These NMR data confirmed that no isomerization at 17-position took place either in glucuronidation or in the saponification reactions.

Table 3
J(H,H) (Hz) for compounds **3a**, **3b**, **4a**, **4b** and **8**.

$J_{H,H}$	**3a** $CDCl_3$	**3b** DMSO	**4a** $CDCl_3$	**4b** $CDCl_3$	**8** $CDCl_3$
Aglycon moiety					
17α, 16α	8.6	8.6	–	–	
17α, 16β	8.6	8.6	–	–	
17β, 16α	–	–	<1	<1	<1
17β, 16β	–	–	5.4	5.4	5.4
Sugar moiety					
1′, 2′	7.7	7.7	7.7	7.7	4.7
2′, 3′	8.6	8.6	8.6	8.6	2.7
3′, 4′	8.6	8.6	Not detectable	8.0	2.7
4′, 5′	9.2	9.2	9.5	9.5	8.3

The orthoacetate **8** can be easily detected by its NMR spectra which show a signal for the methyl group of CCH_3 at δ 1.71 ppm and the orthoester C at δ 122.71 ppm.

Additionally compound **8** has H-1 resonance less shielded than in compound **4a** (4.54 ppm versus 5.80 ppm), whereas H-2 resonance of orthoester **8**, is more shielded than the same signal in the glucuronide **4a** (4.95 ppm versus 4.36 ppm). Finally compound **8** shows the coupling constant between H-1 and H-2 (4.7 Hz) smaller than that of compound **4a** (7.7 Hz).

3.3. Mass spectrometry

The abundant natrium adduct ion $[M + Na]^+$ was detected in positive ion ESI-MS for the protected glucuronides **3a** and **4a**.

In negative ion ESI an intensive deprotonated molecule $[M - H]^-$ was observed as the base peak for glucuronides **3b** and **4b**. In negative ion ESI the most potential site for deprotonation $[M - H]^-$ is the carboxylic acid moiety of the glucuronic acid, as the steroid aglycones were not ionized in negative ion ESI at all and gave evidence on the corrected molecule weights of the synthesized steroid glucuronides. Either the positive or the negative ion MS spectra clearly indicate the presence of glucuronide moiety.

The mass spectrum of the orthoester **8** are identical to the protected glucuronides **3a** and **4a** ones.

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